

**Effect on lipid metabolism of
vegetable lipid interaction with
carbohydrate; a comparative
study with two important marine
aquaculture species, Gilthead sea
bream (*Sparus aurata*) and
European sea bass
(*Dicentrarchus labrax*)**

Carolina Oliveira Castro

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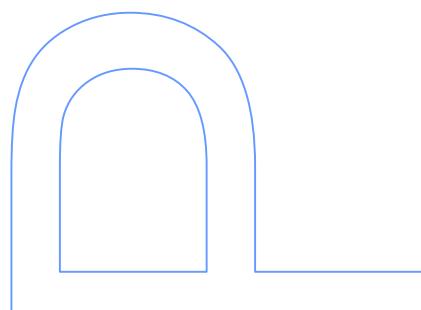
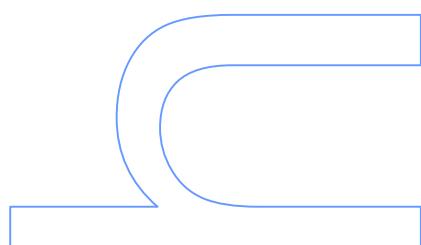
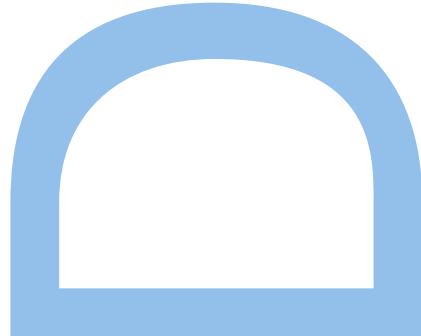
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Orientador

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Previous note

This thesis, was made with paragraph 2 of Article 4 of the General Regulation of Third Cycle of Studies, University of Porto and Article 31 of Decree 74/2006, of March 24, with new wording introduced by Decree 230/2009 of September 14, the total utilization of a coherent set of research papers already published or submitted for publication in international journals indexed and peer review, which comprise some of the chapters of this thesis was made. Given that such work was done in collaboration with some other authors, the candidate clarifies that, in all of them actively participated in its design, obtaining, analysis and discussion of results, as well as in preparing its published form. The presented study was carried out in CIMA (Interdisciplinary Centre for Marine and Environmental Research), Faculty of Sciences (Porto University), specifically in Fish Nutrition and Immunobiology Group (NUTRIMU), and INRA (National Institute of Agricultural Research) in St-Pée-sur-Nivelle (France), specifically in Nutrition Metabolism Aquaculture group (NuMéA).

List of Published Papers

Articles published in peer-reviewed scientific journals, resulting from the present work:

- Castro, C., Corraze, G., Panserat, S., Oliva-Teles, A. 2015. Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, and lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquacult. Nutr.* 21: 592-603.
- Castro, C., Corraze, G., Pérez-Jiménez, A., Larroquet, L., Cluzeaud, M., Panserat, S., Oliva-Teles, A. 2015. Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Br. J. Nutr.* 114:1143-56.
- Castro, C., Peréz-Jiménez, A., Coutinho, F., Díaz-Rosales, P., Serra, C.A., Panserat, S., Corraze, G., Peres, H., Oliva-Teles, A. 2015. Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. *Br. J. Nutr.* 114:1584-93.
- Castro, C., Couto, A., Peréz-Jiménez, A., Serra, C.R., Díaz-Rosales, P., Fernandes, R., Corraze, G., Panserat, S., Oliva-Teles, A. 2016. Effects of fish oil replacement by vegetable oil blend on digestive enzymes and tissue histomorphology of European sea bass (*Dicentrarchus labrax*) juveniles. *Fish Physiol. Biochem.* 42:203-17.

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Castro, C., Corraze, G., Diógenes, A., Larroquet, L., Panserat, S., Oliva-Teles, A. 2016. Regulation of glucose and lipid metabolism by dietary carbohydrate and lipid source in gilthead sea bream juveniles. Submitted to Br. J. Nutr.

Castro, C., Diógenes, A., Coutinho, F., Panserat, S., Corraze, G., Pérez-Jiménez, A., Peres, H., Oliva-Teles, A. 2016. Liver and intestine oxidative status of gilthead sea bream fed in vegetable oil and carbohydrate rich diets. Submitted to Br. J. Nutr.

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Abstract

A sustainable development of aquaculture requires reducing fish meal (FM) and fish oil (FO) use in aquafeeds, by replacing it with eco-friendly ingredients such as vegetable protein and oil sources. Vegetable ingredients are usually rich in carbohydrates (CH) and, contrarily to FO, are abundant in 18-carbon polyunsaturated fatty acids (C18 PUFA) but lack n-3 long-chain polyunsaturated fatty acids (LC-PUFA). These nutritional drawbacks may limit the use of vegetable ingredients in aquafeeds, especially for carnivorous marine fish species, which have specific requirements of n-3 LC-PUFA, as they are not able to efficiently elongate C18 PUFA to n-3 LC-PUFA, and are metabolically adapted to diets almost devoid of CH. For an increased incorporation of vegetable ingredients in aquafeeds at the expense of FM and FO, more knowledge is needed on the effects of dietary vegetable oils (VO) and CH *per se*, and the possible interactive effects between dietary VO and CH on lipid and CH related metabolic pathways and on fish oxidative status. For this purpose, using as models two important Mediterranean aquaculture species - European seabass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) - the experimental work applied in thesis relied on an integrative approach combining classical zootechnical parameters, plasma metabolites, activity of key enzymes and expression of selected genes involved in key metabolic pathways.

In Chapters 2 and 4, diets with FO or a blend of VO as main lipid sources, and with 0 or 20% gelatinized starch as CH source were used to evaluate processes involved in nutrient digestion, absorption and transport in European sea bass and gilthead sea bream juveniles. Moreover, in European sea bass the effects of dietary lipid source in intestinal digestive enzyme activities and in liver and intestine histomorphology were also evaluated (Chapter 3). Afterwards, the effects of the same diets on growth performance, tissue fatty acid (FA) composition, glucose and lipid metabolism (Chapters 5 and 6), and on fish oxidative status (Chapters 7 and 8) were also evaluated for both species.

Replacement of FO by VO in the diets slightly but significantly reduced apparent lipid digestibility in both species. However, this decreased lipid digestibility did not induce marked changes in lipid serum profile, liver and intestine transcriptional mechanisms involved in lipid transport and absorption. In European sea bass, no alterations in digestive enzyme activities and in liver and intestine histomorphology were also noticed. In gilthead sea beam, dietary CH inclusion did not affect lipid digestibility but modulated the transcriptional mechanisms involved in lipid absorption and transport at intestinal level.

As expected, replacement of FO by VO and inclusion of dietary CH led to small effects on growth performance and feed efficiency in both species. In gilthead sea bream, interactions between dietary CH and lipid source were observed in liver lipids and in muscle glycogen deposition. However, lipid and glycogen deposition in muscle and liver was mainly positively related to the dietary CH content in the two target species. Muscle and liver FA profiles markedly reflected the FA composition of dietary lipids, although dietary starch increased tissue saturated FA content. Dietary CH, but not dietary VO, enhanced liver lipogenic activity in both species and promoted a moderated liver transcriptional regulation of the glycolytic (only up-regulation of glucokinase gene expression) and the gluconeogenic (only down-regulation of phosphoenolpyruvate carboxykinase gene expression) pathways.

No increase in liver and muscle n-3 LC-PUFA content were observed in both species, but enhanced hepatic transcript levels of FADS2 - the key enzyme involved LC-PUFA biosynthesis – was observed with dietary VO at 6 hours after feeding in European sea bass and at 18 hours after feeding in gilthead sea bream. In gilthead sea bream fed the dietary CH-rich- diets, enhanced hepatic transcript levels of FADS2 was also observed at 18 hours after feeding.

In European sea bass, but not in gilthead sea bream, dietary CH inclusion enhanced plasma cholesterolemia and, at molecular level, the liver cholesterol biosynthesis capacity (HMGCR, the rate-limiting enzyme of cholesterol biosynthesis).

In European sea bass dietary VO increased transcript levels of key enzymes involved in cholesterol biosynthesis (HMGCR and CYP51A1) liver but did not affect plasma and liver cholesterol levels; whereas in gilthead sea bream no adaptive response in cholesterol biosynthesis pathway was observed at a molecular level, although it was observed a decrease of plasma cholesterol but not of hepatic cholesterol content.

In both species CH-rich diets promoted a reduction of lipid oxidative damage in the liver but not in the intestine. The mechanism by which dietary CH exerted their protective role against oxidative stress in the liver seems to be linked to an increased activity of glucose-6-phosphate dehydrogenase to generate reducing equivalents for the regeneration of reduced glutathione. In gilthead sea bream, dietary VO improved liver and intestine oxidative status, while in European sea bass such effect was only observed at intestinal level. The positive antioxidant effect of dietary VO may be related to the lower degree of unsaturation of the dietary FA and to an enhanced activity of glutathione reductase and glutathione peroxidase.

Overall, in this study no substantial interactions between dietary CH and lipid source were observed. However, we provided for the first time evidence of a transcriptional induction of FADS2, a key enzyme of LC-PUFA biosynthesis pathway, by dietary CH, in addition to VO, in gilthead sea bream. This may open new perspectives for the use of nutritional strategies to induce LC-PUFA biosynthesis in marine fish species. We also highlighted important aspects on the regulation of cholesterol biosynthesis pathway and fish oxidative status that are important in the context of VO and CH rich aquafeeds. In addition, species-specific differences in the regulation of cholesterol and LC-PUFA biosynthesis pathways in fish fed CH and VO were also highlighted, and require further elucidation in future studies.

Keywords: fish nutrition; carbohydrates; vegetable oils; lipid metabolism; glucose metabolism; oxidative status

Resumo

O desenvolvimento sustentável do setor aquícola está fortemente dependente da substituição da farinha de peixe (FP) e óleo de peixe (OP) nas rações para peixes por fontes de proteína e lípidos mais sustentáveis, como as matérias-primas de origem vegetal (MPV). As MPV caracterizam-se por apresentarem em geral grandes quantidades de hidratos de carbonos (HC) e, contrariamente aos OP, apresentarem elevadas quantidades de ácidos gordos polinsaturados (AGPI) de 18 carbonos (18C), mas não possuírem AGPI n-3 de cadeia longa. Estas desvantagens do ponto de vista nutricional podem limitar o uso destas MPV nas rações para peixes, particularmente para espécies carnívoras marinhas, uma vez que estas estão metabolicamente adaptadas a dietas praticamente desprovidas de HC e por terem necessidades específicas de AGPI n-3 de cadeia longa, devido à sua capacidade limitada de converter AGPI 18C em AGPI n-3 de cadeia longa.

Na perspetiva de aumentar os níveis de substituição dos produtos da pesca por MPV nas dietas para peixes, torna-se necessário aprofundar o conhecimento dos efeitos relativos à inclusão nas dietas dos HC e óleos vegetais (OV) *per se*, bem como do potencial efeito de interação entre ambos ao nível das vias metabólicas relacionadas com os hidratos de carbono, lípidos e estado oxidativo dos peixes. Com este propósito, usando como modelos duas espécies de elevada importância para a aquacultura do Mediterrâneo - o robalo Europeu (*Dicentrarchus labrax*) e a dourada (*Sparus aurata*) - o trabalho experimental desta tese teve por base uma abordagem integrada que combina parâmetros clássicos de zootecnia, análises dos metabolitos plasmáticos, e a atividade e expressão génica de enzimas envolvidas em vias metabólicas chave.

Nos Capítulos 2 e 4 foram utilizadas dietas com dois tipos de fontes lipídicas (OP e uma mistura de OV) e dois teores de amido gelatinizado (0 ou 20%), como forma de avaliar o seu impacto nos processos de digestão, absorção e transporte de nutrientes em ambas as espécies. No robalo foi igualmente avaliado o efeito da fonte lipídica da dieta na atividade das enzimas digestivas e na histomorfologia do fígado e do intestino (Capítulo 3). O efeito dessas mesmas dietas no crescimento, composição de ácidos gordos (AG) dos tecidos, metabolismo lipídico e da glucose (Capítulo 5 e 6), e estado oxidativo dos peixes (Capítulo 7 e 8) foi também avaliado em ambas as espécies.

A substituição do OP por OV nas dietas provocou uma ligeira, porém significativa, redução na digestibilidade dos lípidos no robalo e na dourada. Contudo, esta redução da digestibilidade dos lípidos não promoveu alterações significativas no perfil lipídico do plasma nem na expressão génica de proteínas envolvidas no transporte e absorção dos

lípidos no fígado e no intestino de ambas as espécies. No robalo também não foram observadas alterações na atividade das enzimas digestivas e na histomorfologia do fígado e intestino. Na dourada, a incorporação de amido nas dietas não afetou a digestibilidade dos lípidos, porém modulou a expressão génica de proteínas envolvidas na absorção e transporte dos lípidos a nível intestinal.

De acordo com o esperado, a substituição dos OP por OV e inclusão de hidratos de carbono nas dietas não provocaram efeitos significativos no crescimento e na eficiência de conversão alimentar de ambas espécies. Na dourada foi observada uma interação entre os hidratos de carbono e a fonte lipídica na deposição de lípidos no fígado e de glicogénio no músculo. Todavia, nas duas espécies, a deposição lipídica e de glicogénio no fígado e no músculo foram sobretudo relacionadas com o conteúdo de hidratos de carbono na dieta. O perfil de AG do fígado e do músculo refletiu a composição de AG da dieta. Verificou-se também que a incorporação de amido nas dietas provocou o aumento do teor de AG saturados nestes tecidos. A incorporação de HC na dieta, mas não de OV, levou a um aumento da atividade lipogénica e regulação moderada da expressão génica das vias glicolítica (apenas a indução da expressão génica da glucoquinase) e gluconeogénica (apenas a redução da expressão génica da fosfoenolpiruvato carboxiquinase) no fígado das duas espécies.

Apesar do teor de AGPI n-3 de cadeia longa não ter aumentado no fígado e no músculo de ambas espécies, a expressão génica da desaturase (FADS2) - enzima chave da via de biossíntese dos AGPI n-3 de cadeia longa - aumentou no fígado dos robalos (após 6 horas da refeição) e das douradas (após 18 horas da refeição) alimentados com dietas ricas em OV. Para além disso, verificou-se a indução da expressão génica da desaturase (FADS2) no fígado de dourada alimentadas com dietas ricas HC.

No robalo, mas não na dourada, a incorporação de HC na dieta promoveu um aumento dos níveis de colesterol plasmático e da capacidade, a nível molecular, de biossíntese de colesterol no fígado (HMGCR, a enzima limitante da biossíntese do colesterol). Adicionalmente, os OV promoveram um aumento da expressão génica de enzimas-chave envolvidas na biossíntese do colesterol (HMGCR e CYP51A1) no fígado do robalo sem, no entanto, afetarem os níveis de colesterol no fígado e no plasma. Na dourada, por outro lado, os OV provocaram um decréscimo dos níveis de colesterol no plasma, mas não no fígado, e não induziu, a nível molecular, uma resposta adaptativa da via de biossíntese do colesterol.

Em ambas as espécies, dietas ricas em HC promoveram uma redução da peroxidação lipídica no fígado mas não no intestino. Este efeito antioxidante parece estar associado a um aumento da atividade da glucose 6 fosfato desidrogenase, devido ao seu papel de gerar o poder redutor necessário à reciclagem da enzima glutationa reduzida. A incorporação de OV na dieta também melhorou o estado oxidativo do fígado e do intestino na dourada, enquanto no robalo esse efeito foi apenas observado a nível intestinal. O efeito antioxidante dos OV parece estar relacionado com o menor teor em AG insaturados da dieta e com aumento da atividade das enzimas glutationa redutase e glutationa peroxidase.

De um modo geral, neste estudo não se verificou um número substancial de interações entre os HC e a fonte lipídica das dietas. Contudo, foi possível demonstrar, pela primeira vez em dourada, uma indução da expressão génica da desaturase (FADS2), a enzima chave da via de biossíntese dos AGPI n-3 de cadeia longa, pela adição de HC e OV às dietas. Esta observação abre novas perspetivas ao uso de estratégias nutricionais para induzir a via de biossíntese de AGPI n-3 de cadeia longa em espécies de peixe marinhas. Esta tese contribuiu ainda para esclarecer alguns mecanismos relacionados com a regulação da via de biossíntese do colesterol e do estado oxidativo dos peixes, importantes no contexto de dietas com teores elevados de OV e HC. Para além disso, foram também evidenciadas diferenças interespecíficas na regulação das vias de biossíntese do colesterol e de AGPI n-3 de cadeia longa pelos HC e OV da dieta, aspecto que merece ser aprofundado em estudos futuros.

Palavras-chave: nutrição de peixes; hidratos de carbono; óleos vegetais; metabolismo dos lípidos; metabolismo da glucose; estado oxidativo

Index

	Page
Previous note	v
Acknowledgements	ix
Abstract	xi
Resumo	xv
List of Tables	xxiii
List of Figures	xxv
List of Abbreviations	xxix
Chapter 1 - General Introduction	1
1.1 Aquaculture development and feed formulation: trends and prospects	3
1.2 Carbohydrates and lipids in aquafeeds	6
1.3 Carbohydrate digestion, absorption transport and metabolism	10
1.3.1 <i>Carbohydrate digestion, absorption and transport</i>	10
1.3.2 <i>Carbohydrate metabolism</i>	11
1.3.3 <i>Nutritional regulation of carbohydrate related processes by dietary carbohydrates</i>	15
1.4 Lipid digestion, absorption, transport and metabolism	18
1.4.1 <i>Lipid digestion, absorption and transport</i>	18
1.4.2 <i>Lipid metabolism</i>	23
1.4.3 <i>Nutritional regulation of lipid related processes by dietary lipids</i>	32
1.5 Carbohydrate x lipid interaction	38
1.6 Oxidative stress	40
1.6.1 <i>Nutritional regulation of fish oxidative status</i>	45
1.7 Fish species characterisation	47
1.7.1 <i>Biology of the species</i>	48
1.7.2 <i>Nutritional requirements</i>	50
1.8 Alternative ingredients to FM and FO	53
1.9 Aims	55
Chapter 2- Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, and lipid and glucose metabolism of European sea bass (<i>Dicentrarchus labrax</i>) juveniles	57
Abstract	59

Introduction	59
Material and Methods	60
Results	64
Discussion	65
References	68
Chapter 3- Effects of fish oil replacement by vegetable oil blend on digestive enzymes and tissue histomorphology of European sea bass (<i>Dicentrarchus labrax</i>) juveniles	71
Abstract	73
Introduction	73
Material and Methods	74
Results	78
Discussion	79
References	85
Chapter 4- Dietary lipid and carbohydrate interactions: implications on lipid and glucose absorption, transport in gilthead sea bream (<i>Sparus aurata</i>) juveniles	89
Abstract	91
Introduction	92
Material and Methods	94
Results	100
Discussion	107
References	113
Chapter 5- Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (<i>Dicentrarchus labrax</i>) juveniles	121
Abstract	123
Introduction	123
Material and Methods	124
Results	126
Discussion	128
References	134
Chapter 6- Regulation of glucose and lipid metabolism by dietary carbohydrate levels and lipid sources in gilthead sea bream juveniles	137
Abstract	139
Introduction	140

Material and Methods	142
Results	147
Discussion	158
References	165
Chapter 7- Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (<i>Dicentrarchus labrax</i>) juveniles	175
Abstract	177
Introduction	177
Material and Methods	178
Results	180
Discussion	180
References	183
Chapter 8- Liver and intestine oxidative status of gilthead sea bream fed in vegetable oil and carbohydrate rich diets	187
Abstract	189
Introduction	190
Material and Methods	192
Results	196
Discussion	199
References	204
Chapter 9- General conclusions and final considerations	211
9.1 General conclusions	213
9.2 Final considerations	215
Chapter 10- References	219

List of Tables

Table 1. Plant feedstuffs composition (% dry mater, DM).

Table 2. Fatty acid composition of different marine animal oil (fish oil) and terrestrial plant oil sources used in compound aquafeeds (fatty acid values expressed as % total fatty acids).

Table 3. Fatty acyl desaturases (Fad) investigated in fish. The desaturation activities determined for each characterised Fad enzyme are indicated.

Table 4. Elongases (Elovl) investigated in fish. The type of Elovl is determined by amino acid similarities with mammalian orthologues and, in most cases, by functional characterisation analyses.

Table 5. Total European production (including EU and non-EU countries) of European sea bass and gilthead sea bream in 2014.

Table 6. Amino acid requirements (g/16 g N) of European (E.) sea bass and gilthead (G.) sea bream.

Table 7. Minimal dietary vitamin requirements recommended by NRC (1993) for salmonids.

List of Figures

Fig. 1. World fisheries and aquaculture production (A) and food fish consumption (B). A: Global production excluding aquatic plants; available data from 1960-2013 (adapted from FAO, 2015a); B: food fish, includes fish and seafood; available data from 1961-2011 (adapted from FAO, 2015b).

Fig. 2. Schematic representation of the major metabolic pathways involved in intermediary metabolism. The target metabolic enzyme markers of the glycolytic, gluconeogenic, glycogenesis, glycogenolysis, fatty acid synthesis, pentose phosphate (PPP), oxidation and bioconversion pathways are represented: glucokinase (GK), 6-phosphofructokinase-1 (PFK-1), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), glucose-6-phosphatase (G6Pase), glycogen synthase (GSase), glycogen phosphorylase (GPase), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), carnitine palmitoyl transferase- 1 and 2 (CPT1 and CPT2), ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), fatty acyl desaturases (fads) and elongases (elovls).

Fig. 3. Glycolysis and gluconeogenesis pathways. The reactions of glycolysis are shown on the right side; the opposing pathway of gluconeogenesis is shown on the left side. The key enzymes of the glycolytic pathway are glucokinase (GK), 6-phosphofructokinase-1 (PFK-1) and pyruvate kinase (PK) whereas the key gluconeogenic enzymes are phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase). The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P2ase) is also represented. Adapted from Enes et al. (2009).

Fig. 4. Proposed mechanism of lipoprotein assembly and trafficking. In the intestinal lumen, products of lipid hydrolysis are solubilised in micelles (MI) and then crossed the apical membrane of enterocytes. Fatty acids (FA), monoacylglycerols (MAG) and cholesterol (CHOL) are transported from the intestinal lumen into the enterocytes by protein transporters including cluster of differentiation 36/fatty acid translocase (cd36/FAT), fatty acid transport protein (Fatp), ATP-binding cassette ABCG5/ABCG8 heterodimeric transporter and Niemann–Pick C1-like 1 (npc1l1) or by protein-independent diffusion. Within the cytosol, transport of FA and MAG is facilitated by

intestinal fatty acid-binding proteins (FABP2). In the endoplasmic reticulum (ER), FA and MAG are resynthesized to TAG by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT). CHOL that is absorbed or synthesised locally in the enterocytes is thought to be either expelled into the intestinal lumen through the actions of the ABCG5/ABCG8 for eventual excretion via the faeces or esterified (cholesteryl ester, CE) by the action of Acyl-CoA cholesterol acyltransferase (ACAT) and packaged into chylomicrons for transport into the body. These esterified products are assembled into proximal lipoproteins (PLP) with apolipoprotein (Apo)B in a microsomal TAG transfer protein (MTP)-dependent manner. The PLP is merged with apoA4 to form a nascent lipoprotein (NLP) by core expansion. Resynthesized PL is used in the formation of the polar lipid coat of NLP. Within the Golgi lumen, apoA1 is attached to NLP to form a mature LP- chylomicron (CM). The LP is released from the enterocyte to enter the circulatory system. After entering circulation, CM are hydrolysed by lipoprotein lipase (LPL) to CM remnant (CMR), some of which are recognised by LP receptor-related proteins (●) and taken up by the liver, thereby contributing to new LP synthesis, i.e. VLDL that are assembled in a process identical to that of CM formation. After secretion into circulation, VLDL deliver lipid and CHOL to extrahepatic tissues and are processed into LDL by LPL. Extrahepatic tissues can also release excess CHOL into HDL particles, with the help of lecithin cholesterol acyltransferase (LCAT), which then returns to the liver for further processing (Adapted from Sheridan, 1988; Gu et al., 2014).

Fig. 5. The two biosynthetic pathways involved on the resynthesis of triacylglycerol (TAG) and phospholipids (PL): the monoacylglycerol (MAG) and the glycerol-3-phosphate (G3P) pathways. The target substrates, products and enzyme markers of the pathways are represented: MAG, G3P, lysophosphatidic acid (LPA) phosphatidic acid (PA), diacylglycerol (DAG), PL, TAG, monoacylglycerol acyltransferase (MGAT), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphorilase (PAP), diacylglycerol choline phosphotransferase (DGCPT), diacylglycerol ethanolamine phosphotransferase (DGEPT) and diacylglycerol acyltransferase (DGAT). (Adapted from Shi and Cheng, 2009).

Fig. 6. Pathways of biosynthesis of fatty acid families (n-3, n-6, n-9 and n-7 series) in fish. $\Delta 4$ fad, $\Delta 5$ fad and $\Delta 6$ fad and $\Delta 9$ fad, fatty acyl desaturases; Elovl2, Elovl4 and Elovl5, fatty acid elongases; VLC-PUFA, very long-chain polyunsaturated fatty acids. Adapted from Sargent et al. (2002) and Tocher (2015).

Fig. 7. Summary of the pathways for the generation of reactive oxygen species and of the actions of some of the enzymes involved in antioxidant defences in the cell. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD) reduced glutathione (GSH), oxidized glutathione (GSSG). Adapted from Storey et al. (1996).

Fig. 8. Mechanisms of lipid peroxidation. Adapted from Sargent et al. (2002).

Fig. 9. European sea bass (*Dicentrarchus labrax*), on the left; gilthead sea bream (*Sparus aurata*) on the right. Adapted from FAO (2015c,d).

List of Abbreviations

ABCG5 - adenosine triphosphate binding cassette G5

ABCG8 - adenosine triphosphate binding cassette G8

ACAT - Acyl-CoA cholesterol acyltransferase

ACC - acetyl coA carboxylase

ACLY - adenosine triphosphate citrate lyase

ADC - apparent digestibility coefficient

ATP - adenosine triphosphate

ALA - α-linolenic acid

AMPK - 5' adenosine monophosphate activated protein kinase

Apo – apolipoproteins

ARA – arachidonic acid

CAT – catalase

cDNA - complementary deoxyribonucleic acid

cd36/Fat - cluster of differentiation 36/fatty acid translocase

cm - centimeter

CPT - Carnitine palmitoyl transferase

C18 PUFA - polyunsaturated fatty acids of 18 carbons

DAG - diacylglycerol

DGAT - diacylglycerol acyltransferase

DGCPT - diacylglycerol choline phosphotransferase

DGEPT - diacylglycerol ethanolamine phosphotransferase

DHA - docosahexaenoic acid

DM - dry matter

DNA - deoxyribonucleic acid

EPA - eicosapentaenoic acid

Elov1 - elongase

EFA - essential fatty acids

FA - fatty acid

FABP - Fatty acid-binding proteins

Fad - fatty acyl desaturase

FADH₂ - reduced form of flavin adenine dinucleotide

FAS - fatty acid synthetase

Fat - fatty acid translocase

Fatp - fatty acid transport protein

FIFO - fish-in to fish-out

FM - Fish meal

FO - fish oil

FBPase - fructose-1,6-bisphosphatase

g - gram

GK - glucokinase

GLUTs - glucose transporters

GPAT - glycerol-3-phosphate acyltransferase

GPX - glutathione peroxidase

GPase - glycogen phosphorylase

GR - glutathione reductase

GSase - glycogen synthase

GSH - reduced glutathione

GSSG - oxidized form of glutathione

G3P - glycerol-3-phosphate

G6Pase - glucose-6-phosphatase

G6PD - glucose-6-phosphate dehydrogenase

h - hours

HK - hexokinase

HDL - high density lipoproteins

HMG-CoA - 3-hydroxy-3-methylglutaryl-coA

HMGCR - 3-hydroxy-3-methylglutaryl-CoA reductase

H₂O₂ - hydrogen peroxide

IDL - intermediate density lipoproteins

IU - international units of enzyme activity

kg - kilograms

LA - linoleic acid

LCAT - lecithin-cholesterol-acyl-transferase

LC-PUFA - long-chain polyunsaturated fatty acids

LDH - lactate dehydrogenase

LDL - low density lipoproteins

LPA - lysophosphatidic acid

LPAAT - lysophosphatidic acid acyltransferase

LPL - lipoprotein lipase

m - meter

MAG - monoacylglycerol

ME - malic enzyme

mg - milligram

MTP - microsomal TAG transfer protein

MUFA - monounsaturated fatty acids

N – nitrogen

NADH – reduced form of nicotinamide adenine dinucleotide

NADPH - reduced form of nicotinamide adenine dinucleotide phosphate

npc1l1 - Niemann–Pick C1-like 1

NO₂ - nitrogen dioxide

NO - nitric oxide

O₂ - molecular oxygen

O₂^{·-} - superoxide radical

ONOO[·] - peroxynitrite

PA - phosphatidic acid

PAP - phosphatidic acid phosphorilase

PC - phosphatidylcholine

PEPCK - phosphoenolpyruvate carboxykinase

PFK-1 - 6-phosphofructokinase-1

PK - pyruvate kinase

PL - phospholipids

PPAR - peroxisome proliferator-activated receptor

PPP - pentose phosphate

RNS - reactive nitrogen species

ROS - reactive oxygen species

RNS - reactive nitrogen species

SGLT1 - sodium dependent glucose co-transporter type 1

SFA - saturated fatty acids

SOD - superoxide dismutase

SREBP - sterol regulatory element binding protein

TAG – triacylglycerols

TCA – tricarboxylic acid cycle

TOR - target of rapamycin

VLDL - very low density lipoprotein

VLC-PUFA – very-long-chain polyunsaturated fatty acids

VO - vegetable oils

$^1\text{O}_2$ - singlet oxygen

6PGD - 6-phosphogluconate dehydrogenase

6PF-2-K/Fru-2,6-P2ase - 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

$\cdot\text{OH}$ - hydroxyl radical

$^\circ\text{C}$ – degrees Celsius

\euro - euros

1- General Introduction

1.1 Aquaculture development and feed formulation: trends and prospects

Aquaculture is widely regarded as the fastest world animal-food production sector, generating a total of over 70.2 million tonnes in 2013 (excluding aquatic plants), with an estimated total value of nearly 138.7 billion € (Figure 1). Nowadays, almost half of all seafood used for human consumption is provided by aquaculture (FAO, 2014). The failure of capture fisheries production to meet the growing world demand for fisheries products as well as the increasing awareness of the nutritional benefits and properties of fish to humans have triggered this dramatic increase in fish and shellfish production through the farming of aquatic organisms (Figure 1) (Boyd, 2015).

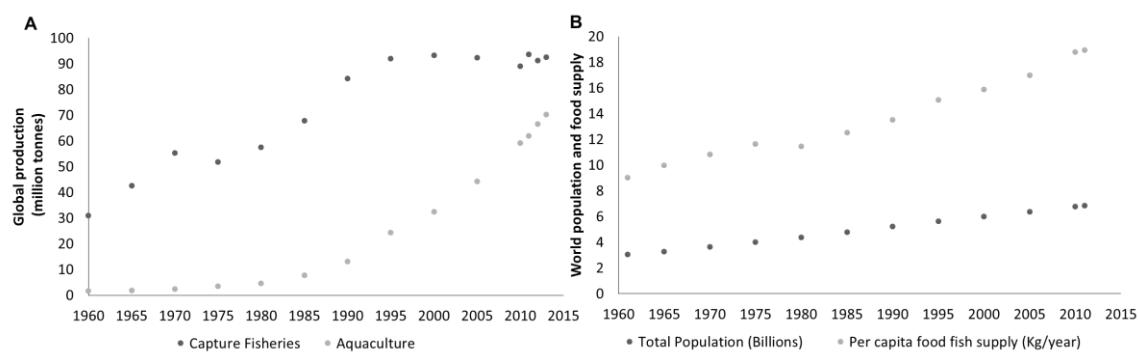


Fig. 1. World fisheries and aquaculture production (A) and food fish consumption (B). A: Global production excluding aquatic plants; available data from 1960-2013 (adapted from FAO, 2015a); B: food fish, includes fish and seafood; available data from 1961-2011 (adapted from FAO, 2015b).

Fish are a source of high quality protein and micronutrients, but are also a rich source of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic (DHA; 22:6 n-3) acids, which are known by the health beneficial effects in a range of human pathologies, including cardiovascular, inflammatory and neurological diseases (Tocher, 2015).

Over the years, major improvements in different aspects of aquaculture management such as improved control of fish reproduction and larval rearing, technological innovations of facilities, and improvements of aquafeed efficiency, have contributed for the expansion of aquaculture production.

However, the increase of aquaculture production is highly dependent on the increased production of aquafeeds. On the other hand, aquafeed formulation is still heavily dependent of both fish meal (FM) and fish oil (FO) as major sources of protein and lipids,

particularly for the farming of carnivorous species of higher trophic levels (such as marine shrimps, salmonids, marine finfishes, eels) (Tacon et al., 2011).

The overexploitation of wild fisheries stocks and the limitations imposed by regulation policies of fishing areas led to restrictions in the supply of these resources. Dramatic price increases of FM and FO, as well as concerns about ethical sustainability of using huge amounts of fish by-products for feeding farmed fish, including the development of sustainability metrics, such as the “fish-in to fish-out” ratio (FIFO) (Naylor et al., 2009; Tacon et al., 2011) led the industry and researchers to explore sustainable alternatives to FM and FO, such as plant feedstuffs, terrestrial animal by-products, single cell proteins, etc. However, for a number of reasons, including the cost of production and food security issues, plant feedstuffs and vegetable oils have been the main alternative sources of proteins and lipids in aquafeeds (NRC, 2011; Olsen and Hasan, 2012; Oliva-Teles et al., 2015).

In herbivorous/omnivorous fish and crustacean species, dietary incorporation of high amounts of plant feedstuffs has been achieved without reducing growth performance and feed efficiency. In carnivorous species such as salmonids and marine fish substantial reduction of FM (above 50-75%) in aquafeeds has also proved to be feasible without affecting performance (Naylor et al., 2009; NRC, 2011; Oliva-Teles et al., 2015). Nevertheless, high incorporation levels of plant feedstuffs in aquafeeds for carnivorous fish has several nutritional drawbacks related to poor palatability, inappropriate amino acid balance, high content of starch or non-starch polysaccharides, indigestible organic matter, and presence of anti-nutritional compounds (protease inhibitors, lectins, saponins, phytic acid, among others). To minimize these potential adverse effects of plant feedstuffs several strategies have been suggested such as supplementation of the diets with feed attractants to improve diet palatability and feed intake; incorporation of enzymes to break down phytic acid and improve phosphorus and cationic mineral availability; use genetically improved grains and oilseeds that have higher amino acid content or low anti-nutrients levels; apply processing treatments to destroy anti-nutritional compounds and improve starch availability (Gatlin et al., 2007; Naylor et al., 2009; NRC, 2011; Olsen and Hasan, 2012).

A number of alternative protein sources exist that can be used for replacing dietary FM. However, there are no commercial substitutes for FO as suppliers of LC-PUFA,

particular of n-3 series, which are indispensable to maintain fish health and nutritional value to consumers (Klinger and Naylor, 2012). Thus, the limited global supply of FO, rather than FM, is currently the most serious problem concerning the aquaculture feed industry.

Vegetable oils (VO) do not contain LC-PUFA but generally have high concentrations of their precursors (C18 PUFA). Thus, in fish species such as freshwater/diadromous fish whose essential fatty acids (EFA) requirements can be satisfied by C18 PUFA, dietary FO can be totally substituted with VO without significantly affecting growth performance and feed efficiency (Turchini et al., 2009; Oliva-Teles et al., 2015). Contrarily, in species that have a requirement for LC-PUFA, such as marine fish, total replacement of FO by alternative lipid sources is not viable. In these species a dietary inclusion of 5-10% FO should be considered to fulfill EFA requirements (Turchini et al., 2009; Tocher el al., 2010; Oliva-Teles et al., 2015).

The main constraint on using alternative lipid sources in aquafeeds is the resultant modification of fish fillet fatty acid (FA) composition and the consequent loss of the health-promoting benefits associated with fish consumption. Nonetheless, several approaches are now being implemented or under investigation in an attempt to maintain the health benefits of fish. These include the use of finishing diets high in LC-PUFA, the use of transgenic or genetically modified plant products rich in LC-PUFA, and the selection of fish with increased potential for endogenous production or retention of n-3 LC-PUFA (Turchini et al., 2009; Tocher, 2015).

It is also important to mention that with the increasing substitution of FM and FO by plant alternatives in aquafeeds, dietary cholesterol and phospholipids (PL) content will also be progressively reduced, while phytosterols, that are present in VO but not in fisheries products, will be increased (Tocher et al., 2008; Turchini et al., 2009). These may become important nutritional issues, particularly in larvae and fingerlings, considering the requirements of cholesterol and PL in these life stages (Tocher et al., 2008; Olsen et al., 2010). Briefly, cholesterol and PL are biological molecules that exhibit within the organism important structural (such as in membranes and lipoprotein structures) and functional roles as precursors of many physiologically active compounds (such as bile acids, vitamin D, adrenal corticoids and sex hormones in the case of cholesterol; eicosanoids, diacylglycerol, inositol phosphates and platelet activating factors in the case of PL) (NRC, 2011; Tocher et al., 2008).

1.2 Carbohydrates and lipids in aquafeeds

Proteins, along with lipids, comprise the major macronutrient classes that provide nutrients for plastic purposes, metabolic functions and for energy production required for growth and maintenance of homeostasis in all vertebrate organisms, including fish (Sargent et al., 2002; Leaver et al., 2008a). On the other hand, carbohydrates, which are components of a number of body metabolites (such as blood glucose, nucleotides, glycoproteins, and glycogen) are not an essential macronutrient, as glucose can be endogenous synthetized from non-glucose precursors (lactate, amino acids), and are not well utilized as energy source by carnivorous fish.

Carbohydrates are usually present in high quantities in plant feedstuffs and comprise both digestible carbohydrates (such as starch) and non-digestible polysaccharides (such as fibres) (Table 1) (NRC, 2011). Thus, presence of carbohydrates in fish feeds tends to rise with the trend to increase dietary incorporation of plant feedstuffs. Also, carbohydrates are the cheapest dietary energy source and its inclusion in the diets allows to spare amino acids and lipids from their energy-providing processes, and direct their use for tissues growth and for the synthesis of biologically important compounds (Wilson, 1994; Stone, 2003).

The ability to use dietary carbohydrates by fish is closely related with their natural feeding habits. Accordingly, fish of low trophic level (such as herbivorous/omnivorous common carp and the Nile tilapia) are able to use diets with relatively high levels (40-60%) of carbohydrates (Wilson, 1994; Hemre et al., 2002a; Enes et al., 2009; Polakof et al., 2012). In contrast, fish of high trophic levels (carnivorous fish such as salmonids and most marine species) usually do not tolerate dietary digestible carbohydrate inclusion levels higher than 20%.

Table 1 Plant feedstuffs composition (% dry mater, DM).

Feedstuffs	Starch(%DM)	Fibres(%DM)
Cereals		
Barley grain	59.7	5.2
Maize grain	73.4	2.5
Maize bran	35.0	12.3
Wheat grain	69.1	2.6
Wheat bran	23.2	10.4
Rye grain	62.2	2.2
Oat bran	48.3	4.0
Rye bran	28.4	6.3
Leguminous		
Pea seeds	51.3	6.0
Lupin seeds	10.3	13.9
Oleaginous		
Soybean seeds	6.4	6.3
Linseeds	5.2	10.4
Rapeseeds	3.7	10.1
Cottonseeds	2.1	28.1
Sunflower seeds	1.3	17.2

Adapted from Feedipedia (2015)

Aside their importance as high-density energy suppliers, dietary lipids are source of EFA and precursors of functional metabolic derivatives (including eicosanoids, docosanoids, hormones, and vitamins) as well as vectors for the absorption and delivery of lipid-soluble nutrients (including vitamins and carotenoid pigments). Lipids can be classified in two main classes: neutral lipids and polar lipids. Neutral lipids serve mainly as energy source and include triacylglycerols (TAG), sterols, sterol esters, free fatty acids and wax esters. Polar lipids are mostly cell membrane constituents and include phosphoglycerides (commonly named PL), sphingolipids, sulpholipids, and glycolipids (Sargent et al., 2002; Bell and Koppe, 2010).

Driven by the protein-sparing effect of energy-dense diets, dietary lipid content in aquafeeds has increased steadily over the years (Sargent et al., 2002; Leaver et al., 2008a). The upper limits of dietary lipid incorporation that maximally exploit their protein sparing effect and support highest fish growth rate differ among species. Accordingly, while in salmonids dietary lipid content can be as high as 40%, in several marine species increasing dietary lipid level above 20% has no benefits in the above mentioned

parameters (Sargent et al., 2002; Turchini et al., 2009; Oliva-Teles et al., 2015). In order to avoid excessive deposition of lipids in the flesh, a major issue relating to fillet quality, optimization of the lipid and digestible energy content of the feeds is of paramount importance (Sargent et al., 2002; Leaver et al., 2008a). In the case of fish that perform well with diets with low dietary lipids, such as Senegalese sole and omnivorous species such as tilapia and catfish, a minimum level dietary lipids should be provided in the feeds to supply the requirements for EFA (NRC, 2011).

It is generally accepted that VO constitute a valuable source of energy. However, contrary to FO, VO have unfavorable EFA composition. FO are rich in LC-PUFA, mainly EPA and DHA (Table 2), while VO are rich in saturated FA (SFA; palm oil, coconut oil), monounsaturated FA (MUFA; rapeseed/canola, olive oil), PUFA of the n-6 series (n-6 PUFA; soybean sunflower oil, cottonseed oil), or PUFA of the n-3 series (n-3 PUFA, linseed/flaxseed oil) (Table 2) (Glencross, 2009; Tacon et al., 2009). While the EFA requirements of freshwater and diadromous fish species can generally be satisfied by C18 PUFA present in VO, namely α -linolenic acid (ALA, 18:3 n-3), or linoleic acid (LA, 18:2 n-6), marine fish have an absolute requirement for EPA and DHA due to their limited ability for elongating and desaturating C18 FA precursors (Sargent et al., 2002; Tocher, 2010).

Table 2 Fatty acid composition of different marine animal oil (fish oil) and terrestrial plant oil sources used in compound aquafeeds (fatty acid values expressed as % total fatty acids).

FA	Marine animal oils						Terrestrial plant oils											
	Anchovy	Jack mackerel	Cod liver	Capelin	Menhaden	Soybean	Palm	Rapeseed	Sunflower	Coconut	Safflower	Linseed	Sesame	Olive	Corn	Cottonseed	Peanut	
08:0	-	-	-	-	-	-	-	-	-	9	-	-	-	-	-	-	-	
10:0	-	-	-	-	-	-	-	-	-	7	-	-	-	-	-	-	-	
12:0	-	-	-	-	-	-	-	-	-	47	-	-	-	-	-	-	-	
14:0	8	6	6	8	8	1	1	-	-	18	-	-	-	-	-	1	1	
16:0	18	17	14	11	19	8	44	6	7	9	7	5	10	11	11	23	10	
18:0	6	4	3	1	4	3	5	3	6	3	3	4	6	4	2	3	2	
20:0	4	-	-	-	-	1	-	1	-	-	-	-	1	-	-	-	-	
SFA	36	26	23	20	31	13	50	9	13	94	10	9	17	15	13	27	12	
16:1 n-7	11	8	8	11	9	2	1	1	-	-	-	-	-	1	-	1	1	
18:1 n-9	15	25	19	17	13	21	39	54	23	7	16	16	41	76	24	17	45	
20:1 n-9	3	4	10	19	2	2	-	2	-	-	-	-	-	-	-	-	-	
22:1 n-11	2	-	13	15	1	-	-	1	-	-	-	-	-	-	-	-	-	
MUFA	30	37	49	62	25	24	40	57	23	7	16	16	41	77	24	18	46	
18:2 n-6	1	4	2	2	2	57	11	20	64	-	73	19	43	7	58	52	32	
20:4 n-6	1	1	1	-	1	-	-	-	-	-	-	-	-	-	-	-	-	
n-6 FA	2	5	3	2	2	57	11	20	64	0	73	19	43	7	58	52	32	
18:3 n-3	1	1	1	1	1	7	-	13	-	-	1	56	-	1	1	1	-	
18:4 n-3	3	2	3	2	3	-	-	-	-	-	-	-	-	-	-	-	-	
20:5 n-3 (EPA)	12	13	9	5	11	-	-	-	-	-	-	-	-	-	-	-	-	
22:5 n-3	2	2	2	1	2	-	-	-	-	-	-	-	-	-	-	-	-	
22:6 n-3 (DHA)	12	8	9	3	9	-	-	-	-	-	-	-	-	-	-	-	-	
n-3 FA	30	26	24	11	26	7	0	13	0	0	1	56	0	1	1	1	0	
PUFA	5	6	6	4	5	64	11	33	64	0	74	75	43	8	59	52	32	
LC-PUFA	26	25	21	8	23	0	0	0	0	0	0	0	0	0	0	0	0	

Note: 18:1 n-7 data is combined with 18:1 n-9, 20:1 n-11 data is combined with 20:1 n-9, 22:1 n-9 data is combined with 22:1 n-12; LC-PUFA, long-chain polyunsaturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids (adapted from Tacon et al., 2009).

The lipid content and FA profile of fish is dependent on the processes involved in digestion, absorption, transport and metabolism of nutrients, processes that are regulated by numerous biotic and abiotic factors (including genetic, nutritional, endocrine and environmental factors) with diet playing a preponderant role. Therefore, the general aspects concerning digestion, absorption, transport and metabolism of carbohydrates and lipids in fish will be briefly introduced in the following chapter, followed by a brief review of the nutritional regulation of these processes. In addition, a concise review on the mechanisms involved in fish oxidative stress, and of the nutritional requirements of the target species, European sea bass and gilthead sea bream, will be presented. Detailed aspects on the role of protein in fish diets and of the mechanisms that regulate the intracellular processes involved in protein digestion, absorption, transport and metabolism in fish fall outside the scope of this work and will not be addressed.

1.3 Carbohydrate digestion, absorption, transport and metabolism

1.3.1 Carbohydrate digestion, absorption and transport

Regardless of the variability in feeding habits and in the anatomy of digestive tract among fish species, all fish species studied to date are equipped with an enzymatic machinery for the hydrolysis and absorption of simple and complex carbohydrates. However, contrary to carnivorous species, herbivorous and omnivorous developed a microbiota and a digestive tract apt for processing a great variety of carbohydrate-containing feedstuffs (Krogdahl et al., 2005). The activity levels of endogenous enzymes involved in carbohydrate digestion (pancreatic α -amylase and disaccharidases produced in the brush border membrane of the enterocytes) also seem to reflect the trophic level of the species, with higher activities being recorded in herbivorous and omnivorous than in carnivorous species (Krogdahl et al., 2005; NRC, 2011). The enzyme α -amylase hydrolyzes $\alpha(1\text{-}4)$ glucoside-linkages of glycogen and starch to shorter oligosaccharides (short chain dextrans, maltotriose and maltose). These molecules are further hydrolysed by disaccharidases into their constituent monosaccharides (mainly glucose). Most fish apparently lack chitinases and cellulases and other enzymes that hydrolyze the β -glycoside bonds found in non-starch polysaccharides such as chitin, cellulose, hemicellulose, pectins and gums, rendering them non-nutritive or detrimental for the animal (Stone, 2003; NRC, 2011). Though some chitinase and cellulase activity has been detected in some species, whether such enzymes are produced by fish or are obtained through food or produced by gut microbiota is still a matter of debate (Enes et

al., 2011; NRC, 2011). In general, proximal regions of the digestive tract (pyloric caeca, proximal and mid intestine) show greater capacity for carbohydrate digestion than the distal region (Krogdahl et al., 2005).

Intestinal absorption of glucose and other monosaccharides released by digestion is thought to be dominated by transcellular transport (simple diffusion or by the aid of specific transporters), rather than paracellular absorption. The existence of glucose transporters, similar to those found in mammals, have been described in fish, namely the brush border and basolateral membrane transporters of the enterocytes, SGLT1 and GLUT2, respectively. The brush border transport is electrogenic and dependent on energy and Na^+ whereas the basolateral transport is facilitated and Na^+ independent (Krogdahl et al., 2005). An alternate mean of exporting glucose to the bloodstream independent of GLUT2 is also possible in fish. This process is dependent on glucose phosphorylation by hexokinase and further dephosphorylation by glucose 6-phosphatase (Mithieux, 2005; Polakof et al., 2010). Although the affinity of transporters for glucose increases from the proximal to the distal region of the intestine, proximal regions seem to have the higher contribution in glucose absorption (Krogdahl et al., 2005).

1.3.2 Carbohydrate metabolism

Following the absorption across the digestive tract, dietary carbohydrates (primarily glucose) are transported into the blood stream and, via the portal vein, to the liver and then to other tissues/organs where glucose is either metabolized for energy purposes, stored as glycogen or converted to lipid or other metabolites such as pentoses (Figure 2). Most of the hepatic cellular glucose uptake occurs passively, through facilitative glucose transporters (such as GLUT2). Other glucose transporters (GLUT) were also reported in different fish tissues, including an insulin-sensitive GLUT4 homologue in muscle and adipose tissues (Planas et al., 2000; Teerijoki et al., 2000; Hall et al., 2004, 2005, 2006; Zhang et al., 2003; Capilla et al., 2004a; Hrytsenko et al., 2010; NRC, 2011; Polakof et al., 2012).

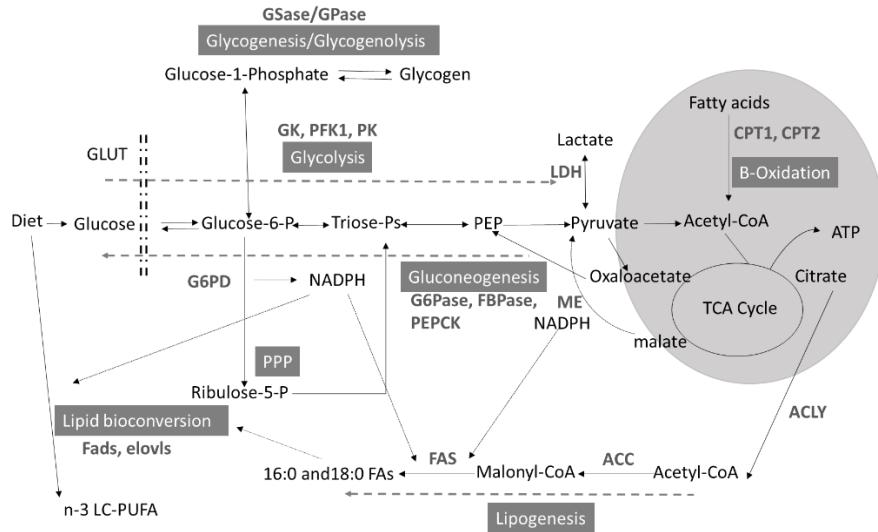


Fig. 2. Schematic representation of the major metabolic pathways involved in intermediary metabolism. The target metabolic enzyme markers of the glycolytic, gluconeogenic, glycogenesis, glycogenolysis, fatty acid synthesis, pentose phosphate (PPP), oxidation and bioconversion pathways are represented: glucokinase (GK), 6-phosphofructokinase-1 (PFK-1), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), glucose-6-phosphatase (G6Pase), glycogen synthase (GSase), glycogen phosphorylase (GPase), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), carnitine palmitoyl transferase- 1 and 2 (CPT1 and CPT2), ATP citrate lyase (ACLY), acetyl CoA carboxylase (ACC), fatty acid synthase (FAS), fatty acyl desaturases (fads) and elongases (elovls).

Glycolysis is the main catabolic pathway for glucose, by which it is converted to pyruvate (Figure 2 and 3). Under anaerobic conditions, which prevail in the white muscle, pyruvate is converted to lactate via lactate dehydrogenase (LDH, EC 1.1.1.27). Under aerobic conditions pyruvate is subsequently converted into acetyl-CoA by the mitochondrial pyruvate dehydrogenase complex. Acetyl-CoA can then enter the mitochondrial citric acid cycle (also called the Krebs or tricarboxylic acid -TCA- cycle) for complete oxidation with the production of energy under the form of ATP. If not directly used for energy purposes, acetyl-CoA may be used for lipid synthesis and/or converted to ketone bodies (Nelson and Cox, 2005). All the enzymes involved in the glycolytic pathway were reported to exist in fish (Enes et al., 2009).

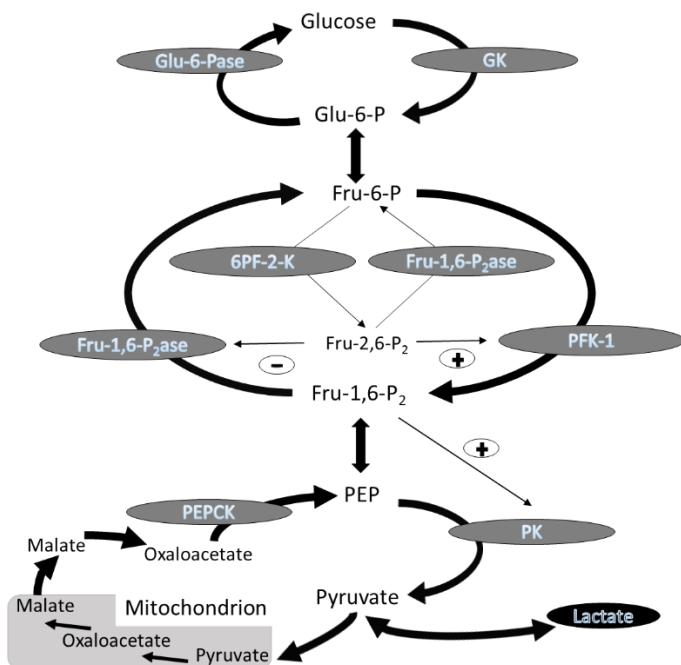


Fig. 3. Glycolysis and gluconeogenesis pathways. The reactions of glycolysis are shown on the right side; the opposing pathway of gluconeogenesis is shown on the left side. The key enzymes of the glycolytic pathway are glucokinase (GK), 6-phosphofructokinase-1 (PFK-1) and pyruvate kinase (PK) whereas the key gluconeogenic enzymes are phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase) and glucose-6-phosphatase (G6Pase). The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PF-2-K/Fru-2,6-P2ase) is also represented. Adapted from Enes et al. (2009).

The pentose phosphate pathway constitutes an alternative catabolic route for glucose that leads to the production of reducing equivalents under the form of NADPH, which is required for lipid biosynthesis, and of ribose-5-phosphate, which is required for nucleotides formation. This pathway involves the enzymes glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) (Engelking, 2010) and is particularly active in tissues that need electron donor NADPH to carry out extensive FA synthesis (liver and adipose tissues), active synthesis of cholesterol and steroid hormones (liver, adrenal gland, gonads) or production of reduced glutathione (see 1.4.2 and 1.6 sections for details) (Nelson and Cox, 2005; Engelking, 2010).

Glucose also takes part in other cellular metabolic pathways as glycogenesis, where excess glucose is converted to its storage polymer, glycogen, by glycogen synthase (GSase; EC 2.4.1.11). Per unit of tissue, liver is by far the major site of glycogen storage in fish, which concentration can reach up to 200mg/g, depending on the feeding status, species, and other factors such as temperature (NRC, 2011). Nonetheless, in terms of whole organism, muscle represents the major reservoir of glycogen in fish.

Requirements of glucose for metabolic purposes may be satisfied by glycogen degradation into glucose (glycogenolysis) or by de novo synthesis of glucose (gluconeogenesis) from non-glucose substrates, such as lactate, glycerol (derived from the hydrolysis of TAG) and α -ketoacids (derived from the catabolism of glucogenic amino acids). Glycogen phosphorylase (GPase; EC 2.4.1.1) catalyzes the rate-limiting step in glycogenolysis. The enzymes involved in glycogen metabolism are activated through phosphorylation (GPase) or dephosphorylation (GSase) reactions (Enes et al., 2009).

Glycolysis and gluconeogenesis share several enzymes that catalyze reversible reactions. However, three reactions are considered irreversible and alternative routes are used in glycolysis and gluconeogenesis (Figure 3). The conversion of glucose to glucose 6-phosphate is the first step for capturing glucose in the cell and it is catalyzed by hexokinases (HK; EC 2.7.1.1), including the HK-IV or glucokinase (GK; EC 2.7.1.2). Glucose 6-phosphate can then be used as substrate for glycolysis, glycogenesis or the pentose-phosphate pathway. The other key steps in glycolysis involve the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate by 6-phosphofructokinase-1 (PFK1; EC 2.7.1.11) and the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase (PK; EC 2.7.1.40). These three enzymes are considered to be the limiting and unidirectional steps in glycolysis.

In gluconeogenesis, the three irreversible steps are bypassed by a separate set of enzymes, catalyzing opposing reactions: phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32) catalyses the conversion of oxaloacetate to phosphoenolpyruvate (the first step of gluconeogenesis), fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11) catalyses the hydrolysis of fructose-1,6-bisphosphate to fructose-6-phosphate; and glucose-6-phosphatase (G6Pase; EC 3.1.3.9) catalyses the dephosphorylation of glucose-6-phosphate to glucose. This is the last step of gluconeogenesis as well as glycogenolysis, so this microsomal enzyme plays a key role in glucose homeostasis. Gluconeogenesis is particularly active in the liver, kidney and intestine (Enes et al., 2009).

Although fish are equipped with the whole set of enzymatic machinery required for the digestion and metabolism of carbohydrates, a prolonged postprandial hyperglycemia is generally observed, particularly in carnivorous species, after the ingestion of carbohydrate-rich diets or after a glucose tolerance test. This suggests that nutritional and hormonal regulatory mechanisms of glycemia may be inadequate (Enes et al., 2009). Several hypotheses have been proposed to explain the poor utilization of dietary carbohydrates by fish. The more feasible hypotheses up to know are: a lower potency of glucose than amino acids as insulin secretagogue; inhibition of insulin secretion by other hormones such as somatostatin; relatively low number of insulin receptors and lower affinity of insulin to the receptors; poor regulation of signaling pathways of metabolic sensors (AMPK and TOR); lack of an adequate balance between hepatic glucose uptake (glycolysis) and de novo production (gluconeogenesis); poor induction of lipogenesis by glucose; low capacity to use glucose in muscle and adipose tissues (Enes et al., 2009; Polakof et al., 2012; Panserat et al., 2013).

1.3.3 Nutritional regulation of carbohydrate related processes by dietary carbohydrates

Regardless of carbohydrate source, a general reduction in carbohydrate digestibility is observed in a number of fish species when dietary inclusion of carbohydrates is increased (Aksnes et al., 1995; Hemre et al., 1995; Grisdale-Helland et al., 1998; Webb et al., 2010). This reduction in digestibility may be due to substrate overload and subsequent saturation of digestive carbohydrases and/or glucose transporters (Stone, 2003; Krogdahl et al., 2005). This is particularly marked in carnivorous species which, contrary to omnivorous and herbivorous species, apparently have limited ability to modulate digestive enzymes and transporters according to dietary carbohydrate levels (Soengas and Moon, 1998; Stone, 2003; Krogdahl et al., 2005; Couto et al., 2012; Kamalam et al., 2013a). Indeed, whereas a glucose load has been reported to enhance the activity of glucose transporters SGLT1 and GLUT2 in the enterocytes of black bullhead catfish, an omnivorous fish (Soengas and Moon, 1998), the intake of a starch rich diet did not induce intestinal amylase activity in gilthead sea bream (Couto et al., 2012) or the expression of glucose transporters in rainbow trout (Kamalam et al., 2013a), which are carnivorous species.

The effect of botanical origin, molecular complexity and physical state of carbohydrates on its digestibility in a given species is directly related to the activity of carbohydrases (mainly amylase) (Stone, 2003).

Briefly, considering botanical origin of starch: granules of smaller size have higher surface area for action of digestive enzymes; higher amount of amylopectin relatively to amylose increases the availability of α -glycosidic bonds for the action of enzyme α -amylase; this results in increased digestibility of starch (Degani et al., 1997; Venou et al., 2003; Stone, 2003; Enes et al., 2006a; NRC, 2011; Couto et al., 2016). Similarly, digestibility usually decreased with the increase of molecular complexity of the molecule (i.e. digestibility of maltose>dextrin>starch) (Singh and Nose, 1967; Storebakken et al., 1998). Processing conditions (such as cooking, steaming, expansion and extrusion) lead to gelatinization of starch and consequently turns it more accessible to digestive enzymes, and making it more digestible, particularly in carnivorous species (Peres and Oliva-Teles, 2002; Venou et al., 2003; Amirkolaie et al., 2006; NRC, 2011). Heat processing also inhibits α -amylase inhibitors that are present in some cereals and that were shown to impair α - amylase activity of carp and tilapia (Natarajan et al., 1992).

Knowledge about dietary regulation of glucose transporters is limited. However, in a number of studies a carbohydrate-rich diet or a glucose load were reported not to induce modifications on GLUT4 expression in the white muscle of rainbow trout, suggesting a poor ability of the peripheral tissues to adapt to a high influx of glucose (Capilla et al., 2002; Panserat et al., 2009; Jin et al., 2014).

On the other hand, the ability to deal with a glucose load or a carbohydrate rich diet does not seem to be linked to an inefficient regulation of the key enzymes involved in glycolysis, at least at hepatic level.

In most fish species, including carnivorous species, the expression and activity of hepatic glucokinase was induced by a carbohydrate rich diet (Enes et al., 2009; Polakof et al., 2012). However, the adaptation of hepatic GK activity to a carbohydrate intake may be restricted to a maximum dietary carbohydrate level, at least in some species. Accordingly, Moreira et al. (2008) observed in European sea bass, a carnivorous species, a lack increase of hepatic GK activity as dietary carbohydrate increased from 20 to 30%. Findings concerning other enzymes of glycolysis (such as HK, PFK1 and PK) suggest that they are not as responsive to dietary carbohydrates as GK. As in higher vertebrates, the activity of HK does not seem to be under nutritional regulation in fish (Enes et al., 2009). On the other hand, while the activities or expression of PFK1 and PK were reported to be induced by dietary carbohydrate in several species (Metón, et al. 1999; Borrebaek and Christophersen, 2000; Enes et al., 2006a; Fernández et al., 2007),

this does not seem to be the case in other species (Dias et al., 2004; Enes et al., 2008a; Kamalam et al., 2012; Skiba-Cassy et al., 2013; Castro et al., 2015a).

Regarding gluconeogenesis, a carbohydrate-rich diet was reported to have little effects on the activity or gene expression of key enzymes such as G6Pase, FBPase and PEPCK in several carnivorous species such as the Atlantic salmon, rainbow trout, European sea bass or gilthead sea bream (Tranulis et al., 1996; Panserat et al., 2001; Caseras et al., 2002; Fernández et al., 2007; Moreira et al., 2008; Skiba-Cassy et al., 2013; Bou et al., 2014). In European sea bass, studies using deuterated water revealed that plasma glucose appearance was almost entirely derived from gluconeogenesis, regardless of the fish nutritional status (Viegas et al., 2011). On the other hand, in herbivorous or omnivorous fish such as the Nile tilapia and common carp, glucogenesis enzyme activities or gene expressions are often inhibited after feeding carbohydrates (Shimeno et al., 1993; Shikata et al., 1994; Panserat et al., 2002a; Figueiredo-Silva et al., 2013). Therefore, the absence of gluconeogenesis inhibition after feeding a carbohydrate-rich diet may be one of the reasons behind the inefficient use of dietary carbohydrates in carnivorous fish.

Despite the limit number of studies assessing the nutritional regulation by dietary carbohydrates of GSase, the key enzyme involved in glycogen synthesis, it was reported that it was unresponsive to a carbohydrate stimulus (Leung and Woo, 2012, Rocha et al., 2015). Nevertheless, increased glycogen concentration in the liver and other tissues such as muscle and intestine with the increase of dietary carbohydrate content or in response to a glucose load, have been reported in a number of fish species (Tranulis et al., 1996; Borrebaek and Christoffersen, 2000; Hemre et al., 2002b; Capilla et al., 2004b; Lee and Lee, 2004; Polakof et al., 2010; Kamalam et al., 2012; Leung and Woo, 2012; Ekmann et al., 2013; Castro et al., 2015a). For instance, Ekmann et al. (2013) using ¹³C isotope-labelled starch demonstrated in gilthead sea bream that up to 69% of liver glycogen was originated by glycogenesis from dietary starch. Data on the effect of dietary carbohydrates on expression and activity of GPase, the key enzyme involved in glycogen breakdown, demonstrated that it is negative regulated by dietary inclusion of carbohydrates (Borrebaek and Christoffersen, 2000; Leung and Woo, 2012).

Although an increased intestinal glycolytic potential was recorded in rainbow trout fed a carbohydrate rich diet or subjected to a glucose load (Kirchner et al. 2008; Polakof et al., 2010), in other extra-hepatic tissues such as white muscle or adipose tissue such changes were not detected (Panserat et al., 2009; Kamalam et al., 2012; Jin et al., 2014).

In addition, lack of gluconeogenesis regulation in the intestine was also reported (Kirchner et al. 2008; Polakof et al., 2010). This data also confirms the relatively meagre utilisation of glucose in peripheral tissues, thus contributing to the persistent postprandial hyperglycaemia that occurs in carnivorous species (Enes et al., 2009; Polakof et al., 2012).

Although simple carbohydrates are more digestible than complex carbohydrates in most species, there is no discernable pattern in the metabolic ability of different species to utilize simple versus complex carbohydrates. Indeed, in European sea bass and gilthead sea bream, glucose induced higher liver and PK and/or GK activity than starch (Enes et al., 2006b; Enes et al., 2008b; Enes et al., 2010), while in gibel carp and Chinese Longsnout catfish, PK activity was similar when fish were fed diets containing glucose, dextrin or starch (Tan et al., 2006).

1.4 Lipid digestion, absorption, transport and metabolism

1.4.1 *Lipid digestion, absorption and transport*

Fish digest dietary lipids efficiently, with apparent lipid digestibility coefficients above 90% being commonly reported in a number of species (Olsen and Ringo, 1997; Tocher, 2003). This highlights the pivotal role of lipid (generally TAG with smaller amounts of PL, wax and sterol esters) in the natural diet of fish as well as a digestive enzymatic apparatus well suited for lipid digestion (Olsen and Ringo, 1997). In the digestive tract of a range of fish species the presence of two types of lipases (pancreatic lipase, EC 3.1.1.3; and non-specific carboxyl ester lipase, EC 3.1.1.1), likely of a pancreatic origin, was reported. While pancreatic lipase predominantly hydrolyzes TAG, carboxyl ester lipase has broader substrates specificity and can hydrolyze a wide range of lipid classes (tri-, di-, and monoglycerols, cholesteryl esters, esters of vitamins A and E, aryl esters, PL, lysophospholipids, wax esters, and ceramide) (Kurtovic et al., 2009). Although few studies are available in fish, based on the wide variety of lipid classes in the marine environment there are clues that carboxyl ester lipase-type lipase may prevail in marine fish, while in freshwater fish pancreatic lipase-type lipase seems to be the predominant enzyme (Kurtovic et al., 2009). Evidence of a pancreatic lipase-colipase in fish, as described in mammals, is scarce but some studies described a protein with similar weight and properties of lipase-colipase in rainbow trout (Leger et al., 1977). Additional sources of digestive lipases in fish, such as enterocytes, intestinal allochthonous and autochthonous bacteria, have also been suggested (Olsen and Ringo, 1997; Tocher,

2003). Moreover, although the presence of phospholipase A2, a specific enzyme with hydrolytic activities for PL has also been reported in a number of fish species (Tocher et al., 2008; Sæle et al., 2011; Fujikawa et al., 2012), its role in lipid digestion in fish is still a matter of debate (Tocher, 2003). Furthermore, the existence of specific enzymes that hydrolyse several carboxylic acid esters (such as wax esters and sterol esters) remains uncertain despite the attempts to elucidate it (Olsen and Ringo, 1997; Tocher, 2003).

The pattern of lipase activity along the digestive tract seems to differ among fish species, mainly in relation to differences in the gastrointestinal tract anatomy. Indeed, while in most fish species lipid hydrolysis occurs to a greater extent in the pyloric caeca and proximal intestine (Olsen et al., 1998; Tocher, 2003; De la Parra et al., 2007; Castro et al. 2013, 2016), in other species the posterior intestine seems to play a major role in lipid hydrolysis (Chakrabarti et al., 1995; Izquierdo and Henderson, 1998). Izquierdo and Henderson (1998) hypothesized that in turbot, a short intestine and the presence of only two rudimentary caeca may explain the higher role of posterior intestine in lipid digestion. In fish, information regarding the physical processes occurring in intestinal lumen during lipid digestion is limited, but is presumed to occur as in mammals, with the main hydrolytic products being solubilized or emulsified in bile salt micelles. The main products of lipid digestion (free FA, acyl glycerols and glycerol, 1-acyl-lyso-glycerophospholipids, cholesterol and long chain alcohols from the hydrolysis of cholestryll, and wax esters) are then taken up by enterocytes mainly by passive diffusion, although also some FA and cholesterol may be transported by a carrier-mediated transport mechanism involving membrane proteins (such as Fatp, and cd36/fat; ABCG5/G8 and npc111 for cholesterol) (Figure 4) (Olsen and Ringo, 1997; Tocher, 2003; Bakke et al., 2010; Kortner et al., 2013, 2014; Gu et al., 2014).

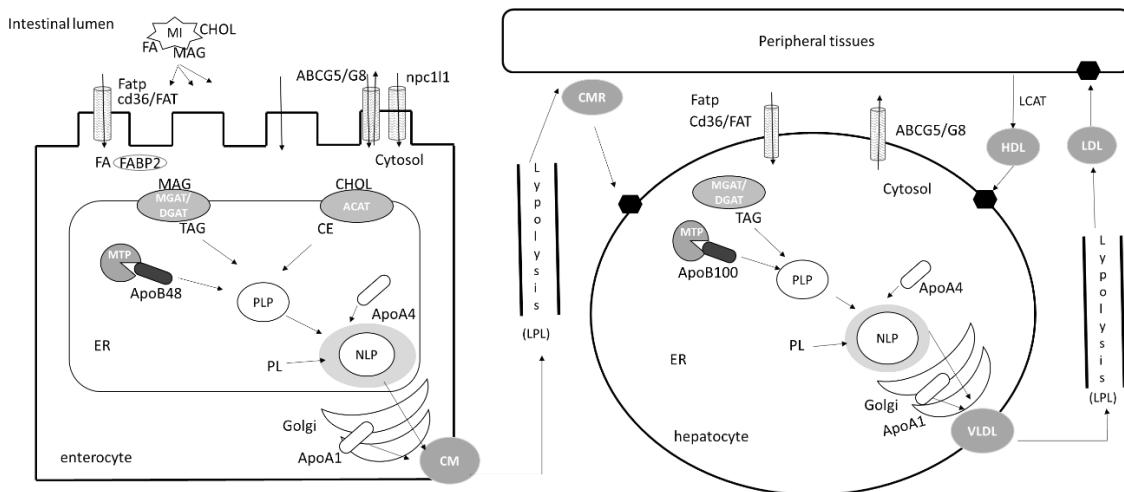


Fig. 4. Proposed mechanism of lipoprotein assembly and trafficking. In the intestinal lumen, products of lipid hydrolysis are solubilised in micelles (MI) and then crossed the apical membrane of enterocytes. Fatty acids (FA), monoacylglycerols (MAG) and cholesterol (CHOL) are transported from the intestinal lumen into the enterocytes by protein transporters including cluster of differentiation 36/fatty acid translocase (cd36/FAT), fatty acid transport protein (Fatp), ATP-binding cassette ABCG5/ABCG8 heterodimeric transporter and Niemann–Pick C1-like 1 (npc1l1) or by protein-independent diffusion. Within the cytosol, transport of FA and MAG is facilitated by intestinal fatty acid-binding proteins (FABP2). In the endoplasmic reticulum (ER), FA and MAG are resynthesized to TAG by monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT). CHOL that is absorbed or synthesised locally in the enterocytes is thought to be either expelled into the intestinal lumen through the actions of the ABCG5/ABCG8 for eventual excretion via the faeces or esterified (cholesteryl ester, CE) by the action of Acyl-CoA cholesterol acyltransferase (ACAT) and packaged into chylomicrons for transport into the body. These esterified products are assembled into proximal lipoproteins (PLP) with apolipoprotein (Apo)B in a microsomal TAG transfer protein (MTP)-dependent manner. The PLP is merged with apoA4 to form a nascent lipoprotein (NLP) by core expansion. Resynthesized PL is used in the formation of the polar lipid coat of NLP. Within the Golgi lumen, apoA1 is attached to NLP to form a mature LP- chylomicron (CM). The LP is released from the enterocyte to enter the circulatory system. After entering circulation, CM are hydrolysed by lipoprotein lipase (LPL) to CM remnant (CMR), some of which are recognised by LP receptor-related proteins (black hexagons) and taken up by the liver, thereby contributing to new LP synthesis, i.e. VLDL that are assembled in a process identical to that of CM formation. After secretion into circulation, VLDL deliver lipid and CHOL to extrahepatic tissues and are processed into LDL by LPL. Extrahepatic tissues can also release excess CHOL into HDL particles, with the help of lecithin cholesterol acyltransferase (LCAT), which then returns to the liver for further processing (Adapted from Sheridan, 1988; Gu et al., 2014).

Similarly to lipid digestion, lipid absorption also seem to predominate in the proximal region of the intestine in most fish species studied (Diaz et al., 1997; Olsen et al., 1999; Nordrum et al., 2000; Denstadli et al., 2004; Hernandez-Blazquez et al., 2006). This assumption is supported by isotope and histomorphological studies made along the digestive tract of fish. Histological investigations reporting an accumulation of lipid deposits in the proximal region but not in the distal region of the intestine (Nordrum et al., 2000; Olsen et al., 1999; Denstadli et al., 2004). Studies with radiolabelled FA substrates also recorded higher substrate concentrations in the proximal region than in

the distal region of the intestine (Denstadli et al., 2004). These lipid deposits are generally considered to be temporary storage forms of lipids that have been transported into the enterocytes and are on their way towards the circulatory system (Babin and Vernier, 1989; Olsen et al., 1999).

Due to its hydrophobic nature, transport of lipids through the circulatory system of fish is thought to occur as in mammals, through a dynamic and complex transport system involving lipoproteins (Corraze, 2001; Vance and Adeli, 2008; Xiao et al., 2011). Lipoproteins are lipid complexes with a neutral and hydrophobic lipid core, consisting of TAG and cholesteryl esters, surrounded by a surface monolayer of PL and small amounts of unesterified cholesterol, and specialized proteins: apolipoproteins (Apo) (Xiao et al., 2011). The association of lipids and proteins during lipoprotein synthesis is complex and involves multi-intracellular processes including: resynthesis of TAG and PL; synthesis of various non-exchangeable (apoB) and exchangeable (apoA1, apoA4 and apoCs) apolipoproteins; assembly of lipids and apolipoproteins into lipoproteins; secretion of lipoproteins to circulation (van Greevenbroek and de Bruin 1998; Mu and Hoy 2004; Xiao et al., 2011). These apolipoproteins were shown to be highly expressed both in the liver and the intestine of different fish species (Borges et al., 2013; Kamalan et al., 2013a,b; Gu et al., 2014; Castro et al., 2015b).

Two biosynthetic pathways, as in mammals, seem to be involved on the resynthesis of TAG and PL in fish: the monoacylglycerol (MAG) and the glycerol-3-phosphate (G3P) pathways (Figure 5) (Oxley et al., 2005, 2007; Caballero et al., 2006a). Despite having different precursors (MAG and G3P), both pathways converge at the synthesis of diacylglycerol (DAG). Then, an important branch point occurs, into TAG synthesis, through diacylglycerol acyltransferase (DGAT, EC 2.3.1.20), or into PL production, such as phosphatidylcholine (PC; catalysed by diacylglycerol choline phosphotransferase, DGCPT EC 2.7.8.2) or phosphatidylethanolamine (catalysed by diacylglycerol ethanolamine phosphotransferase, DGEPT EC 2.7.8.1). In fish, it is almost certain that the MAG pathway predominates over the G3P in the biosynthesis of TAG, but the main pathway for PL biosynthesis is not so clear, at least at intestinal level (Oxley et al., 2007; Caballero et al., 2006a).

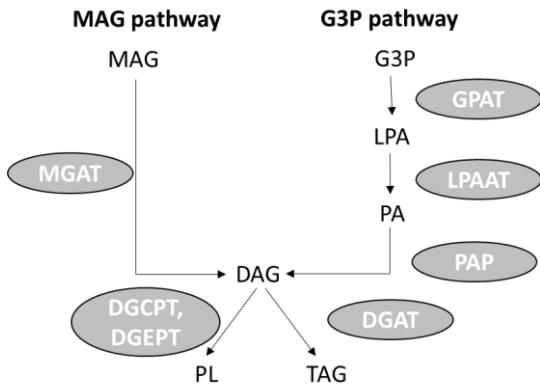


Fig. 5. The two biosynthetic pathways involved on the resynthesis of triacylglycerol (TAG) and phospholipids (PL): the monoacylglycerol (MAG) and the glycerol-3-phosphate (G3P) pathways. The target substrates, products and enzyme markers of the pathways are represented: MAG, G3P, lysophosphatidic acid (LPA) phosphatidic acid (PA), diacylglycerol (DAG), PL, TAG, monoacylglycerol acyltransferase (MGAT), glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT), phosphatidic acid phosphotriesterase (PAP), diacylglycerol choline phosphotransferase (DGCPT), diacylglycerol ethanolamine phosphotransferase (DGEPT) and diacylglycerol acyltransferase (DGAT). (Adapted from Shi and Cheng, 2009).

Lipoprotein particle assembly starts in the endoplasmic reticulum lumen with association of a newly formed apoB molecule (apoB48 in the intestine and apoB100 in the liver) with resynthesised TAG by a specific lipid transfer complex – the heterodimer microsomal TAG transfer protein (MTP). This primordial particle is further lipidated with the addition of core TAG and cholesteryl ester by MTP. Prior to secretion at the basolateral membrane into the circulation, lipoprotein particle acquire additional apolipoproteins (apoA4 and apoA1) and other lipids (mainly PC), which have important functions in the particle assembly or in lipid core expansion, and in the particle export (Figure 4) (Black, 2007; Mansbach II and Gorelick, 2007; Tocher et al., 2008).

Exogenous lipids absorbed by the enterocytes are predominantly integrated into TAG-rich chylomicrons and partly as very low density lipoprotein (VLDL) of intestinal origin and secreted mainly into the lymph but also into the blood. Additionally, absorbed short- and medium-chain FA can be directly used for oxidation in the enterocytes or secreted into circulation (van Greevenbroek and de Bruin 1998; Glencross, 2009).

Besides chylomicrons and VLDL of intestinal origin, studies on the distribution and characterization of plasma lipoproteins in teleost fish have demonstrated the existence, as in mammals, of additional lipoproteins classes, namely VLDL of hepatic origin, intermediate density lipoproteins (IDL), low density lipoproteins (LDL) and high density

lipoproteins (HDL) (Chapman, 1980; Babin and Vernier, 1989; Iijima et al., 1995; Santulli et al., 1996; Tocher, 2003; Nanton et al., 2006).

VLDLs of hepatic origin are TAG-rich lipoproteins formed by mechanisms similar to those described above for chylomicron. However, in VLDLs the lipids incorporated in the particle are predominantly of endogenous source, i.e., lipid synthetized *de novo* (Figure 4).

Lipoproteins are then transported to different tissues for conversion, storage or energy utilization (Sheridan, 1988; Corraze, 2001, Tocher, 2003). Once in circulation, lipoproteins interact with enzymes or cellular receptors and, especially VLDL and chylomicrons, suffer intra-vascular transformations that lead to modifications in TAG, PL, cholesterol and Apos composition and result in the formation of IDL, LDL and HDL by the action of lipoprotein lipase (LPL) and lecithin-cholesterol-acyl-transferase (LCAT) (Figure 4) (Corraze, 2001). HDL is generally the most predominant class of lipoproteins present in the plasma of fish suggesting that it has an important role in the transport of lipids in most fish (Turchini et al., 2009).

General agreement exists that lipid digestion, absorption and transport in fish is essentially similar to that of mammals. However, it is generally assumed that such processes occur at a much slower rate than in mammals, mainly because fish are ectothermic animals (Bakke et al., 2010). Because of their ectothermic nature and the wide range of water temperatures adaptation among species, a high temporal amplitude absorption peaks of dietary lipids have been reported, ranging from 8h to 48h after feeding (Iijima et al., 1985, 1990; Santulli et al., 1988; Babin and Vernier, 1989; Wallaert and Babin, 1994; Geurden et al., 2008; Eliason et al., 2010; Borges et al., 2013; Luo et al., 2014; Castro et al., 2015b). Furthermore, within the same species reared at different temperatures, distinct maximum absorptions peaks were reported. For example in rainbow trout maximum absorptions peaks were reported to occur 10h after feeding at 22°C; 22h after feeding at 14°C; and 48h after feeding at 6°C (Wallaert and Babin, 1994).

1.4.2 Lipid metabolism

Dietary lipid can be used for: structural purposes, mainly for biological membranes assembly; functional purposes, mainly eicosanoids production; deposition in peripheral tissues or liver as energy reserves; energy production (Tocher, 2003; Leaver et al., 2008a). Catabolism of lipids (β -oxidation) takes place mainly in the liver, red muscle and heart, but due to the overall size of the tissue, white muscle is often the major site of β -

oxidation in fish (Nanton et al., 2003). In fish, storage lipids are mainly found in the liver, viscera (especially in marine fish species) or muscle (particularly in salmonids) (Glencross, 2009). Additional to the exogenous (diet) source of lipid, fish lipid deposits are also derived from endogenous sources (*de novo* FA synthesis, or lipogenesis).

It remains to be fully elucidated if lipids are taken up into cells by means of passive uptake mechanism (simple diffusion), and thereby regulated only by lipid physical chemistry, or if are transported into the cytoplasm by protein-mediated transport, such as membrane-bound fatty acid translocase (fat) or fatty acid transport protein (Fatp). In fact, several membrane proteins may be present in the membranes of different tissues of fish (FABPpm, cd36/fat and several types of Fatp). These membrane proteins may dominate in certain cell and tissue types; be regulated by different stimulus; and be responsible for the uptake of different FA over cell membranes (Torstensen et al., 2009). Indeed, Torstensen et al. (2009) reported in Atlantic salmon a tissue-specific expression pattern (liver, heart, red and white muscle and visceral adipose tissue) of genes encoding for Fatp1 and cd36/fat proteins. Inside the cells, transport of FA and other hydrophobic compounds is believed to be carried out as in mammals, by a variety of cytosolic proteins-fatty acid-binding proteins (FABP). In fish, several types of FABPs have been identified (FABP2, FABP3, FABP10, FABP11) and its tissue expression reported (Jordal et al., 2006; Agulleiro et al., 2007; Torstensen et al., 2009; Chen et al., 2012; Venold et al., 2013). Like in mammals, it is generally assumed that due to the diversity of specific FABPs found in different cells and tissues in fish, and its capacity to trigger FA towards different organelles and metabolic pathways, piscines' FABPs fulfill specialized roles in cells where they are expressed (Tocher, 2003; Leaver et al., 2008a; Storch and Thumser, 2010).

The principal pathway of FA catabolism, known as β -oxidation, takes place in two different organelles in the cells, mitochondria and peroxisomes, via completely different set of enzymes (Tocher, 2003). Mitochondrial β -oxidation involves cyclic series of reactions by which FA are sequentially oxidized into acetyl residues in the form of acetyl-CoA. Acetyl-CoA can be used for other metabolic purposes (as previously mentioned in 1.3.2 section) or be further oxidized through the TCA cycle, yielding FADH_2 and NADH, which are transferred to the electron transport chain to generate energy in the form of ATP by oxidative phosphorylation (Figure 3) (Nelson and Cox, 2005). Carnitine palmitoyl transferase- 1 and 2 (CPT1 and CPT2, EC 2.3.1.21) are key enzymes for mitochondrial β -oxidation. Contrarily to mitochondrial β -oxidation, which is linked to electron-transfer and ATP-generating systems, peroxisomal β -oxidation occurs via an oxygen-dependent

oxidase that generates hydrogen peroxide instead of ATP. There is evidence that peroxisomal β -oxidation may act mainly as chain shortening of long-chain FA (such as 22:1 n-9 or DHA) with the shorter chain derivatives generated being then transferred to the mitochondria for oxidation (Henderson, 1996).

FA synthesis (lipogenesis) from non-lipid precursors (glucose and amino acids) in fish is assumed to be basically similar to that operating in mammals (Henderson, 1996). Both liver and adipose tissues are capable of FA synthesis; however, contrary to mammals, liver seems to play a greater role than adipose tissue in FA synthesis in fish (Henderson and Sargent, 1985). Consistent with this view, the activity of lipogenic enzyme is substantially higher in hepatocytes than in adipocytes, which suggest that liver is the main site for lipogenesis in fish, while adipose tissue seems to be better adapted for the uptake and storage of FA (Henderson and Sargent, 1985).

Lipogenesis occurs in the cytosol and requires acetyl-CoA as primary substrate donor and sequential addition of malonyl-CoA units (obtained by carboxylation of acetyl-CoA by acetyl-CoA carboxylase, ACC EC 6.4.1.2), by the fatty acid synthetase (FAS, EC 2.3.1. 38) multienzymes complex. The final products of lipogenesis in the cytosol are 16:0 (palmitic acid) or 18:0 (stearic acid). In this process, a considerable amount of reducing power in the form of NADPH is required. NADPH is supplied by the action of dehydrogenases of the pentose phosphate pathway (principally G6PD) or malic enzyme (ME, EC 1.1.1.40), (Figure 3) (Henderson and Sargent, 1985). Irrespective of nutritional conditions, the activity of these NADPH-forming enzymes is thought to vary between species. Accordingly, in rainbow trout (Walzem et al., 1991), European sea bass (Dias et al., 1998; Castro et al., 2015a), Senegalese sole (Dias et al., 2004) and gilthead sea bream (Menoyo et al., 2004), NADPH reducing equivalents are mainly provided by G6PD. On the other hand, in Atlantic salmon (Menoyo et al., 2003) and white sturgeon (Hung et al., 1989; Fynn-Aikins et al., 1992) NADPH is mainly provided by ME. The process by which acetyl-CoA is converted into FA is reversible, allowing regeneration of metabolites (such as acetyl-CoA, NADH and FADH₂) from deposit lipids via β -oxidation.

The main products formed in lipogenesis, 16:0 and 18:0 FAs, can then be converted into longer and more unsaturated FA by specific fatty acyl desaturases (fad)s and elongases (eloval)s in microsomes. Indeed, fish, as all animals, can produce 16:1 n-7 (palmitoleic acid) and 18:1 n-9 (oleic acid) from 16:0 and 18:0 by the action of a Δ 9 fad (EC 1.14.19.1), sometimes termed stearoylCoA desaturase (Figure 6). The extent to which 16:1 n-7 and, especially, 18:1 n-9 are chain elongated to higher homologs by the

conventional microsomal elongation pathway is not well studied in fish (Sargent et al., 2002). On the other hand, all vertebrates are unable to biosynthesize de novo 18C FAs of the n-3 and n-6 series (18:3 n-3 and 18:2 n-6, respectively) from 18C SFAs or MUFA precursors. The enzymes responsible for these reactions, $\Delta 12$ and $\Delta 15$ fads (marked in white in Figure 6), are apparently only present in primary producers (such as higher plants and photosynthetic microalgae), some invertebrates and other lower animals (Monroig et al., 2013).

As precursors of the physiologically essential LC-PUFA (EPA, DHA, arachidonic acid-ARA), 18:2 n-6 and 18:3 n-3 must be obtained in vertebrates through the diet. Biosynthesis of LC-PUFA from 18:2 n-6 and 18:3 n-3 precursors are achieved by an alternating succession of desaturation and elongation reactions (Figure 6). Synthesis of ARA and EPA, from 18:2 n-6 and 18:3 n-3 precursors, respectively, is accomplished by two desaturation reactions through a $\Delta 6/\Delta 8$ fad (depending upon whether or not 18:2 n-6 or 18:3 n-3 are first elongated) and a $\Delta 5$ fad (Cook and McMaster, 2004; Tocher, 2015). DHA synthesis from EPA can proceed by two further elongation steps, a second $\Delta 6$ desaturation and a peroxisomal chain shortening step; or directly via elongation followed by $\Delta 4$ desaturation (Sprecher, 2000; Sargent et al., 2002; Tocher, 2015).

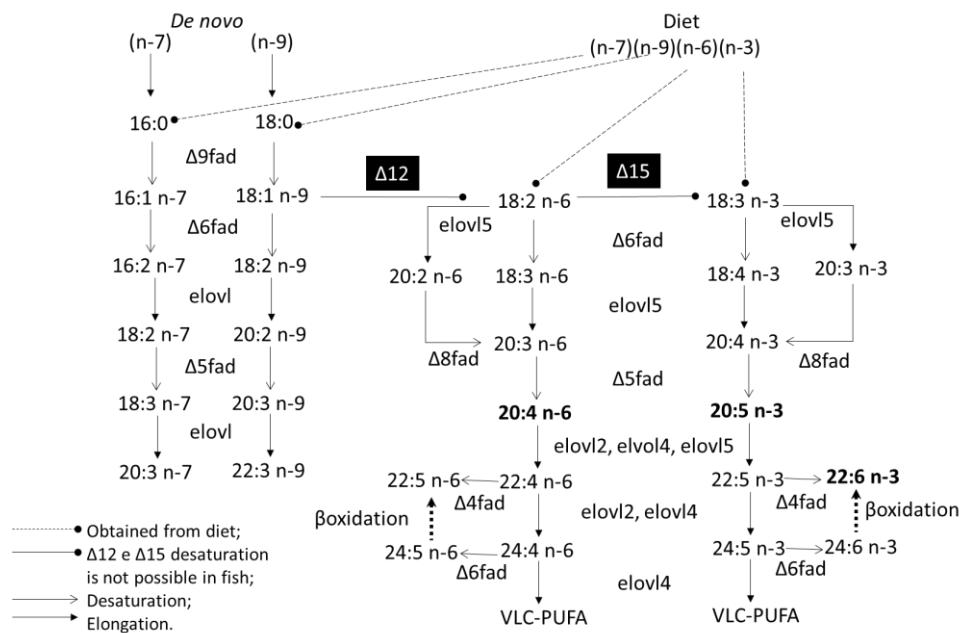


Fig. 6. Pathways of biosynthesis of fatty acid families (n-3, n-6, n-9 and n-7 series) in fish. $\Delta 4$ fad, $\Delta 5$ fad and $\Delta 6$ fad and $\Delta 9$ fad, fatty acyl desaturases; Elov12, Elov14 and Elov15, fatty acid elongases; VLC-PUFA, very long-chain polyunsaturated fatty acids. Adapted from Sargent et al. (2002) and Tocher (2015).

However, as stated before in 1.2 section the ability to convert C18 PUFA (18:3 n-3 and 18:2 n-6) to LC-PUFA varies within species, depending upon the presence and expression of fad and elovl genes (Tocher, 2003, 2010). Evidence suggests that the capabilities of different fish to biosynthesize LC-PUFA are mainly related to the trophic-specific LC-PUFA abundance in which fish evolved. As a consequence, this originated differences among fish species as regards their EPA requirements (Sargent et al., 2002; Vagner and Santigosa, 2011; Castro et al., 2012; Tocher, 2015).

Indeed, freshwater species have essential requirement for C18 FAs (18:2 n-6 and 18:3 n-3), and are capable to convert C18 PUFA into the corresponding LC-PUFA. In contrast, marine species require LC-PUFA as EPA, as they are assumed to have a deficient capacity to bioconvert C18 PUFA into LC-PUFA. Even so, EPA requirements are also dependent on fish developmental stage (larvae, juvenile, adult, or broodstock), environmental conditions (season and temperature, for instance), health and immune status (Turchini et al., 2009).

Several fads and elovls cDNAs have been isolated and functionally characterized from a relatively wide range of freshwater, marine and diadromous fish species (Table 3 and 4). In all freshwater and marine fish species studied so far a cDNA fad with $\Delta 6$ -desaturation functionality has been isolated, but unifunctional or bifunctional enzymes possessing the $\Delta 5$ activity have only been found in diadromous and freshwater species, such as Atlantic salmon, zebrafish and pike silverside (Hastings et al., 2001, 2005, Monroig et al., 2014). In most teleosts, especially marine species, the $\Delta 6$ fad (EC 1.14.19.3) was also demonstrated to have $\Delta 8$ activity (Monroig et al., 2011b).

Table 3 Fatty acyl desaturases (Fad) investigated in fish. The desaturation activities determined for each characterised Fad enzyme are indicated.

Species	Gene name*	Common name	Reported activities	References
<i>Danio rerio</i>		Zebrafish	Δ6, Δ5, Δ8	Hastings et al. (2001) Seiliez et al.(2001); Zheng et al. (2004a)
<i>Oncorhynchus mykiss</i>		Rainbow trout	Δ6, Δ8	Hastings et al. (2005)
<i>Salmo salar</i>	Δ5Fad	Atlantic salmon	Δ5	Zheng et al. (2005a)
<i>S. salar</i>	Δ6Fad_a	Atlantic salmon	Δ6	Monroig et al. (2010a)
<i>S. salar</i>	Δ6Fad_b	Atlantic salmon	Δ6, Δ8	Monroig et al. (2010a)
<i>S. salar</i>	Δ6Fad_c	Atlantic salmon	Δ6, Δ8	Zheng et al. (2004a) Seiliez et al.(2003); Zheng et al. (2004a)
<i>Cyprinus carpio</i>		Common carp	Δ6	Zheng et al. (2004a)
<i>Sparus aurata</i>		Giltthead sea bream	Δ6, Δ8	Not determined
<i>Psetta maxima</i>		Turbot	Δ6, Δ8	Alimuddin et al. (2005)
<i>Oncorhynchus masou</i>	Δ6-like	Masu salmon	Not determined	Alimuddin et al. (2007)
<i>O. masou</i>	Δ5-like	Masu salmon	Δ6, Δ8	Tocher et al. (2006)
<i>Gadus morhua</i>		Atlantic cod	Δ6, Δ8	Zheng et al. (2009)
<i>Rachycentron canadum</i>		Cobia	Δ6, Δ8	Li et al.(2008);Li et al.(2010)
<i>Siganus canaliculatus</i>	Fad1	Rabbitfish	Δ6,Δ5, Δ8	Li et al. (2010)
<i>S. canaliculatus</i>	Fad2	Rabbitfish	Δ4, Δ8	González-Rovira et al. (2009); Santigosa et al. (2011a)
<i>Dicentrarchus labrax</i>		European sea bass	Δ6	Mohd-Yusof et al. (2010)
<i>Lates calcarifer</i>		Asian sea bass/Barramundi	Δ6	Yamamoto et al. (2010)
<i>Nibea mitsukurii</i>		Nibe croaker	Not determined	Jaya-Ram et al. (2011)
<i>Channa striata</i>		Striped snakehead fish	Not determined	Morais et al. (2011a)
<i>Thunnus thynnus</i>		Northern bluefin tuna	Δ6	Sarker et al. (2011)
<i>Pagrus major</i>		Red sea bream	Not determined	Morais et al. (2012a)
<i>Solea senegalensis</i>		Senegalese sole	Δ4	Monroig et al. (2014)
<i>Chiostoma estor</i>		Pike silverside	Δ6,Δ5, Δ4	

*In species in which more than one desaturase has been isolated (*Salmo salar* and *Siganus canaliculatus*), the name of the specific gene is indicated according to published information. Adapted from Monroig et al. (2011a).

Elovl5 (EC 2.3.1.199)-encoding cDNAs have also been isolated and characterized from many species of freshwater and marine environments, while elovl2 (EC 2.3.1.199) cDNAs appear to be lacking in many marine species. The low LC-PUFA biosynthetic ability of marine fish has been explained by the lack of genes encoding for Δ5 Fad and

elovl2, due to evolutionary adaptation to a DHA-rich marine ecosystem (Morais et al., 2009; Monroig et al., 2011a; NRC, 2011). However, the discovery of the bifunctional Δ6/Δ5 Fad in an herbivorous marine fish species, the rabbitfish, and of elovl2 in a carnivorous marine fish species, Senegalese sole, demonstrate that the assumption that marine fish lack such genes cannot be generalized (Li et al., 2010; Morais et al., 2012a). More recently, identification of an elovl4 in marine species, as well as its potential to participate in DHA synthesis in a way similar to elovl2, suggest that this functional capacity of elovl4 may be critical in marine species such as cobia and Atlantic cod that apparently lack ElovL2-like proteins (Morais et al., 2009; Monroig et al., 2011a, c; Xue et al., 2014). Furthermore, the demonstration of a gene encoding a Δ4 Fad in some marine and freshwater species also reveals an alternative pathway and more direct route for DHA synthesis from EPA, at least in some species (Li et al., 2010; Morais et al., 2012a; Monroig et al., 2014).

Table 4 Elongases (Elov1) investigated in fish. The type of Elov1 is determined by amino acid similarities with mammalian orthologues and, in most cases, by functional characterisation analyses.

Species*	Common name	Elongase type	References
<i>Danio rerio</i>	Zebrafish	Elov1	Agaba et al.(2005)
<i>D. rerio</i>	Zebrafish	Elov1 ²	Monroig et al. (2009)
<i>D. rerio</i>	Zebrafish	Elov1 ⁴ (isoforms a and b)	Monroig et al. (2010b)
<i>Salmo salar</i>	Atlantic salmon	Elov1 ⁵ (isoforms a and b)	Agaba et al. (2005); Morais et al. (2009)
<i>S. salar</i>	Atlantic salmon	Elov1 ²	Morais et al. (2009)
<i>S. salar</i>	Atlantic salmon	Elov1 ⁴	Carmona-Antoñanzas et al. (2011)
<i>Oreochromis niloticus</i>	Nile tilapia	Elov1 ⁵	Agaba et al.(2005)
<i>Clarias gariepinus</i>	African catfish	Elov1 ⁵	Agaba et al.(2005)
<i>Sparus aurata</i>	Gilthead sea bream	Elov1 ⁵	Agaba et al.(2005)
<i>Psetta maxima</i>	Turbot	Elov1 ⁵	Agaba et al.(2005)
<i>Gadus morhua</i>	Atlantic cod	Elov1 ⁵	Agaba et al.(2005)
<i>G. morhua</i>	Atlantic cod	Elov1 ^{5*} Elov1 ^{4*} (isoforms c1, c2) and partially (isoforms a,b)	Xue et al. (2014)
<i>G. morhua</i> (elov1 ² -like)	Atlantic cod	not determined	Xue et al. (2014)
<i>Oncorhynchus masou</i>	Musu salmon	Elov1 ^{5*}	Alimuddin et al. (2008)
<i>Rachycentron canadum</i>	Cobia	Elov1 ⁵	Zheng et al. (2009)
<i>R. canadum</i>	Cobia	Elov1 ⁴	Monroig et al.(2011c)
<i>Lates calcarifer</i>	Asian sea bass/ Barramundi	Elov1 ⁵	Mohd-Yusof et al. (2010)
<i>Thunnus maccoyii</i>	Southern bluefin tuna	Elov1 ⁵	Gregory et al. (2010)
<i>Nibea mitsukurii</i>	Nibe croaker	Elov1 ^{5*}	Yamamoto et al. (2010)
<i>Thunnus thynnus</i>	Northern bluefin tuna	Elov1 ⁵	Morais et al. (2011a)
<i>Pagrus major</i>	Red sea bream	Elov1 ^{5*}	Sarker et al. (2011)
<i>Solea senegalensis</i>	Senegalese sole	Elov1 ²	Morais et al. (2012a)
<i>Esox lucius</i>	Northern pike	Elov1 ⁵	Carmona-Antoñanzas et al. (2013)

* Not functionally characterised; Elov1 annotation is based on amino acid sequence homology. Adapted from Monroig et al. (2011a).

The tissue distribution profile of fads and elovls is apparently also different between freshwater and marine species. Accordingly, in a number of studies with marine species fad and elovl transcripts were predominantly expressed in the brain, with much lower expression being detected in the liver and the intestine, and even less in other tissues (Tocher et al., 2006; Zheng et al., 2009; Mohd-Yusof et al., 2010; Xiu et al., 2014); whereas in diadromous and freshwater species the transcript expression levels were highest in the intestine (i.e. pyloric caecum), liver and brain within the tissues tested (Zheng et al., 2005a; Morais et al., 2009). These findings suggest that the biosynthetic machinery for LC-PUFA is probably functional in the brain of most teleost fish, including marine species, to supply the high requirements of this tissue. In salmonids, the high expression levels observed in the liver and intestine, the main tissues responsible for dietary fatty acid uptake, biosynthesis and distribution, may be a result of adaptation to a freshwater environment relatively poor in LC-PUFA.

As stated before in 1.1 section, cholesterol is a vital molecule within the organism due its structural and functional roles (NRC, 2011; Tocher et al., 2008). However, as vertebrates are capable of synthesizing it, cholesterol is not considered to be an essential nutrient (Nelson and Cox, 2005). Cholesterol synthesis occurs mainly in liver and, to a lesser extent, in intestine (Engelking, 2010). It is synthesized from acetyl-CoA in a complex and energy expensive process, involving more than twenty enzymatic reaction steps and requiring 18 acetyl-CoA, 18ATP, 16 NADPH and 4 O₂ molecules per molecule of cholesterol produced (Engelking, 2010). Synthesis takes place in four stages that involve the formation of mevalonate, conversion to isoprenoids, condensation to squalene, and conversion to cholesterol. The first step, reduction of HMG-CoA to mevalonate catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), is the major point of regulation of the cholesterol pathway (Liscum, 2008).

1.4.3 Nutritional regulation of lipid related processes by dietary lipids

A relationship between lipid digestibility of a given lipid source and its FA composition, specifically the degree of unsaturation and the chain length of FA, was previously established (Torstensen et al., 2000; Caballero et al., 2002; Menoyo et al., 2003; Ng et al., 2003; Francis et al., 2007; Martins et al., 2009). Accordingly, in numerous fish species higher apparent FA digestibility was described for LC-PUFA, followed by C18 PUFA, MUFA and SFA; short-chain FA compared to long-chain FA with the same degree of unsaturation (Torstensen et al., 2000; Caballero et al., 2002; Menoyo et al., 2003; Ng et al., 2003; Francis et al., 2007; Martins et al., 2009). Among the potential explanations of

the differences in digestibility of individual FA are the lipolytic enzyme specificities towards different FA and factors related to emulsification and micelle incorporation (Koven et al., 1994; Olsen and Ringo, 1997; Tocher, 2003).

Many studies have also highlighted the importance for lipid digestibility the presence in dietary lipids of non-TAG (such as PL and wax esters), the individual FA concentration and the position of the first double bond in FA (Geurden et al., 2008; Bøgevik et al., 2009). Accordingly, beneficial effects of dietary PL administration on lipid digestibility was recorded in common carp juveniles, probably linked to the emulsifying properties of PL (Geurden et al., 2008). In opposition, diets rich in wax-esters seemed to decrease lipid digestibility in Atlantic salmon (Bøgevik et al., 2009). FA digestibility may also decrease when the position of the first double bond moves from the methyl end of the carbon chain ($n-3 > n-6 > n-9$) (Francis et al., 2007). An increased concentration of individual FA may also decrease their own digestibility and consequently overall lipid digestibility (Caballero et al., 2002; Ng et al., 2004; Hansen et al., 2008). The latter must receive particular attention given the increased trend to use high-lipid formulations in aquafeeds (Bell and Koppe, 2010).

In vitro studies in salmonids demonstrated that dietary lipid source affects intestinal FA absorption and this may also disturb lipid digestibility (Jutfelt et al., 2007; Geurden et al., 2009). Indeed, Jutfelt et al. (2007) observed that dietary FO replacement by sunflower oil led to higher uptake of free FA in Atlantic salmon intestine. In rainbow trout, *in vitro* FA uptake was correlated with *in vivo* FA digestibility, being higher in fish fed a diet with linseed oil than in fish fed FO or rapeseed oil diets (Geurden et al., 2009). It remains uncertain the mechanisms by which dietary FA composition modulates intestinal absorption. It is suggested that in the case of protein mediated transport, a change in FA substrate may change the affinity or expression of membrane transporters to dietary FA. Furthermore, an altered membrane FA composition may also affect FA absorption (Geurden et al., 2009).

In addition to effects on lipid digestibility, dietary lipid source has been reported to affect plasma lipid classes and lipoprotein concentrations in some fish species, as well as the lipid proportion and composition of each lipoprotein. The mechanisms involved in lipid absorption, lipoprotein formation and uptake, lipid transport and uptake and, consequently modulators of the lipid proportion and composition of each lipoprotein in

plasma, were also reported to be regulated by dietary lipid source. Indeed, total plasma cholesterol, LDL- and VLDL- cholesterol, and PL were recorded to decrease with dietary FO replacement by VO (Jordal et al., 2007; Richard et al., 2006a,b; Morais et al., 2011b), while the amounts of plasma protein, TAG, VLDL and LDL remained unaltered (Torstensen et al., 2000, 2004; Jordal et al., 2007; Richard et al., 2006a,b). Contrarily, in gilthead sea bream high dietary replacement of FO by soybean or linseed oil (80%) was reported to increase HDL and LDL cholesterol, and TAG concentration in VLDL (Caballero et al., 2006b). According to Caballero et al. (2006b) this effect might be linked to the high n-3 LC-PUFA content of FO, which is reported to decrease plasma cholesterol level. Concomitantly, the FA composition of Atlantic salmon and gilthead sea bream lipoproteins was reported to reflect the dietary FA profile, with HDL being less influenced by dietary FA composition (Lie et al., 1993; Torstensen et al. 2000, 2004; Caballero et al., 2006b). Although differences in plasma lipid and lipoprotein concentration or composition are linked with the dietary FA profile, plant anti-nutrients, particularly phytosterols, and dietary cholesterol content are also determinant factors (see below).

VO were reported to affect: intestinal TAG and PL biosynthesis; expression of the major proteins associated with chylomicron and VLDL assembly (apoB, apoA4, apoA1 and MTP); uptake of FA by tissues via LPL, lipoprotein receptors and FA transporters activity or expression. An *in vitro* study with gilthead sea bream intestinal microsomes reported that the two pathways (MAG and G3P) involved in TAG and PL biosynthesis, a key step in lipoprotein assembly, were differently modulated depending on the dietary lipid source (Caballero et al., 2006a). Diets with 60 % substitution of FO by rapeseed oil had no effects on PL resynthesis but induced a reduction in TAG resynthesis in both G3P and MAG pathways, whereas an increase in PL synthesis in the two pathways was observed in fish fed diets containing soybean oil replacing 80 % of FO. As consequence, lower TAG in VLDL/chylomicron and in lipoprotein concentration may be expectable with diets rich in rapeseed oil. Likewise, soybean oil rich diets may promote higher production of VLDL/chylomicron which may also contain higher amounts of PL (Caballero et al., 2006a). On the other hand, in Atlantic salmon the total dietary replacement of FO by a VO blend or rapeseed oil induced no changes on the intestinal key enzyme activities involved in TAG and PL biosynthesis (Oxley et al., 2005) and on hepatic DGAT activity, an enzyme involved in the last step of TAG resynthesis (Kjaer et al., 2008). Likewise, in European sea bass diets with 60% FO replaced by a VO blend induced no modifications on the intestinal and hepatic expression of DGAT (Castro et al., 2015b).

The expression of proteins known to be involved in the multistep process of chylomicron and VLDL assembly in the intestine and liver was also proved to be under nutritional regulation. In Atlantic salmon, substitution of FO by rapeseed oil promoted a decrease in the hepatic expression of MTP and ApoA1 (Kjaer et al., 2008a). Contrary to Leaver et al. (2008b), that observed an up-regulation of hepatic transcript levels of ApoB with the replacement of FO with rapeseed oil or linseed oil in Atlantic salmon diets, in other studies in the same species (Kjaer et al., 2008a; Morais et al., 2011b) or in European sea bass (Castro et al., 2015b) no such effect was observed with the replacement of FO by rapeseed oil or VO blend.

In red seabream, diets supplemented with n-3 LC-PUFA increased the hepatic expression of LPL, while diets supplemented with oleic acid had an inverse effect in the adipose tissue (Liang et al., 2002). Total replacement of FO by a VO blend had no effect on LPL activity in visceral adipose tissue, white muscle and liver of rainbow trout (Richard et al., 2006a). On the other hand, while partial substitution of FO by two VO blends induced no impact on LPL activity in the liver and adipose tissues of European sea bass, LPL activity in white muscle decreased in fish fed one of the VO blends (Richard et al., 2006b). Furthermore, complete replacement of FO by soybean oil induced an up-regulation of the hepatic LPL expression but not activity in turbot (Peng et al., 2014). LDL and VLDL receptors and scavenger receptor class B1 are the major lipoproteins receptors assessed in fish (Richard et al., 2006a; Kjaer et al., 2008; Morais et al., 2011b) but only the expression of LDL receptor was demonstrated to be under nutritional regulation by dietary lipid source in rainbow trout (Richard et al., 2006a). In the perivisceral adipose tissue and in the liver LDL receptor gene expression was depressed by dietary inclusion of VO blend (Richard et al., 2006a).

In vitro studies using isolated hepatocytes from Atlantic salmon demonstrated that the uptake efficiency differs among FA (Torstensen and Stubhaug 2004; Stubhaug et al., 2005a). According to Stubhaug et al. (2005a) this probably indicates that transporters have distinct selectivity for different FA. Indeed, by replacing 75% of dietary FO with VO, a general decrease in the uptake capacity of several FA classes (from saturated FA to LC-PUFA) was reported in Atlantic salmon hepatocytes (Stubhaug et al., 2005a). However, the gene expression pattern of FA transport proteins (Fatp and cd36) and of intracellular fatty acid-binding proteins (FABP3, FABP10 and FABP11) in several tissues of Atlantic salmon (liver, red and white muscle, heart and adipose tissues) revealed that only white muscle, adipose tissue and heart were responsive to a complete replacement

of dietary FO by a VO blend (Torstensen et al., 2009). Total replacement of FO by a VO blend decreased the expression of FABP3, FABP11 and Fatp genes in white muscle and of FABP11 or Fatp in adipose tissue, while increased the expression of cd36 in heart and adipose tissue (Torstensen et al., 2009).

Despite the reported decrease of cholesterol concentration in lipoproteins and in plasma with the replacement of FO by VO, regulation of cholesterol metabolism in fish is largely unknown (Kortner et al., 2014). Apart from the general cholesterol deficit typical of most VO diets, it is believed that phytosterols present in most VO may impair cholesterol uptake from the intestinal lumen and thereby further decrease plasma cholesterol levels. In Atlantic salmon fed plant based diets, lower expression of hepatic npc1l1 was reported in rapeseed oil-fed fish than in fish fed the other VO types (soybean and olive oils) (Lilland et al., 2013). Npc1l1 is known to be important for sterol uptake, and the high phytosterol content of rapeseed oil-based diet may have negatively affected npc1l1 thus leading to lower cholesterol uptake. However, available studies in Atlantic salmon and European sea bass suggest that fish fed VO diets apparently respond to a deficit in absorbed cholesterol by up-regulating genes involved in cholesterol biosynthesis, this way fully compensating the cholesterol deficit at tissue level (Leaver et al., 2008b; Castro et al., 2015a).

In vitro studies with Atlantic salmon hepatocytes have also demonstrated that lipid catabolism seems to be regulated at the level of FA transport and uptake by the cells rather than by regulation of mitochondrial and peroxisomal enzymes involved in β oxidation (Torstensen and Stubhaug, 2004). Indeed, in Atlantic salmon white muscle downregulation of β -oxidation-related genes were positively correlated to the expression of FABP3 and FABP11 genes (Torstensen et al., 2009). Even so, contradictory results have been reported on the effects of dietary VO on lipid β -oxidation in fish. With FO substitution by VO, either an increase (Menoyo et al., 2004, 2006; Stubhaug et al., 2005a), a decrease (Torstensen et al., 2009) or lack of effects (Torstensen et al., 2000; Stubhaug et al., 2005b, 2006, 2007; Morais et al., 2011b) were observed in the liver or muscle of different species. Furthermore, replacing FO with either rapeseed oil (Stubhaug et al., 2005b) or a VO blend (Stubhaug et al. 2006, 2007) in the diet for Atlantic salmon induced no major effects on total β -oxidation capacity in the muscle (red and white) and liver. However, modifications of dietary FA composition led to a shift in substrate preference for β -oxidation from EPA and DHA that were catabolized in fish fed FO diets, towards shorter-chain FA (18:1 n-9) in fish fed VO diets (Stubhaug et al., 2007).

Regarding lipogenesis, it is known that dietary lipid level is well correlated with the inhibition of hepatic FAS and G6PD activities (Bonamusa et al., 1992; Arnesen et al., 1993; Shikata and Shimeno, 1994; Alvarez et al. 1998, 2000; Figueiredo-Silva et al., 2010). However, the regulation of lipogenesis by different types of FA or lipid sources is still not clear.

Indeed, Alvarez et al. (2000) showed *in vitro*, using of rainbow trout hepatocytes, that EPA and DHA induced a higher reduction of lipogenic enzyme activities than linolenic acid. Moreover, while DHA strongly inhibited FAS, EPA strongly inhibited G6PD and acetyl-COA carboxylase. *In vivo*, FO replacement by VO was reported to had no effects (Regost et al., 2003; Richard et al., 2006a,b; Borges et al., 2014a; Castro et al. 2015a), increase (Morais et al. 2011c; Peng et al. 2014), or reduce (Menoyo et al., 2004; Jordal et al., 2007; Panserat et al., 2008) the expression or the activity of hepatic FAS in several fish species. Alongside, lack of effect (Regost et al., 2003; Richard et al., 2006a,b) or an increase (Menoyo et al., 2004; Jordal et al., 2007; Peng et al., 2014; Betancor et al., 2015) in the lipid content of liver or muscle was reported with dietary FO substitution by VO.

The degree of nutritional regulation of LC-PUFA biosynthesis seems to reflect the differences between diadromous and freshwater species and marine species in EFA requirements. Accordingly, the expression of fad and elovl genes (such as Δ5 fad, Δ6 fad, elovl5 and elovl2) and activity of LC-PUFA synthesis enzymes in diadromous and freshwater species were reported to be up-regulated in fish fed VO-based diets (Leaver et al., 2008b; Morais et al., 2009; Torstensen and Tocher, 2010; Vagner and Santigosa, 2011; Zheng et al. 2004b, 2005b; Torstensen and Tocher, 2010).

Contrarily to diadromous and freshwater fish species, VO administration induced small or no effects on LC-PUFA synthesis activities in hepatocytes and enterocytes of marine fish (Mourente and Dick, 2002; Mourente et al., 2005a; Almaida-Pagán et al., 2007). Furthermore, conflicting data regarding the effects of dietary VO on the expression of Δ6 fad and other fad and elovl genes in several marine fish species have also been reported (Seiliez et al., 2003; González-Rovira et al., 2009; Castro et al., 2015a,b; Tocher et al., 2006; Morais et al., 2012a,b; Borges et al., 2014a; Xue et al., 2014). It is not yet clear if it is the reduction of product availability, the increase of substrate supply, their role as ligands of key transcription factors (such as sterol regulatory element binding proteins, SREBP; and peroxisome proliferator-activated receptor, PPAR), or all factors simultaneously, that regulate LC-PUFA biosynthesis (Vagner and Santigosa,

2011; Tocher, 2015). Nonetheless, nutritional modulation of LC-PUFA biosynthesis whether freshwater, diadromous, or marine fish does not seem enough for enhancing LC-PUFA synthesis at appreciable levels, as FA composition of all fish predominantly reflect dietary FA composition (Tocher, 2015).

1.5 Carbohydrate and lipid interaction

The current knowledge on interactions between dietary lipids and carbohydrates in processes such as digestion, absorption, and metabolism that take place in fish is still limited. There are, however, evidences that these macronutrients have the ability to interact to each other and regulate metabolic pathways were they are not directly involved.

Indeed, dietary lipid content and lipid source have been reported to modulate starch digestibility, possibly because lipids may interfere with the velocity of nutrients transit through the digestive tract. Thus, high lipid diets were reported to reduce starch digestibility in Atlantic salmon (Grisdale-Helland and Helland, 1997) while replacement of FO, rich in LC-PUFA, by medium chain TAG was reported to improve starch digestibility (Nordrum et al., 2000).

Furthermore, dietary carbohydrates were reported to interfere with the expression of proteins associated with chylomicron/VLDL assembly (Kamalan et al., 2013a,b). For instance, in rainbow trout fed FO-based diets, carbohydrate intake induced an increase of midgut ApoA1 and of hindgut ApoA1 and ApoB transcript levels (Kamalan et al., 2013a). Likewise, in rainbow trout fed VO-based diets, carbohydrate intake increased intestinal transcript levels of MTP, ApoA1 and ApoA4, along with higher serum cholesterol concentrations (Kamalan et al., 2013b).

Increasing dietary carbohydrate was also reported to increase lipid deposition in the liver, muscle, or whole body, in a number of fish species (Brauge et al., 1994; Ekmann et al., 2013; Castro et al., 2015a). Accordingly, the hepatic expression or the activity of enzymes involved in lipogenesis (FAS) (Likimani and Wilson, 1982; Gélineau et al., 2001) and in NADPH production, mainly G6PD (Hilton and Atkinson, 1982; Likimani and Wilson, 1982; Shimeno et al., 1993; Gélineau et al., 2001; Dias et al., 2004; Fernández, et al., 2007; Kamalam et al., 2012; Castro et al., 2015a), were reported to respond to carbohydrate intake in several fish species. On the other hand, while a similar nutritional regulation by dietary carbohydrates was observed in the adipose tissue of channel catfish and gilthead sea bream (Likimani and Wilson, 1982; Bou et al., 2014) that was

not the case in rainbow trout (Barroso et al., 2001; Figueiredo-Silva et al., 2012; Kamalam et al., 2012).

Conversely, a regulation of carbohydrate utilization and metabolism by dietary lipids was also reported. A large body of evidence suggest that lipid digestibility was negatively affected by dietary starch levels (Hemre et al., 1995; Storebakken et al., 1998; Fountoulaki et al., 2005). This may be due to an increased intestinal transit, as undigested starch may behave like non-starch polysaccharides or fiber (Hemre et al., 1995; Storebakken et al. 1998; Fountoulaki et al., 2005). Hence, lipases action and substrate availability may be hampered due to the viscous nature of undigested starch; or to starch binding to digestive enzymes and bile acids; or to bile acids desconjugation by starch (Storebakken et al., 1998; Fountoulaki et al., 2005).

Further, hyperglycaemia also seems to be influenced by a metabolic interaction between dietary lipids and carbohydrates. For instance, in rainbow trout fed carbohydrate-rich diets and high dietary lipids levels a prolonged hyperglycaemia and reduced plasma glucose clearance was observed in response to glucose tolerance and insulin tolerance tests (Figueiredo-Silva et al., 2012). This glucose intolerance was rapidly reversed by reducing dietary lipid content. In Senegalese sole, fish fed a high fat/low carbohydrate diet presented higher plasma glucose levels than fish fed a low fat/high carbohydrate diet, suggesting an impaired glucose regulation due to dietary lipid (Borges et al., 2014b). Furthermore, glucose and lipid tolerance tests in rainbow trout challenged with single FA (oleic acid or octanoic acid) suggest that post-prandial changes in glycaemia were mainly related to interactions between FA and glucose rather than to FA alone (Librán-Pérez et al., 2013).

The increase of dietary lipid supplied as FO or as a mixture of FO and VO was also reported to increase the gluconeogenic (G6Pase expression or activity) or to reduce the glycolytic (GK activity) potential in the liver of rainbow trout (Panserat et al., 2002b; Figueiredo-Silva et al., 2012). A lower glycolytic potential (PK activity) was also observed in rainbow trout liver after receiving a single load of oleic acid or of octanoic acid, respectively a long-chain and a medium chain FA (Librán-Pérez et al., 2013).

Furthermore, hepatic gluconeogenesis was differently regulated by these single FAs. Whereas an intraperitoneal injection of oleic acid increased FBPase activity, an octanoic acid injection decreased FBPase activity (Librán-Pérez et al., 2013). Increase of hepatic glycogen and decrease of GPase activity were also reported in rainbow trout fed high fat diet or an oleic acid load (Figueiredo-Silva et al., 2012; Librán-Pérez et al., 2013).

The above mentioned studies highlight the importance of studying metabolic interactions between dietary macronutrients. This deserves particular attention in the context of sustainable aquafeeds, as increased incorporation of plant feedstuffs in aquafeeds at the expense of fisheries-related feedstuffs will increase the dietary content of carbohydrates and VO. Also of practical interest in this context, is the optimization of the LC-PUFA and cholesterol biosynthesis pathways. Induction of these pathways may ultimately enable fish to maintain tissue n-3 LC-PUFA levels and cholesterol body pools.

New insights on the transcriptional regulation of LC-PUFA biosynthetic pathway by dietary carbohydrate were recently obtained in salmonids, where fads and elovls were shown to be up-regulated by dietary carbohydrates (Seiliez et al., 2001; Kamalam et al., 2013b). This would be of practical importance for marine fish, which as pointed out before have specific requirements of n-3 LC-PUFA, as they are not able to elongate efficiently C18 FA to n-3 LC-PUFA. Nevertheless, to date no information exists on the effects of VO-carbohydrate interaction in LC-PUFA biosynthetic pathway in marine fish species. Therefore, this thesis aimed to gain further knowledge on the impact of dietary VO-carbohydrate interaction on mechanisms involved in LC-PUFA metabolism in marine fish species.

1.6 Oxidative stress

Fish, like all aerobic organisms continuously generate free radicals and reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radical ($\cdot OH$), and reactive nitrogen species (RNS) such as nitric oxide (NO), peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2), as natural by-products of reactions involving oxygen in several cellular components (mitochondria, peroxisomes, cytochrome P450, etc). Such molecules may also have significant roles in cell signaling and homeostasis (Storey, 1996; Halliwell and Gutteridge, 2015; Ozcan and Ogun, 2015).

One of the major endogenous sources of ROS is the mitochondrial electron-transfer chain. The electron-transfer chain comprises an enzymatic sequence of electron donors and acceptors that through the flow of electrons between donor and acceptor generates a proton gradient that is used to supply energy for the synthesis of ATP (Nelson and Cox, 2005). Molecular oxygen (O_2) is the terminal electron acceptor of this chain, which is ultimately reduced to water during the process of oxidative phosphorylation. Under

normal conditions, leakage of electrons from the electron transfer chain directly to oxygen results in the formation of 1-2% superoxide radical ($O_2^{-\cdot}$), which is then reduced to hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) (Storey, 1996; Ozcan and Ogun, 2015) (Figure 7). Contrarily to superoxide radical and hydrogen peroxide, hydroxyl radical ($\cdot OH$) is highly reactive and is the main responsible for numerous forms of damage to cellular macromolecules (Storey, 1996).

Much of the hydroxyl radical is generated *in vivo* from the reduction of H_2O_2 by superoxide radical (Haber-Weiss reaction). This is, in fact, a two-step process catalyzed by transition metals (mainly Fe^{3+}) and involving the Fenton reaction (Figure 7). Other important activated oxygen species that is exceptionally reactive is the singlet oxygen (1O_2), that can be generated either enzymatically or non-enzymatically by photosensitization, in the presence of O_2 and visible light (Halliwell and Chirico, 1993; Sargent et al., 2002).

In addition to mitochondrial electron transport and photosensitization, other sources of endogenous ROS production include the electron transfer chain of microsomes, the respiratory burst associated with phagocytosis by leukocytes, and the activities of enzymes such as xanthine oxidase, tryptophan dioxygenase, diamine oxidase or prostaglandin synthase (Sargent et al., 2002; Mourente et al., 2007a; Ozcan and Ogun, 2015). In addition, in response to an inflammatory stimulus RNS are also produced in macrophages, neutrophils and dendritic cells, denoting a particular role of RNS in the immune response (Ozcan and Ogun, 2015). Exogenous factors, such as ionizing and non-ionizing radiation, xenobiotics, ozone, oxygen concentration (hypo- and hyperoxia), nutrition and environmental conditions (temperature, salinity, etc.) can also contribute to ROS and RNS production (Noori, 2012; Mehta and Gowder, 2015).

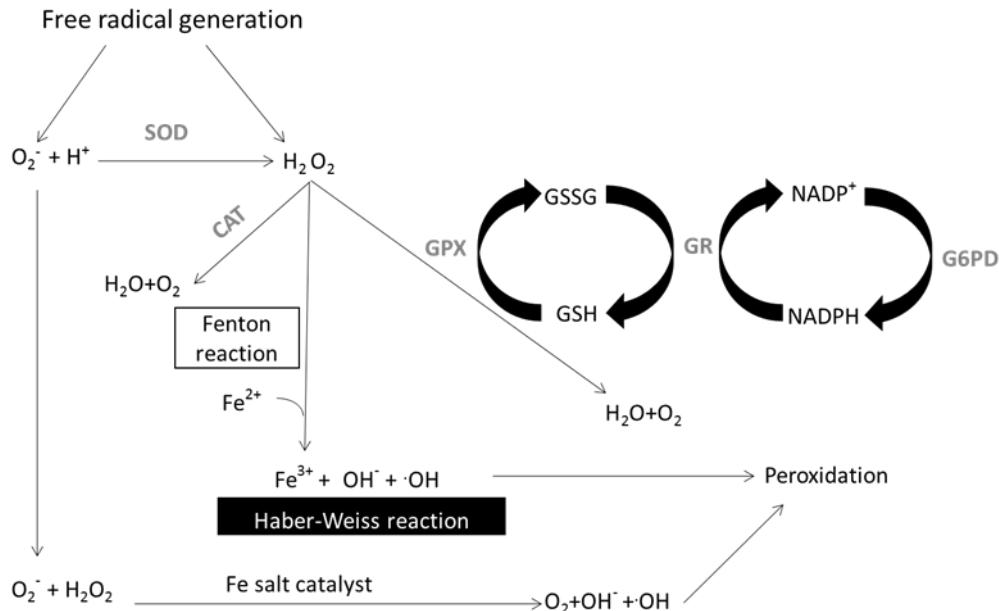


Fig. 7. Summary of the pathways for the generation of reactive oxygen species and of the actions of some of the enzymes involved in antioxidant defences in the cell. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD) reduced glutathione (GSH), oxidized glutathione (GSSG). Adapted from Storey et al. (1996).

Since these by-products of metabolism may harm biological molecules (lipids, proteins, carbohydrates, DNA) as well as macromolecular assemblages (biological membranes), aerobic animals have evolved very effective antioxidant defence systems (Halliwell and Gutteridge, 2015). According to Halliwell and Gutteridge (1990), the term antioxidant comprises compounds, both of non-enzymatic and enzymatic nature that, when present at low concentrations compared to those of an oxidizable substrate, are able to delay or inhibit the oxidation of that substrate. Regardless of localization (cytosol, mitochondria, cell membranes), antioxidants may exert their action by different mechanisms (breaking the chain of an initiated sequence, scavenging or decreasing local ROS and RNS concentration) and even at different stages of the oxidation process (Mehta and Gowder, 2015).

Enzymes involved in antioxidant defence have been detected in most fish species and include the copper/zinc-dependent (cytosolic) and manganese-dependent (mitochondrial) superoxide dismutase (SODs; EC 1.15.1.1). SOD catalyzes the dismutation of superoxide radicals to molecular oxygen and hydrogen peroxide and is thus the first enzyme responding to oxygen radicals, preventing the initialization of the

chain reaction triggered by superoxide radical. The hydrogen peroxide formed is then degraded by peroxisomal and mitochondrial catalase (CAT; EC 1.11.1.6), which convert it to molecular oxygen and water. Hydrogen peroxide may also be degraded by cytosolic and mitochondrial glutathione peroxidase (GPX; EC 1.11.1.19) which, by a glutathione-dependent reaction, reduces both hydrogen peroxide and organic peroxides to water and the corresponding alcohols, respectively (Storey, 1996; Morales et al., 2004; Pérez-Jiménez et al., 2009; Halliwell and Gutteridge, 2015). There are four main GPX isoenzymes: cytosolic (GPX1), gastrointestinal (GPX2), plasmatic (GPX3) and phospholipid hydroperoxidase (GPX4). Other associated enzymes, including glutathione reductase (GR) and G6PD, play a crucial role in supporting the proper functioning of the primary antioxidant enzyme activities (Mourente et al., 2002; Morales et al., 2004; Mehta and Gowder, 2015). GR regenerates reduced glutathione (GSH), which is the substrate for GPX, from oxidized glutathione (GSSG). G6PD generates the NADPH that is crucial for the normal functioning of CAT, GPX and GR (Storey, 1996; Morales et al., 2004; Halliwell and Gutteridge, 2015).

Together with these enzymes, low-molecular-weight antioxidants that are endogenously produced (NADH/NADPH, GSH, protein sulphydryl groups) have also been detected in antioxidant defences in fish. Some of these compounds are provided by the diet, namely nicotinic acid, that is required for the synthesis of NADH/NADPH, and sulfur- and non-sulfur amino acids (cysteine, glutamate, glycine) for the synthesis of GSH. Other exogenous dietary micronutrients (vitamins C and E, and selenium), amino acids (taurine, methionine) and bioactive compounds of plant origin (phenolic and polyphenolic compounds) play also important roles in antioxidant defence (Storey, 1996; Martínez-Álvarez et al., 2005; Noori, 2012; Pérez-Jiménez et al., 2012; Bañuelos-Vargas et al., 2014; Mehta and Gowder, 2015).

This antioxidant system, comprising both enzymatic and non-enzymatic mechanisms, is normally considered the primary defence of an organism to an oxidative stress and is mainly preventive. Secondary defence mechanisms complement the primary defence and include lipolytic (lipases), proteolytic (peptidases or proteases) and other enzymes (DNA repair enzymes, polymerase, ligases, phospholipases, nucleases) that are responsible for the excision and repair of damaged cellular components once they occur, therefore avoiding alterations in cellular metabolism or apoptosis (Halliwell and Gutteridge, 2015).

Under normal physiological conditions, ROS and RNS production is closely matched by antioxidant responses (Halliwell and Gutteridge, 2015). In addition, at low concentrations, these reactive species may be beneficial or even indispensable in defence against microorganisms, by contributing to the phagocytic bactericidal activity. However, under several situations (such as changes in nutritional or environmental factors) reactive species generation may exceed that of antioxidant defences capacity and lead to oxidative stress and, consequently, to an impairment of the animal's health status (Tocher et al., 2002; Morales et al., 2004; Puangkaew et al., 2005; Grim et al., 2010; Halliwell and Gutteridge, 2015).

As stated above the detrimental effects of oxidative stress include oxidative damage to molecules of great biological importance, including proteins, DNA, carbohydrates and lipids (Halliwell and Gutteridge, 2015). Briefly, at protein level, reactive species attack can induce amino acids modification, such as oxidation of sulfhydryl groups, leading to conformational changes, altered enzymatic activity, crosslinking, peptide bond cleavage, carbohydrate modification in glycoproteins, loss of metals in metalloproteins, altered antigenicity, and increased proteolytic susceptibility. The occurrence of strand breaks and base modifications (leading to point mutations) may be a consequence of reactive species attack to DNA (Storey, 1996). Regarding lipids, particular attention has been devoted to lipid peroxidation, an autocatalytic process initiated by reactive species attack that results in deterioration of PUFA. This is particularly important in fish which are PUFA-rich organisms, and PUFA are very prone to suffer lipid peroxidation (Sargent et al., 2002; Mourente et al., 2007a). Furthermore, the resultant damage to PUFA can decrease membrane fluidity or structure, increase membrane permeability to normally impermeable substances, and inactivate membrane-bound enzymes, with potential pathological effects on cells and tissues (Sargent et al., 2002). Indeed, *in vivo* lipid peroxidation caused by reactive species has been linked to a variety of disorders reported in fish, including jaundice, liver degeneration, hemolysis, anaemia or skeletal alterations (Sargent et al., 2002; Mourente et al., 2007a).

Briefly, the process of lipid peroxidation proceeds by a chain-reaction whereby a single radical species (such as hydroxyl radical) extracts a hydrogen atom from a methylene group of a FA, resulting in the formation of an unstable lipid radical. This lipid radical can have several fates but the most likely in aerobic cells is to suffer molecular rearrangement, followed by reaction with O₂ to give a peroxy radical. This peroxy radical can then react with the methylene group of another FA and so exponentially propagate the chain reaction of lipid peroxidation (Figure 8). The length of the propagation chain

will depend on the amount of PUFA available for oxidation, the presence of chain-breaking antioxidants and other peroxy radical radicals. By reacting with other peroxy radicals or by abstracting an easily-donatable hydrogen atom from antioxidants, the lipid peroxidation chain is interrupted by the formation of non-radical products, lipid hydroperoxides. Antioxidants can also prevent lipid peroxidation by acting as quenchers of singlet oxygen (Sargent et al., 2002; Mourente et al., 2007a; Mehta and Gowder, 2015). These lipid hydroperoxides can eventually undergo reductive degradation to alkoxy and hydroxyl radicals, and the alkoxy radical can be converted to secondary products, including aldehydes, ketones and alcohols, which may themselves either diminish or enhance cytotoxic potential (Sargent et al., 2002; Mourente et al., 2007a). Malonyldialdehyde is one of the final products of lipid peroxidation and is usually used as marker for oxidative stress, being commonly assayed with thiobarbituric acid (Halliwell and Chirico, 1993).

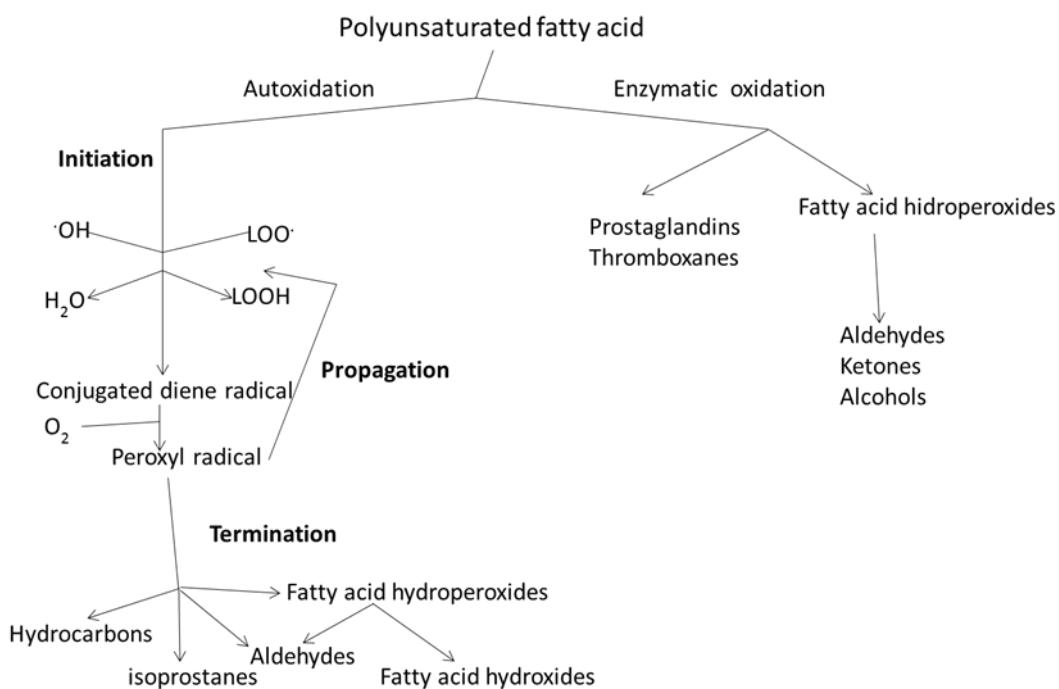


Fig. 8. Mechanisms of lipid peroxidation. Adapted from Sargent et al. (2002).

1.6.1 Nutritional regulation of fish oxidative status

Knowledge on the impact of nutritional factors on fish oxidative status is still limited and sometimes contradictory. There is growing evidence that inclusion of high levels of LC-PUFA in the diets, due to their high degree of unsaturation, induce lipid oxidative damage in the muscle of rainbow trout and European sea bass (Álvarez et al., 1998; Luo et al.,

2012). On the other hand, dietary substitution of FO by single VO at different inclusion levels resulted in decreased susceptibility to lipid peroxidation in the liver of turbot, grouper and black seabream (Stéphan et al., 1995; Lin and Shiau, 2007; Peng et al., 2008) and in the muscle of turbot, gilthead sea bream, Japanese sea bass, and tilapia (Stéphan et al., 1995; Menoyo et al., 2004; Gao et al., 2012; Ng et al., 2013). However, there were no reported differences in hepatic lipid peroxidation of Japanese sea bass, Atlantic cod and European sea bass (Gao et al., 2012; Kjaer et al., 2014; Castro et al., 2015c) and in the muscle of Atlantic salmon (Østbye et al. 2011) when FO was replaced by VO in the diets. Although dietary FA were reported to affect tissue lipid peroxidation susceptibility in fish, their role in the regulation of enzymatic and non-enzymatic antioxidant defence mechanisms is not clearly understood, as studies simultaneously assessing enzymatic and non-enzymatic antioxidant responses and lipid peroxidation damages are still scarce (Østbye et al. 2011; Kjaer et al. 2014; Castro et al. 2015c).

The potential effects of dietary carbohydrate on fish oxidative status has also received limited attention. However, it seems that dietary carbohydrates protect tissues against oxidative damage (Sagone et al., 1983; Álvarez et al., 1999; Lygren and Hemre, 2001; Peréz-Jiménez et al., 2009; Castro et al., 2015c). This possibly occurs through: increased activity of the pentose phosphate pathway (G6PD) to generate the NADPH, which is required for the activity of oxidative stress enzymes; the decrease in the dietary lipid levels; or due to the nature of the glucose molecule, which can scavenge ROS by itself. Indeed low susceptibility to lipid peroxidation was observed in muscle of common dentex (Peréz-Jiménez et al., 2009), rainbow trout and European sea bass (Álvarez et al., 1999) fed carbohydrate-rich diets. Recently, we also observed that the intake of carbohydrates was followed by an increase of reduced GSH concentration, GR and G6PD activities, and a decrease of lipid peroxidation in the liver of European sea bass (Castro et al., 2015c).

Additionally, most studies on macronutrient role in oxidative status focused on liver and muscle, the target tissues involved in lipid deposition. However, the intestine, being an organ with high cell turnover, is also highly susceptible to oxidative stress and an increasing number of studies indicate that intestinal oxidative status may be affected by changes in dietary macronutrients composition (Olsvik et al., 2007; Gao et al., 2012; Morais el at., 2012c; Castro et al., 2015c). Indeed, replacement of FO by palm oil or by a VO blend was reported to decrease the intestinal lipid peroxidation in Japanese sea

bass and European sea bass, respectively (Gao et al., 2012; Castro et al., 2015c). Alongside, increased GPX and GR activities was observed in the intestine of European sea bass fed VO based diets (Castro et al., 2015c). Transcriptional and proteomic studies have also evidenced that intestinal oxidative status may be affected by changes in dietary lipid source (Olsvik et al., 2007; Morais et al., 2012). Accordingly, Morais et al. (2012c) reported in Atlantic salmon that dietary FO substitution by VO led to an up-regulation of transcripts and proteins involved in detoxification and protection from oxidative stress, namely of CAT and selenoprotein, as well as haemopexin-like protein and peroxiredoxin-1 proteins. On the contrary, also in Atlantic salmon, dietary FO replacement by VO decreased the transcription levels of glutathione S-transferase and GR (Olsvik et al., 2007).

To the best of our knowledge, the impact of dietary carbohydrate content on intestinal oxidative status of fish was only evaluated in European sea bass (this thesis) and data indicate that intestine was not responsive to dietary carbohydrates (Castro et al., 2015c).

1.7 Fish species characterisation

European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are the main marine fish species produced in aquaculture of Southern Europe and other countries of the Mediterranean region, where over 150 thousand tonnes of each species were produced in 2014, mainly in Greece, Turkey, Spain and Italy (Table 5) (FEAP, 2015). Although the industry has been suffering from price decreases in the last few years, both species are still the most commercially valuable species in the region and are very much appreciated by consumers. The price of farmed medium-sized fish is around 5 to 8€ per kg (estimates prices for 2011) and fish are mostly sold with a commercial size of 200 to 800 g as whole fish, ungutted, fresh or chilled (EUMOFA, 2014).

Table 5 Total European production (including EU and non-EU countries) of European sea bass and gilthead sea bream in 2014.

Country	Production in 2014 (tonnes)	
	European sea bass	Gilthead sea bream
Greece	42000	71000
Turkey	74653	41873
Spain	17376	16230
Italy	6500	8200
Croatia	3500	3640
Cyprus	1817	2919
France	2021	1105
Portugal	500	1500
Total	148367	146467

Adapted from FEAP, 2015.

Before the development of reliable mass production techniques during the late 1960's, both species were traditionally cultivated in coastal lagoons and tidal reservoirs, under extensive or semi-intensive polyculture conditions. Today, large-scale production of these species is mainly farmed in intensive cages, with only a few semi-intensive farms remaining, mainly in some parts of southern Europe, including in Portugal and Spain. In the latter case, confined areas of coastal lagoons or coastal ponds are generally stocked with fry from commercial hatcheries and fed with pellets (FAO, 2015c,d).

1.7.1 Biology of the species

European sea bass and gilthead sea bream are marine teleosts belonging to the Moronidae and Sparidae families, respectively (Figure 9). European sea bass is found in the coastal area from Norway to Morocco, Canary Islands and Senegal, as well as in the Mediterranean and Black Sea. Gilthead sea bream is also found in the Mediterranean and along the Eastern Atlantic Sea, from British Isles to Cape Verde and around Canary Islands, being rare in the Black Sea.

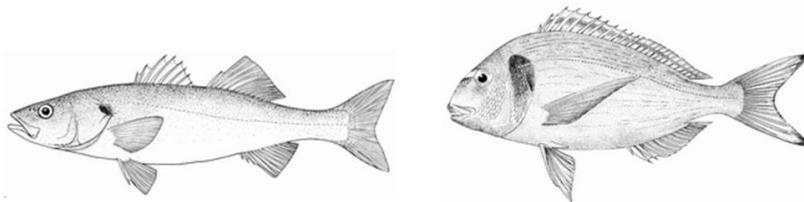


Fig. 9. European sea bass (*Dicentrarchus labrax*), on the left; gilthead sea bream (*Sparus aurata*) on the right. Adapted from FAO (2015c,d).

Both species are benthopelagic (demersal behaviour) inhabiting shallow waters as well as various kinds of bottoms of coastal areas up to 100-150 m depth. Being eurythermic (2/5-32°C) and euryhaline (3‰-to full strength sea water) species, they are recurrent in coastal inshore waters, estuaries, brackish water lagoons and, occasionally, in rivers. European sea bass is a carnivorous species belonging to the trophic level 3.8 (FishBase) that typically feeds on small fish, prawns, crabs and cuttlefish. Gilthead sea bream is also a carnivorous species, but with a lower trophic level 3.3–3.5 (FishBase), that feeds preferentially on shellfish (mussels and oysters), crustaceous, fish and sometimes algae.

European sea bass is a gonochoric species and sexual maturity generally occurs at an age of 2 to 4 years in the Mediterranean populations, while in the Atlantic populations sexual maturity occurs later (males between 4-7 years of age, and 30 to 40 cm of length; and females between 5-8 years of age, with 36-46 cm of length). Spawning season occurs just once a year, mostly in winter for the Mediterranean population (December to March), extending up to June for the Atlantic populations.

Gilthead sea bream is a protandric hermaphrodite species, being functional male until the age of sexual maturity is typically reached (at 2 years, 20-30 cm) and, at 2-3 years become females (33-40 cm). The spawning season takes place typically in the open sea between October and December, with sequenced spawning during the whole period (FAO, 2015c,d).

The current success of European sea bass and gilthead sea bream farming was greatly supported by considerable amount of scientific experiments conducted in the laboratory or in pilot or commercial facilities, and published over the years. That contributed significantly for the definition of optimal environmental conditions, control of all production phases, cultivation techniques and nutritional requirements of the species. As the topic of this thesis is related to nutrition, we will describe below in more detail the current knowledge on the nutritional requirements of these species.

1.7.2 Nutritional requirements

Reflecting their carnivorous nature, the protein requirements of both species are high (>40%) (Oliva-Teles, 2000). Many studies focused on defining the minimum percentage of dietary protein required to obtain satisfactory growth rates for each species and thus reduce feed production costs and environmental impact related to N excretion. However, discrepant results for the optimum protein level were reported, possibly related with differences in fish size, feedstuff composition and protein to energy ratio of the experimental diets used. For European sea bass juveniles the optimum level of dietary protein was first established as ranging from 52 to 60% (Alliot et al., 1974; Metailler et al., 1981). Later studies demonstrated however that dietary protein levels could be decreased to 48-54% (Hidalgo and Alliot, 1988; Ballestrazzi et al., 1994; Peres and Oliva-Teles, 1999a) or even lower values 43-45% (Pérez et al., 1997; Dias et al., 1998). In gilthead sea bream juveniles the optimum level of dietary protein was estimated to be 40% using semi-purified diets (Sabaut and Luquet, 1973) and 45-46% with practical diets (Santinha et al., 1996; Vergara et al., 1996).

Quantitative data for amino acid requirements of European sea bass and gilthead sea bream, estimated based on dose-response (Luquet and Sabaut, 1974; Tibaldi and Lanari, 1991; Tibaldi et al., 1993, 1994; Tibaldi and Tulli, 1999; Marcouli et al., 2006), is only available for arginine, lysine, methionine and cysteine, tryptophan, and threonine (Table 6). For other amino acids, indirect estimations were made based on the ideal protein (Kaushik, 1998a; Peres and Oliva-Teles, 2009) and amino acid deletion (Peres and Oliva-Teles, 2009) methods (Table 6).

Table 6 Amino acid requirements (g/16 g N) of European (E.) sea bass and gilthead (G.) sea bream.

Methods	Dose response		Ideal protein		Amino acid deletion G.sea bream
	E.sea bass	G.sea bream	E.sea bass	G.sea bream	
Species					
Amino acid					
Arginine	3.9–4.6	2.6	-	-	5.5
Histidine	-	-	1.6	1.7	1.89
Isoleucine	-	-	2.6	2.6	2.55
Leucine	-	-	4.3	4.5	4.75
Lysine	4.8	4.9-5	-	-	5.13
Methionine	-	-	-	-	2.6
Methionine + Cystine	4	2.8-4	-	-	-
Phenylalanine + Tyrosine	-	-	2.6	2.9	5.76
Threonine	2.3-2.6	-	-	-	2.98
Tryptophan	0.5	0.6	-	-	0.75
Valine	-	-	2.9	3	3.21

Adapted from: Luquet and Sabaut (1974); Tibaldi and Lanari (1991); Tibaldi et al. (1993, 1994); Kaushik (1998a); Tibaldi and Tulli (1999); Marcouli et al. (2006); Peres and Oliva-Teles (2009).

As for protein, the optimum dietary lipid levels for both species is still a matter of debate. In European sea bass, a number of studies reported that increasing dietary lipid levels above 12% has no beneficial effects on growth performance (Alliot et al., 1974; Metailler et al., 1981; Pérez et al., 1997; Peres and Oliva-Teles, 1999b), whereas other studies recorded better growth performances with increasing dietary lipids up to 18-19% (Dias et al., 1998; Lanari et al., 1999). Nonetheless, it appears clear that a dietary lipid level of 30% is not recommended for juveniles, either because of its negative effects on growth performance (Peres and Oliva-Teles, 1999b) or on voluntary feed intake (Boujard et al., 2004). For gilthead sea bream, an optimum dietary lipid level of 15–16% was established by Vergara and Jauncey (1993) and Vergara et al. (1996). This value was reevaluated to 21-22% by Santinha et al. (1999) and Vergara et al. (1999). Although the elevation of dietary lipid levels up to 28% appears to promote the best growth and effectively spare dietary protein when low quality FM was used, the excess of lipid may have been the cause of hepatocyte abnormalities recorded in fish fed the highest lipid levels (Vergara et al., 1999). In this sense, the authors concluded that it may be advisable not to use such high dietary lipid levels to avoid unwanted adiposity and adverse effects.

As for other marine fish species, essential FA requirements of European sea bass and gilthead sea bream are only satisfied by the n-3 series LC-PUFA, namely EPA and DHA (Tocher, 2010). Available data regarding quantitative FA requirements is scarce;

however, estimates suggest that dietary requirements of n-3 LC-PUFA are 0.7-1% for European sea bass juveniles (Coutteau et al., 1996; Skalli and Robin, 2004) and 0.5-1.9% gilthead sea beam juveniles (Kalogeropoulos et al., 1992; Ibeas et al. 1994, 1997). Regardless the developmental physiological stage of the fish, and probably also the dietary lipid content, the DHA:EPA ratio is reported to greatly affect quantitative EFA requirements (Tocher, 2010). Accordingly, in gilthead sea bream requirements for total n-3 LC-PUFA of 0.9% and 1.9% were established with dietary DHA: EPA ratios of 1 and 0.5, respectively (Kalogeropoulos et al., 1992; Ibeas et al., 1994). It was also advanced that gilthead sea bream seems to require preformed 20:4 n-6 (Mourente and Tocher, 1993). However, although n-6 LC-PUFA are most certainly required by marine fish species, quantitative requirements were not yet determined (NRC, 2011).

Fish, as other vertebrates, do not have specific carbohydrate requirements. However, within limits carbohydrate inclusion in the diets spares the use of proteins and lipids for energy purposes (Wilson, 1994; Stone, 2003). Incorporation of digestible carbohydrates in diets for European sea bass and gilthead sea bream juveniles is recommended to be limited to 20%, as high levels of starch (> 30 %) appear to reduce growth and feed utilization (Enes et al., 2011). Despite the reported high starch digestibility coefficients of starch (native starch above 70%; processed starch above 90%) and the potential to efficiency regulate high levels of plasma glucose within 12h, the relatively limited capacity to metabolize dietary glucose by both species is apparently linked to an ineffective regulation of the gluconeogenic pathway (Enes et al. 2009, 2011).

Data on vitamin and mineral requirements of European sea bass and gilthead sea bream is very scarce. Kaushik et al. (1998b) suggested that NRC (1993) requirement levels for vitamins established for salmonids could be used in practical diets for juvenile European sea bass but not in purified diets, which would require a surplus of vitamins (Table 7). Although the minimal requirement levels of the NRC (1993) to support an adequate growth performance of European sea bass were established around 50 mg /kg diet for ascorbic acid, 2500IU /kg diet for vitamin A, 2400 IU /kg diet for vitamin D₃, later studies recommended different dietary vitamin requirement levels. Indeed, the supplementation levels suggested by that studies were for ascorbic acid 20 mg/kg (Merchie et al., 1996), for vitamin A 5-31 mg(16667- 103333 IU) kg/diet (Villeneuve et al. 2005a,b; Mazurais et al., 2009) and for vitamin D₃ 27600 IU kg/diet (Darias et al. 2010). For gilthead sea bream, quantitative requirements for some vitamins were estimated as follows: 0.5 to 10 mg/kg feed for thiamine (vitamin B₁) (Morris and Davies, 1995a); 63 to 83 mg/kg for

nicotinic acid (Morris and Davies, 1995b), and 1.97 mg/kg for pyridoxine (vitamin B₆) (Kissil et al., 1981).

Table 7 Minimal dietary vitamin requirements recommended by NRC (1993) for salmonids

<i>Vitamins, min IU/kg</i>	
A (acetate)	2500
D ₃	2400
<i>Vitamins, min mg/kg</i>	
E	50
K (menadione)	10
Thiamine (B ₁)	1
Riboflavin (B ₂)	4
Pyridoxine (B ₆)	3
Pantothenic acid	20
B ₁₂	0.01
Niacin	10
Biotin	0.15
Folic acid	1
Ascorbic acid	50
Choline	1000
Inositol	300

Adapted from Kaushik et al. (1998b).

Regarding mineral requirements, data is only available for phosphorous, with estimated dietary requirements of 0.65% for European sea bass and of 0.75% for gilthead sea bream (Pimentel-Rodrigues and Oliva-Teles, 2001; Oliva-Teles and Pimentel-Rodrigues, 2004). In European sea bass, Fountoulaki et al. (2010) further observed that zinc provided by the raw materials in practical diets (90.8 mg/kg) was sufficient for normal performance, and that increasing dietary zinc up to 250.3 mg/kg did not improve growth, feed utilization efficiency or immune-competence of fish.

1.8 Alternative ingredients to FM and FO

In the context of the development of sustainable aquaculture, much progress has been made in developing feeds for European sea bass and gilthead sea bream with low FM and FO and rich in plant feedstuffs. Several studies have shown that around 20 to 60% of dietary FM protein can be replaced with several single plant protein sources (soybean meal, corn gluten, lupin seed meal, pea seed meal, rapeseed protein concentrate) in European sea bass (Gouveia and Davies, 1998, 2000; Lanari and D'Agaro, 2005) and gilthead sea bream (Kissil et al., 1997; Pereira and Oliva-Teles, 2002, 2003, 2004; Martínez-Llorens et al. 2007a,b) without affecting growth and/or nutrient utilization. Almost total, or total, replacement of FM protein by an appropriate mixture of plant protein sources and amino acid supplementation has also been achieved without affecting

growth performance of European sea bass (Kaushik et al., 2004; Messina et al., 2013) and gilthead sea bream (Gómez-Requeni et al., 2004; Kissil and Lupatsch, 2004; De Francesco et al., 2007; Santigosa et al., 2008).

Regarding FO replacement by VO sources, data obtained so far demonstrate that in diets with 20-25% lipids, substitution more than 60% FO by VO reduced growth performance of both species (Caballero et al., 2004; Izquierdo et al., 2005; Mourente et al., 2005b; Montero et al., 2008, 2010). This seems to be related to an EFA deficiency in the low FO diets. Indeed, providing a minimum content of EFA in the diet it is possible to replace up to 60-66% of the FO by a single source (soybean oil, rapeseed oil and linseed oil) or a blend (rapeseed, linseed, palm, olive soybean, sunflower, and/or cottonseed oils at different proportions) of VO without compromising growth performance of European sea bass (Izquierdo et al., 2003; Figueiredo-Silva et al., 2005; Mourente et al., 2005b; Richard et al., 2006b; Castro et al., 2015a) and gilthead sea bream (Izquierdo et al., 2003, 2005; Caballero et al. 2004; Benedito-Palos et al. 2007, 2008; Wassef et al., 2007; Fountoulaki et al. 2009; Montero et al., 2010).

Studies addressing the impact of a totally plant-based diet supplemented with amino acids on growth performance of both species are scarce and deserve further investigation. Indeed, in European sea bass, total replacement of fish-based diet with plant-based diet led to a negative impact on growth performance (Geay et al., 2011). In gilthead sea bream fed plant protein based diets, total replacement of FO by VO resulted either in depression (Benedito-Palos et al. 2007, 2008) or had no effects (Watson et al., 2013) on fish performance.

Although, studies support that an adequate growth performance can be achieved in both species when the needs of essential amino acids and FA are met, independently of the feedstuffs used, there are ample evidences that these kind of alternatives ingredients negatively affect tissue histomorphology (Caballero et al., 2003; Benedito-Palos et al., 2008; Santigosa et al., 2011b; Kokou et al., 2012), carcass composition, fillet nutritional value (Izquierdo et al., 2003; Martínez-Llorens et al. 2007b; Geay et al., 2011), lipid body allocation (Kaushik et al., 2004; Sitjà-Bobadilla et al., 2005), plasma cholesterol (Gómez-Requeni et al., 2004; Kaushik et al., 2004; Sitjà-Bobadilla et al., 2005; Richard et al., 2006b; Messina et al., 2013), immune status (Mourente et al., 2005b, 2007b; Sitjà-Bobadilla et al., 2005; Montero et al., 2008; Geay et al., 2011), and stress response (Montero et al., 2003; Ganga et al., 2011).

1.9 Aims

For increased incorporation of plant feedstuffs in aquafeeds at the expense of fisheries-related feedstuffs, more knowledge is needed on the effects of plant feedstuffs macronutrients, namely carbohydrates and VO, and the potential interaction between nutrients, on processes that control the quality and quantity of fish lipid deposits. In order to fill this gap of knowledge, the overall aim of this thesis was to assess these potential interactive effects of dietary carbohydrate and lipid source on a broad zootechnical perspective (growth performance, feed efficiency, whole-body composition and nutrient digestibility) to more detailed aspects related to nutrient metabolism and tissue lipid deposition at compositional, enzymatic and gene expression levels. In addition, the implications of macronutrient interaction on fish oxidative status was also evaluated. The studies were done comparatively in two important aquaculture fish species, European seabass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*), that although being both carnivorous species, seem to have dissimilarities in the digestive and metabolic utilization of macronutrients.

Specific objectives of this thesis was to assess the effects of dietary lipid source (FO and VO blend) and dietary starch (gelatinized starch) level (0 and 20%) on the:

- Diet digestibility;
- Growth performance, feed utilization, whole body composition;
- Processes involved in lipids digestion, absorption and transport;
- Tissue fatty acid composition;
- Modulation of LC-PUFA biosynthesis pathway;
- Glucose and lipid metabolism;
- Tissue histomorphology;
- Oxidative status;

Considering the importance of LC-PUFA biosynthesis pathway from human health perspective and also as a mean to provide the essential LC-PUFA for marine fish, particular emphasis was given to the study of the nutritional modulation of LC-PUFA biosynthesis pathway.

This approach will provide not only a deeper knowledge on the metabolic, physiological and health consequences of diet manipulation, but also provide insights for improving the utilization of diets rich in alternative feed ingredients that may improve the use of VO in aquafeeds.

Chapter 2-

Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, and lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles

Aquaculture Nutrition



Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles

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Abstract

A digestibility trial was conducted to evaluate the effects of dietary fish oil (FO) replacement by a vegetable oil blend (VO) (rapeseed, linseed and palm oils) on nutrient digestibility, lipid absorption and transport and lipid and carbohydrate metabolic pathways of *Dicentrarchus labrax* juveniles. Following 36 days, the liver and intestine of fish were sampled 6 h after the last meal. Remaining fish were fed one more week and blood was collected at different postprandial time intervals. Dietary VO decreased apparent digestibility coefficients (ADC) of lipid and energy. Among the serum parameters analysed, only high-density lipoprotein was affected, being higher in VO group. Hepatic and intestinal expression of genes related to lipid transport and lipoprotein metabolism (FABP, DGAT, ApoB, ApoA4, ApoA1, MTP) showed no differences in transcript levels. No alteration in genes related to cholesterol biosynthesis (HMGCR), glucose transport (GLUT2) or metabolism (GK, PK, G6Pase and PEPCK) in the liver and intestine was found. However, hepatic transcript levels of FADS2 and elovl5 were up-regulated in VO group. In conclusion, replacement of FO by VO did not induce major alterations at molecular and biochemical levels, although a nutritional modulation of LC-polyunsaturated fatty acids biosynthesis pathway was observed.

KEY WORDS: *Dicentrarchus labrax*, fatty acid bioconversion, gene expression, glucose and lipid metabolism, lipid source, nutrient transport

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Introduction

Nowadays, substitution of fish oil (FO) and fish meal (FM) in aquafeeds has become an imperative issue in aquaculture. Regarding FO, the only practical alternatives at present are vegetable oils (VOs). However, the main drawback of using VO in aquafeeds is related to their fatty acid (FA) profiles and, particularly, the lack of n-3 long-chain polyunsaturated fatty acids (LC-PUFA): eicosapentaenoic (EPA) and docosahexaenoic (DHA), which are abundant in FO and are required by marine fish. Different studies in mammals (Harris 1989, 1997; Nestel 1990) and also in fish (Torstensen *et al.* 2000; Richard *et al.* 2006a,b; Jordal *et al.* 2007; Morais *et al.* 2011b) reported that dietary lipid source is associated with modifications in the level and composition of plasma lipoproteins, triacylglycerol (TAG) and other lipids. Such alterations are probably linked with modifications of lipoprotein metabolism (Harris 1989; Ranheim *et al.* 1994; Brown *et al.* 1999; Madsen *et al.* 1999).

In fish, as in mammals, transport of hydrophobic lipids is thought to occur through a dynamic and complex transport system involving lipoproteins (Corraze 2001; Vance & Adeli 2008; Xiao *et al.* 2011). Both in enterocytes and in hepatocytes, association of lipids with proteins for lipoprotein assembly/synthesis (chylomicron, CM, and very low-density lipoproteins, VLDL, respectively) involves multi-intracellular processes such as intra-

cellular binding and transport of FA; resynthesis of TAG; synthesis of various non-exchangeable (apoB) and exchangeable (apoA1, apoA4 and apoCs) apolipoproteins; assembly of lipids and apolipoproteins into lipoproteins and secretion to circulation, either directly (in the liver) or via the lymphatic system (in the intestine) (van Grevengroek & de Bruin 1998; Mu & Hoy 2004; Xiao *et al.* 2011).

The exact transcriptional and translational mechanisms by which dietary FA affects lipoprotein metabolism and induces the reported changes in plasma lipid profile have not yet been completely elucidated (Sundaram & Yao 2010; Pan & Hussain 2012).

In recent years, increased attention has been given in fish to cellular and molecular mechanisms involved in the assembly and secretion of lipoproteins and its nutritional regulation (Oxley *et al.* 2005, 2007; Caballero *et al.* 2006; Jordal *et al.* 2006; Kjaer *et al.* 2008; Torstensen *et al.* 2009; Geay *et al.* 2011; Morais *et al.* 2011a,b, 2012; Borges *et al.* 2013; Kamalam *et al.* 2013). However, available information is still fragmented and controversial. Under a VO challenge, proteins participating in lipoprotein assembly, such as ApoB, ApoA1 and ApoA4, MTP, may exhibit or not a nutritional regulation by dietary lipid source at both transcriptional and translational levels (Kjaer *et al.* 2008; Morais *et al.* 2011a,b, 2012).

Besides the effects on mechanisms of lipid transport, dietary FAs were also shown to affect different intracellular events of lipid absorption and FA bioconversion processes (Torstensen *et al.* 2000; Seiliez *et al.* 2003; Morais *et al.* 2009).

Thus, in this study, expression levels of key genes involved in lipid transport and lipoprotein metabolism of European sea bass (*Dicentrarchus labrax*) in response to modifications of dietary FA profile will be assessed to elucidate some of the reported changes in plasma lipid profile.

Along with gene expression analysis in the intestine and liver, complementary data on diet digestibility and of selected serum metabolites and lipoproteins at different postprandial time intervals will also be obtained to elucidate short-term effects of dietary FO replacement by VO on mechanisms involved in lipid absorption and blood transport. Given the previous evidence of the modulator role of dietary lipids in carbohydrate metabolism (Panserat *et al.* 2002; Menoyo *et al.* 2006; Figueiredo-Silva *et al.* 2012), the impact of dietary modulation on glucose metabolism and on LC-PUFA biosynthesis pathway in sea bass juveniles was also assessed.

Materials and methods

Experimental diets

Two isoproteic (450 g kg⁻¹ crude protein) and isolipidic (180 g kg⁻¹ CL) diets were formulated to contain a high level of carbohydrate (200 g kg⁻¹ gelatinized starch) and FO or VO blend as lipid sources (Table 1). The VO blend was composed of rapeseed (200 g kg⁻¹), linseed (500 g kg⁻¹) and palm (300 g kg⁻¹) oils and replaced circa 700 g kg⁻¹ of dietary lipids provided by cod liver oil and fish meal. The diets were similar in saturated fatty acids (SFA) content but, comparatively to the VO diet, the FO diet had higher

Table 1 Proximate analysis of the experimental diets

Diets	Fish oil	Vegetable oils (VO)
Ingredients (g kg ⁻¹ dry weight)		
Fish meal ¹	630.6	630.6
Gelatinized starch ²	200	200
Cod liver oil ³	120.4	4
VO blend ⁴	0	120
Cellulose	0	0
Vitamin premix ⁵	10	10
Mineral premix ⁶	10	10
Choline chloride (50%)	5	5
Binder ⁷	10	10
Chromium	5	5
Proximate analyses (g kg ⁻¹ dry weight)		
Dry matter (g kg ⁻¹ , DM)	870.5	870.2
Crude protein (CP)	460.2	470.1
Crude fat (CF)	170.9	180.2
Ash	130.9	130.7
Starch	200.4	190.8
Cholesterol	3	3
Gross energy (MJ kg ⁻¹)	21.4	21.0

¹ Steam-dried LT fish meal, Pesquera Diamante, Perú (CP:700.2 g kg⁻¹ DM; GL: 80.8 g kg⁻¹ DM).

² C-Gel Instant-12018, Cerestar, Mechelen, Belgium.

³ Fagron Iberica S.A.U., Spain.

⁴ 300 g Kg⁻¹ palm oil (Colmi, Malasia), 500 g Kg⁻¹ linseed oil (Sociedade Portuense de Drogas, S.A., Portugal) and 200 g Kg⁻¹ rapeseed oil (Huilerie Emile Noël S.A.S., France).

⁵ Vitamins (mg kg⁻¹ diet): retinol acetate, 18 000 (IU kg⁻¹ diet); cholecalciferol, 2000 (IU kg⁻¹ diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400. Premix, Viana do Castelo, Portugal.

⁶ Minerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.40 (g kg⁻¹ diet). Premix, Viana do Castelo, Portugal.

⁷ Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, UK.

monounsaturated fatty acids (MUFA), EPA acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and eicosenoic acid (20:1) and lower levels of n-6 PUFA, linoleic acid (LA, 18:2n-6), linolenic acid (LNA, 18:3n-3) and oleic acid (OA, 18:1) (Table 2).

All ingredients were finely ground, well mixed and dry-pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA), through a 3-mm die. The pellets were air-dried and stored in a refrigerator until used.

Digestibility trial

The digestibility trial was performed at the Marine Zoology Station, Porto University, in a thermoregulated recirculation water system equipped with a battery of six fibreglass tanks of 60 L capacity designed according to Cho *et al.* (1982) and with a faeces settling column connected to the outlet of each tank.

Table 2 Fatty acid (FA) composition (10 g kg^{-1} of total FAs) of the experimental diets

Diets	Fish oil	Vegetable oils
14:0	5.3	1.4
15:0	0.4	0.1
16:0	13.7	14.1
17:0	0.3	0.2
18:0	3.0	3.7
20:0	0.1	0.3
Sum saturates	22.9	19.9
16:1	7.6	2.1
18:1	18.7	27.0
20:1	7.1	0.8
22:1	5.4	0.2
Sum monoenes	39.0	30.2
16:2 n-4	0.5	0.2
16:3 n-4	0.9	0.3
16:4 n-1	0.8	0.4
Sum other	2.4	1.0
polyunsaturated fatty acids (PUFA)		
18:2 n-6	2.1	10.6
18:3 n-6	0.2	0.1
20:2 n-6	0.2	0.1
20:4 n-6	0.7	0.5
Sum n-6 PUFA	3.3	11.4
18:3 n-3	0.8	21.1
18:4 n-3	2.0	0.6
20:4 n-3	0.7	0.2
20:5 n-3	10.2	5.3
21:5 n-3	—	0.2
22:5 n-3	0.8	0.7
22:6 n-3	12.3	7.6
Sum n-3 PUFA	26.9	35.6
Sat/PUFA	0.7	0.4
n3/n6	8.2	3.1

The trial was performed with European sea bass (*D. labrax*) juveniles with a mean initial body mass of 96 g. Before the trial, the fish were allocated to the system and acclimatized for 15 days to the tanks and water temperature and during this period were fed a commercial diet. Thereafter, 16 fish were randomly distributed to each tank and each diet was randomly assigned to triplicate groups of these fish. During the trial, water temperature averaged $24.1 \pm 0.9^\circ\text{C}$, salinity averaged $34.7 \pm 0.8 \text{ g L}^{-1}$ and dissolved oxygen was kept near saturation. Fish were hand-fed to satiation twice a day. The first 15 days of the trial were used for fish to adapt to the diets; then, faeces were collected once a day during the following 19 days. Immediately after the collection, faeces from each tank were centrifuged, pooled and stored at -20°C until analysis.

Tissues and blood sampling

After 36 days of being fed with the experimental diets, two fish from each tank were randomly sampled to collect intestine (midgut just after the last pyloric caecum) and liver samples for the analysis of gene expression. Fish were sampled 6 h after the last feeding as this time corresponds to the peak interval of postprandial nutrient absorption (between 6 and 8 h, Fig. 1). Immediately after collection, tissue samples were frozen in liquid nitrogen and then stored at -80°C until analysis. The remaining fish were fed one more week and then blood samples were collected at 2, 4, 6, 8, 12 and 24 h after feeding for the analysis of serum metabolites. Blood was collected from the caudal vein by puncture with a heparinized syringe and immediately centrifuged at 1800 g for 12 min, and serum aliquots were stored at 4°C until analysis by a clinical laboratory. In order to minimize stress due to sampling, at each sampling point blood was collected from five fish from one tank and that tank was only sampled again after at least 6 h.

Analytical methods

Chemical analysis Diets and faeces were analysed using the following procedures: dry matter after drying at 105°C in an oven until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein content ($N \times 6.25$) according to the Kjeldahl method after acid digestion, using a Kjeltec digester and distillation units (Tecator Systems, Höganäs, Sweden); gross energy by direct combustion in an adiabatic bomb calorimeter

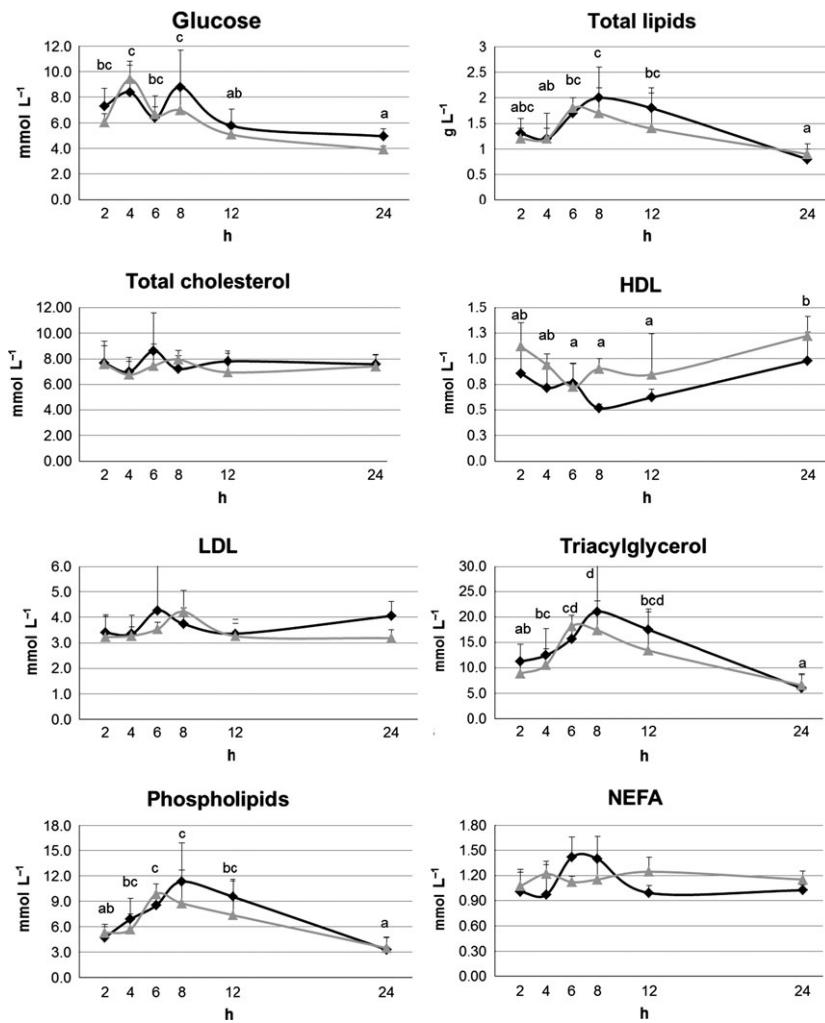


Figure 1 Postprandial evaluation of serum glucose, total lipids, total cholesterol, high-density lipoprotein, low-density lipoprotein, triacylglycerol, phospholipids and non-esterified fatty acids levels in European sea bass (*Dicentrarchus labrax*) for 24 h following feeding period of 43 days with the fish oil diet (◆) or the vegetable oils diet (▲). Values are means \pm SD ($n = 5$). Significant differences ($P < 0.05$) among sampling times are indicated by different lowercase letters. Statistical differences related to diets are described in the text.

(PARR model 1261; PARR Instruments, Moline, IL, USA); starch according to Beutler (1984); chromic oxide by acid digestion according to Furukawa and Tsukahara (1966). Lipids in faeces were determined according to Folch *et al.* (1957) and in diets by petroleum ether extraction in a SoxTec extraction system (Tecator Systems). Total cholesterol in the diets was assayed by the Liebermann–Burchard method (Stadtman 1957). For fatty acid (FA) composition analyses of diets, total lipids were extracted and measured gravimetrically according to Folch *et al.* (1957) using dichloromethane instead of chloroform. Fatty acid methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride methanol according to Santha and Ackman (1990) and were analysed in a Varian

3900 gas chromatograph (Varian, Les Ulis, France). The chromatograph was equipped with a DB WaxTR-fused silica capillary column (30 m \times 0.25 mm i.d., film thickness: 0.25 μm ; J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas (1 mL min⁻¹), and the thermal gradient was 100–180 °C at 8 °C min⁻¹, 180–220 °C at 4 °C min⁻¹ and a constant temperature of 220 °C during 22 min. Injector and flame ionization detector temperatures were 260 and 250 °C, respectively. Fatty acid methyl esters were identified by comparison with known standard mixtures (Sigma 189-19, St Louis, MO, USA) and quantified using the STAR version 5 computer package (Varian).

Apparent digestibility coefficients were calculated according to the formula:

$$\text{ADC} = 100 - [100 \times (\text{Cr}_2\text{O}_7 \text{ diet}/\text{Cr}_2\text{O}_7 \text{ faeces})]$$

$$\times (N_{\text{faeces}}/N_{\text{diet}})]$$

where N is nutrient or energy.

Serum metabolites Serum total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), total lipids, TAG, phospholipid (PL), non-esterified fatty acids (NEFA) and glucose analyses were performed on a Clinical Laboratory-certified NP EN ISO 9001-2000 by Bureau Veritas, following standard clinical methods and using an auto-analyzer (Architect ci8200; Abbot Diagnostics, Saint-Laurent, Canada).

Gene expression analysis: real-time polymerase chain reaction (q-PCR) Total RNA was extracted from the livers and intestines using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations, and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000; Nanodrop Labtech, Palaiseau, France). Complementary DNA (cDNA) synthesis was performed with 1 µg of the resulting total RNA using the SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen) and random primers (Promega, Charbonnières, France). Gene expression levels were determined by real-time quantitative PCR (q-PCR) using the iCycler iQ™ (Bio-Rad, Hercules, CA, USA).

Analyses were performed on 5 µL of the diluted cDNA (1:76) using iQ™SYBR® Green Supermix (Bio-Rad), in a total PCR volume of 15 µL, containing 200 nM of each primer. Primers were designed to overlap an intron if possible [Primer3 software (Rozen and Skaletsky 2000)] using known sequences in European seabass nucleotide databases (Public Sigenae Contig Browser, Ensembl; <http://public-contigbrowser.sigenae.org:9090/index.html>) (Table 3). Thermal cycling was initiated with the incubation at 95 °C for 3 min for hot-start iTaq™DNA polymerase activation. Thirty-five steps of PCR were performed, each one consisting of heating at 95 °C for 20 s for denaturation and at 60 °C for 30 s for annealing and extension. Following the final PCR cycle, melting curves were systematically monitored (55 °C temperature gradient at 0.5 °C 10 s⁻¹ from 55 to 94 °C) to ensure that only one fragment was amplified. Each PCR run included duplicates of reverse transcription for each sample and also negative controls (reverse transcriptase-free samples, RNA-free samples). The PCR run for reference gene included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and also negative controls. Quantification of the target gene transcripts was performed using the elongation factor 1α (EF1α) gene expression as a reference as previously used in European sea bass by Geay *et al.* (2011), which was stably expressed in the present study. Relative

Table 3 Sequences of the primer pairs used for real-time quantitative PCR determination of the transcript level of several European sea bass (*Dicentrarchus labrax*) genes involved in hepatic and intestinal lipid and glucose metabolism

Gene	5'-3' Forward primer	5'-3' Reverse primer	Accession number
ApoA1	TGGATGCCCTGAAGGTAAG	CTTCAGGGTGGTGATCTCGT	CV186176
APoA4	GCTGATGACACCCTCCAAAT	GGGTCACTCGTTCTCTCAGC	CV186119
ApoB	GCTGGCTGCATTTATGACA	GAGCAGATGGAACATGCAGA	CV186215
DGAT	CAAGCCAACATCCGAGTTT	GCTGATGACCCACTGTAGCA	FL486573
EF1α	GCTTCGAGGAAATCACCAAG	CAACCTCCATCCCTGAAC	AJ866727
elovl5	GCACGGTGGTACAACATTCT	TGTCCATGAACTCGATGAGC	*
FABP	AGCGGATTTCAAGAACCAA	TCACCCCATCACACTGAA	FM004624
GLUT2	GAGCCCACGGTACCTTACA	CGGATCAAAGAAAGGATGGA	EF014277
GK	ATCGTCAGGGAACTCACACC	GAGTTCAGGCTTGCTTCACC	AM986860
G6Pase	TGAGACCCGGTTTATGGAG	CATGCAGACCAACCAGCTTA	AM987970
HMGCR	TCTACATCCGCTTCCATTCC	GAGCTCTGTCGTGTTTC	AAR02862
MTP	CAAAGGTTGGGTATCGTCT	GCAAACGACTCCAGGTCTTC	AM985588
PK	CTGTTCTGTGGAGGCAGT	CAGCACAGCATTGAAGGAG	AM981422
PEPCK	GCGCCATCAACACTAAAGGT	TTGTGCACTGTGCTTCCAG	DV217087
SREBP1	CTGGAGCCAAAACAGAGGGAG	GACAGGAAGGGAGGGAGGAAG	FN677951
FADS2	CCTTCACTGCTTCATCCCAA	CCCAGGTGGAGGCAGAAGAA	EU439924

Sigenae accession no. in parentheses: EF1α, elongation factor 1α; GK, glucokinase; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase; GLUT2, glucose transporter type 2; ApoA1, apolipoprotein A1; ApoA4, apolipoprotein A4; ApoB, apolipoprotein B; DGAT, diacylglycerol acyltransferase; elovl5, elongase 5 (*Dr D Mazurais, personal communication); FABP, fatty acid-binding protein; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; MTP, microsomal triglyceride transfer protein; SREBP1, sterol-response element-binding protein-1; FADS2, Δ6 fatty acyl desaturase.

quantification of the target gene transcript with the EF1 α reference gene transcript was performed using the mathematical model described by Pfaffl (2001). The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and the C_T deviation (ΔC_T) of the unknown sample compared with a control sample and expressed in comparison with the EF1 α reference gene:

$$R = \frac{[(E_{\text{target gene}})^{\Delta C_T \text{ target gene}(\text{mean control} - \text{mean sample})}]}{[(E_{\text{ef1}\alpha})^{\Delta C_T \text{ EF1}\alpha(\text{mean control} - \text{mean sample})}]}$$

Efficiency of PCR was measured by the slope of a standard curve using serial dilutions of cDNA.

Statistical analysis

Data are presented as means \pm standard deviation. Data were checked for normal distribution and homogeneity of variances and when appropriate were normalized. The ADC and gene expression data were analysed by unpaired two-tailed Student's t -tests. The effects of diet and sampling time on serum metabolites level were analysed by two-way ANOVA, followed by one-way ANOVA in case of significant interaction. Significant differences between means were evaluated by the Tukey's multiple range test. The significance level of 0.05 was used for rejection of the null hypothesis. All statistical analyses were carried out using the SPSS 20.0 software package (IBM Corp., New York, NY, USA) for Windows.

Results

The ADC of dry matter, protein and starch were not affected by diet composition, but ADC of lipid and energy

Table 4 Apparent digestibility coefficients (ADC) (%) of the experimental diets¹

ADC (%)	Diets		
	Fish oil	Vegetable oils	P-value ²
Dry matter	88.6 \pm 0.1	88.6 \pm 0.9	0.864
Protein	95.1 \pm 0.1	95.0 \pm 0.1	0.132
Lipid	99.5 \pm 0.1	99.1 \pm 0.3	0.040*
Starch	99.6 \pm 0.0	99.6 \pm 0.0	0.643
Energy	97.0 \pm 0.2	96.4 \pm 0.2	0.027*

¹ Values presented as means ($n = 3$) and standard deviation (SD).

² P-values assessed by Student's t -test: values not showing an asterisk are non-statistically significant ($P > 0.05$); * $P < 0.05$.

were lower in the VO diet (Table 4). Nonetheless, irrespective of dietary treatments, ADC of all nutrients and energy were very high.

Postprandial serum glucose, total lipids, cholesterol, TAG, PLs, non-esterified fatty acids (NEFA), HDL and LDL levels at 2, 4, 6, 8, 12 and 24 h after a meal are presented in Fig. 1. Excluding serum HDL levels, no differences in serum metabolites promoted by dietary treatments were observed. Average HDL levels were higher in VO (1.0 ± 0.3 mM) than in FO (0.7 ± 0.2 mM) groups.

Postprandial serum glucose level increased to a first peak 4 h after feeding and then a second peak was observed 8 h after feeding; thereafter, glucose decreased to a basal value which was attained 12 h after feeding. Serum total lipids attained a peak 8 h after feeding and then slowly decreased to a minimum 24 h after feeding. No postprandial differences in serum total cholesterol and LDL were observed, and postprandial HDL levels were only lower at 8, 6 and 12 h, comparatively to the value observed 24 h after feeding. Serum TAG increased to a peak at 8 h after feeding and thereafter decreased to a basal value which was attained 24 h after feeding. Serum PL levels also increased to a peak 6–8 h after feeding and then decreased to a basal value 24 h after feeding. A significant interaction between diet \times time was observed for serum NEFA. While no differences between sampling times were observed for the VO group, for the FO group significant differences were found (one-way ANOVA, $P < 0.01$) although Tukey's test was unable to detect such differences.

Data on mRNA levels of key proteins involved in lipid transport (fatty acid-binding protein, FABP), TAG resynthesis (diacylglycerol acyltransferase, DGAT), lipoprotein assembly (apolipoproteins B, A4, A1: ApoB, ApoA4, ApoA1; microsomal triglyceride transfer protein, MTP), cholesterol biosynthesis (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, HMGCR) and LC-PUFA biosynthesis ($\Delta 6$ fatty acyl desaturase, FADS2; elongase 5, elovl5; and sterol-response element-binding protein-1, SREBP1) in the liver and intestine of fish fed the experimental diets are reported in Table 5.

Regardless of the experimental diets, no variation in mRNA levels for hepatic and intestinal proteins involved in lipid transport (FABP), TAG and cholesterol synthesis (DGAT and HMGCR) and lipoprotein assembly (ApoB, MTP, ApoA4 and ApoA1) was observed. Further, FABP was not expressed in liver. Within the actors involved in the LC-PUFA-biosynthesis pathway (FADS2, elovl5, SREBP1), only hepatic FADS2 and elovl5 transcript levels increased in the VO group comparatively to the FO group

Table 5 Gene expression of selected proteins involved in lipid transport (FABP), TAG resynthesis (DGAT), lipoprotein assembly (ApoB, MTP, ApoA4, ApoA1), cholesterol biosynthesis (HMGCR) and LC-PUFA biosynthesis (FADS2, elovl5 and SREBP1) in the liver and intestine of European sea bass (*Dicentrarchus labrax*) fed diets with vegetable blend 6 h after feeding, compared to fish fed the fish oil (FO) diet assayed by RT-qPCR¹

Genes	FC in liver	P-value ²	FC in intestine	P-value ²
FABP	–	–	–1.1	0.815
DGAT	+1.6	0.272	+1.4	0.197
ApoB	–1.2	0.649	+1.2	0.410
MTP	1.0	0.861	+1.3	0.586
ApoA4	+1.5	0.088	+1.2	0.548
ApoA1	+1.2	0.147	+1.2	0.465
HMGCR	+1.3	0.549	–1.9	0.308
SREBP1	+1.7	0.079	1.0	0.898
FADS2	+2.5	0.005**	+1.2	0.539
elovl5	+3.3	0.015*	+1.6	0.501

FABP, fatty acid-binding protein; DGAT, diacylglycerol acyltransferase; ApoB, apolipoprotein B; MTP, microsomal triglyceride transfer protein; ApoA4, apolipoprotein A4; ApoA1, apolipoprotein A1; HMGCR, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; SREBP1, sterol-response element-binding protein-1; FADS2, Δ6 fatty acyl desaturase; elovl5, elongase 5; PUFA, polyunsaturated fatty acids; TAG, triacylglycerol; –, no detectable gene expression.

¹ Values presented as fold changes, FC ($n = 6$ fish per treatment), in fish fed vegetable blend diet relative to the FO diet. Expression results were normalized by EF1 α mRNA levels.

² P-values assessed by Student's *t*-test. Asterisks indicate fold changes, assessed by RT-qPCR, that are statistically significant: * $P < 0.05$ ** $P < 0.01$. Values not showing an asterisk are non-statistically significant ($P > 0.05$).

(2.5- and 3.3-fold higher, respectively). Hepatic and intestinal mRNA levels of proteins involved in glucose transport (glucose transporter type 2, GLUT2), glycolysis (glucokinase, GK, and pyruvate kinase, PK) and gluconeogenesis (phosphoenolpyruvate carboxykinase, PEPCK; glucose 6-phosphatase, G6Pase) showed no changes between dietary treatments (Table 6).

Discussion

Apparent digestibility coefficients of nutrients determined in this trial are within the range of values reported in previous studies for this species (Dias *et al.* 1998; Peres & Oliva-Teles 1999; Enes *et al.* 2006; Martins *et al.* 2006). Similarly to other studies (Ng *et al.* 2003; Bahurmiz & Ng 2007; Francis *et al.* 2007), lower ADC of lipids in fish fed VO compared with fish fed FO diets were also found in the present study. This is most probably due to differences in FA composition of the two lipid sources, as ADC of lipids

Table 6 Gene expression of selected glycolytic (GK, PK), gluconeogenic (PEPCK, G6Pase) enzymes and of protein involved in glucose transport (GLUT2) in the liver and intestine of European sea bass (*Dicentrarchus labrax*) fed diets with vegetable blend 6 h after the last meal and compared to fish fed the FO diet assayed by RT-qPCR¹

Genes	FC in liver	P-value ²	FC in intestine	P-value ²
GK	+1.8	0.115	–	–
PK	1.0	0.896	+1.1	0.201
PEPCK	1.0	0.907	–	–
G6Pase	1.0	0.863	–	–
GLUT2	–1.3	0.071	–1.1	0.353

GK, glucokinase; PK, pyruvate kinase; PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase; GLUT2, glucose transporter type 2; FO, fish oil; –, no detectable gene expression.

¹ Values presented as fold changes, FC ($n = 6$ fish per treatment), in fish fed vegetable blend diet relative to the FO diet. Expression results were normalized by EF1 α mRNA levels.

² P-values assessed by Student's *t*-test. Values not showing an asterisk are non-statistically significant ($P > 0.05$).

were shown to be related to the degree of unsaturation and the chain length of FA. In numerous fish species, higher ADC of FA were observed for LC-PUFA, followed by C18 PUFA, MUFA and SFA or, within the same degree of unsaturation, for short-chain than for long-chain FA (Torstensen *et al.* 2000; Caballero *et al.* 2002; Menoyo *et al.* 2003; Ng *et al.* 2003; Francis *et al.* 2007; Martins *et al.* 2009). Possible reasons for the ADC differences in individual FA are lipolytic enzyme specificities towards different FA and factors related to fat emulsification and micelle incorporation (Koven *et al.* 1994; Olsen & Ringo 1997; Tocher 2003). However, in the present study, the differences in ADC of lipids between diets were small, and thus, any reduction in individual FA digestibility was probably also quantitatively small and therefore of no biological significance for the fish (Ng & Gibon 2010). As a consequence of differences in lipid digestibility, energy digestibility was also affected by FO replacement with VO. However, as for lipids, differences in energy digestibility were also negligible.

Within serum metabolites and lipoproteins analysed, only HDL levels were affected by dietary treatments, being higher in VO group than in FO group. A relationship between plasma HDL concentration and lipoprotein lipase (LPL) activity, a key enzyme involved in TAG hydrolysis, was previously established (Frenais *et al.* 2001; Engelking 2010). It is accepted that LPL, via its hydrolytic activity over TAG of both CM and VLDL, contributes to nascent HDL formation through transference of surface protein and

lipid from CM and VLDL remnant particles (Cheung *et al.* 2003; Engelking 2010). Although additional information is required, previous data seem to support the assumption that reverse transport via HDL of cholesterol not used by tissues to the liver might have been changed under VO challenge through variations in the rate of LPL-mediated lipolysis of TAG-rich lipoproteins. Accordingly, Geay *et al.* (2011) found that in European sea bass fed an vegetable diet, there was a stimulation of liver LPL and of apolipoproteins ApoA1 genes. ApoA1 is the major protein constituent of HDL and increased expression of it was also observed in the current study although it was not statically significant.

Lack of variation in serum cholesterol levels is in accordance with the similar dietary cholesterol levels in both diets. Likewise, diets where FO was replaced by VO but that had the same cholesterol levels induced no changes in plasma cholesterol in Atlantic salmon (*Salmo salar*) (Torstensen *et al.* 2004). On the contrary, diets with VO inclusion and reduction in cholesterol content were reported to promote hypocholesterolaemia in rainbow trout (*Oncorhynchus mykiss*) (Richard *et al.* 2006a).

The postprandial serum lipid profile of fish fed the two experimental diets indicates that peak of lipid absorption occurs earlier and its magnitude is lower in VO group than in FO group. Further studies are needed to clarify this but because VO diets have lower SFA/PUFA ratios than FO diets, we can hypothesize that this fast absorption may be due to higher lipase specificity towards PUFA and/or that PUFA released by lipolysis are more effectively absorbed by the enterocytes than MUFA and SFA (Koven *et al.* 1994; Olsen & Ringø 1997).

In the present study, postprandial serum TAG reached maximum values (17.4–21.0 mM) at 8 h after feeding. Also in this species, Santulli *et al.* (1988) previously reported a postprandial peak of 9.0 mM 24 h after feeding at 14.3–15.2 °C. Different temperatures and dietary lipid levels in both studies may contribute to explain differences in peak intensity and time of appearance. In the present trial, TAG concentration at 12 h was still high, and lower levels were attained only at 24 h after feeding. Unlike total lipids and PL, which followed a postprandial profile similar to that of TAG, cholesterol levels did not show postprandial fluctuation, as previously observed (Robaina *et al.* 1999), but cholesterol levels were identical to values reported in other studies in this species (Dias *et al.* 2005; Figueiredo-Silva *et al.* 2005; Richard *et al.* 2006b).

Studies conducted in mammals, both *in vitro* and *in vivo*, suggest that intracellular processes involved in the assembly and secretion of lipoproteins may constitute potential tar-

gets for dietary FA regulation (Mu & Hoy 2004; Pan & Hussain 2012). Intracellular transport of FA is mediated by cytosolic FABPs that bind non-covalently long-chain FA and other hydrophobic ligands (Tocher 2003) and transport them throughout the cytoplasm to intracellular organelles. FABPs are tissue specific and several FABPs have been identified and characterized in different fish tissues (Jordal *et al.* 2006; Chen *et al.* 2012; Venold *et al.* 2013), and its nutritional control has also been demonstrated (Jordal *et al.* 2006; Torstensen *et al.* 2009; Geay *et al.* 2011; Borges *et al.* 2013). In the liver, FABP expression was not detected and a nutritional modulation over transcript levels of what seems to be a FABP isoform present in the intestine was not observed. To the authors' knowledge, regulation of FABPs by lipid source in the intestine of fish has never been evaluated.

Re-esterification of TAG, which is crucial for lipoprotein assembly, occurs via two different pathways (monoacylglycerol acyltransferase, MAG; and glycerol-3-phosphate, G-3-P). Diacylglycerol acyltransferase is the intrinsic membrane-bound enzyme associated with the endoplasmic reticulum that is involved in the last step of TAG resynthesis that is common to both pathways (Caballero *et al.* 2006). In the present study, no differences were observed on DGAT mRNA transcript levels in fish fed either FO or VO blend. In other studies, replacing FO with VO either increased (Caballero *et al.* 2006) or did not affect (Oxley *et al.* 2005; Kjaer *et al.* 2008) the activity of TAG-synthesizing enzymes in fish intestine.

In this study, expression of the main apolipoproteins (ApoB, ApoA1 and ApoA4), known to be involved in the multistep process of CM and VLDL lipoproteins assembly in the intestine and liver (van Greevenbroek & de Bruin 1998; Mu & Hoy 2004; Xiao *et al.* 2011), respectively, suggests that they are not regulated by diet composition.

Lack of response of transcript levels of ApoB, which is a major structural protein involved in the first step of lipoprotein assembly, was expected because it is believed that, as it happens in mammals, the primary site of ApoB secretion regulation occurs at the stage of its intracellular degradation rather than at transcriptional level (Xiao *et al.* 2011). In the liver of Atlantic salmon fed either FO- or VO-based diets, Morais *et al.* (2011a) also found no nutritional modulation of ApoB transcript levels. In both liver and intestine, the newly synthesized ApoB is proteolytically degraded via the ubiquitin-dependent proteosomal pathway in the absence of initial incorporation of lipids into ApoB by MTP (van Greevenbroek & de Bruin 1998; Iqbal & Hussain 2009). MTP is a lumen protein that facilitates lipid

addition to lipoproteins by acting as a chaperone to assist in ApoB folding and, contrary to ApoB, seems to be under regulation at the transcriptional level (Iqbal & Hussain 2009). Contrary to Kjaer *et al.* (2008), who observed a nutritional modulation of transcript levels of MTP in Atlantic salmon fed either FO or rapeseed oil, the present results did not evidence such effect.

Regarding cholesterol biosynthetic pathway, there are evidences from human and animal studies that plant sterols/phytosterols present in VOs have an inhibitor effect on intestinal cholesterol absorption, and this may stimulate *de novo* hepatic cholesterol synthesis (Moghadasian & Frohlich 1999; Normén *et al.* 2000; Liland *et al.* 2013). In European sea bass, complete replacement of FM and FO by vegetable alternatives resulted in an increased expression of hepatic hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), the rate-limiting enzyme of cholesterol biosynthesis (Geay *et al.* 2011). However, in the present study, no modulation of transcript levels of this enzyme was found both at hepatic and at intestinal levels. Dietary phytosterol content, VO source and/or identical dietary and serum cholesterol levels observed in two groups may explain both the lack effect in cholesterollaemia and in cholesterol biosynthesis.

In the present study, an up-regulation of both Δ6 fatty acyl desaturase (FADS2) and elongase 5 (elovl5) gene expression levels at hepatic, but not at intestinal, level was observed in fish fed the VO diet. González-Rovira *et al.* (2009) also found in European sea bass that diets where 60% FO was replaced with rapeseed or linseed oils induced an up-regulation of the hepatic FADS2 gene expression, although the pattern of FADS2 gene expression was influenced by dietary VO source. At a protein level, *in vitro* studies also demonstrated that FO replacement by VO led to a significant increase in FADS2 enzymatic activity in the enterocytes, but not in the hepatocytes, of European sea bass (Mourente & Dick 2002; Mourente *et al.* 2005a,b). To the authors' knowledge, no data currently exist about nutritional regulation of elovl5 gene expression by lipid source in European sea bass. However, in another marine species (*Gadus morhua*), replacement of FO by VO induced little differences in the expression and activity of elovl5 at intestinal level (Tocher *et al.* 2006; Morais *et al.* 2012), but increased expression of elovl5 in the liver was observed (Tocher *et al.* 2006). A long-term trial is therefore required to see whether the observed up-regulation of both FADS2 and elovl5 gene expression levels is maintained and has benefit effects on whole fish and tissues lipid composition.

Fatty acid and their derivatives are known to regulate the activities of a variety of transcription factors, such as

SREBPs, which in turn have been shown to be regulators of many genes involved in FA, cholesterol and carbohydrate metabolism (Jump 2008). As in mammals (Nakamura & Nara 2002; Mullen *et al.* 2004), transcription of cholesterol and LC-PUFA biosynthesis genes in fish seems to be mediated by SREBP activation (Geay *et al.* 2010; Kortner *et al.* 2012). Consistent with hepatic FADS2 and elovl5 transcript levels, although no statistically significant different ($P = 0.08$), an increase in transcript levels of SREBP1 in the liver of fish fed the VO diet was also observed.

In both experimental groups, glycaemia peaks were achieved 4 h after feeding, which is in accordance with previous observations in this species (Peres *et al.* 1999; Enes *et al.* 2011). In glucose tolerance tests, Peres *et al.* (1999) and Enes *et al.* (2011) observed that maximum glucose levels of 14.2–15.5 and 23.0–25.0 mM, respectively, were attained between 2 and 6 h after peritoneal injection of 1 g glucose per kg body weight. Further, similarly to previous works (Peres *et al.* 1999; Peres & Oliva-Teles 2002; Enes *et al.* 2011), transitory hyperglycaemia also disappeared within 12 h after feeding. A second peak on glycaemia was also observed in the present study, and it may be related to an anticipation of meal time associated with the previous feeding schedule imposed to the animals. Indeed, fish maintained under a periodic feeding regime were shown to synchronize physiological variables involved in food intake and digestive processes to expected meal times (López-Olmeda *et al.* 2009, 2012; Montoya *et al.* 2010).

Several studies suggest that levels of lipids may have an important role in the modulation of carbohydrate metabolism (Panserat *et al.* 2002; Menoyo *et al.* 2006; Figueiredo-Silva *et al.* 2012). However, there are few studies relating the effects of dietary lipid source on carbohydrate metabolism. For instance, dietary lipid source was shown to influence mRNA levels of the muscle glucose transporter GLUT4 in Atlantic salmon, with concomitant modifications of plasma glucose levels (Menoyo *et al.* 2006). Present results indicate, however, that dietary lipid source did not promote major alterations on glucose metabolism.

Conclusions

Fish oil replacement by a VO blend induced slight differences in lipid digestibility which, by its magnitude, do not seem to be of significance to the animals. Data provided in this short-term trial demonstrate a lack of alterations on metabolite postprandial profile due to dietary treatments, which is in accordance with the observed lack of transcriptional modifications of proteins related to lipid transport, lipoprotein

metabolism (FABP, DGAT, ApoB, ApoA4, ApoA1, MTP), cholesterol biosynthesis pathway (HMGCR) and glucose metabolism (GLUT2; GK, PK, PEPCK; G6Pase).

The observed nutritional regulation of FADS2 and elovl5 genes by VO is of potential interest for practical marine fish nutrition, especially regarding the necessity of optimizing LC-PUFA biosynthesis pathways in marine species and the urgent need of reducing FO use in aquafeeds. It is also necessary to confirm if the stimulation of FADS2 mRNA expression induced by the substitution of FO by VO may compensate HUFA deficiency as apparently it was not the case in the studies by (Geay et al. 2010, 2011). Also, confirmation of this nutritional modulation in long-term studies is required to confirm maintenance of the observed effects on whole fish and tissues lipid composition.

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References

- Bahurmiz, O.M. & Ng, W.K. (2007) Effects of dietary palm oil source on growth, tissue fatty acid composition and nutrient digestibility of red hybrid tilapia, *Oreochromis* sp., raised from stocking to marketable size. *Aquaculture*, **262**, 382–392.
- Beutler, H.O. (1984) Starch. In: Methods of Enzymatic Analysis, Vol. 6 (Bergmeyer, H.U. ed.), pp. 2–10. Verlag Chemie Weinheim, Basel.
- Borges, P., Medale, F., Veron, V., Pires Mdos, A., Dias, J. & Valente, L.M.P. (2013) Lipid digestion, absorption and uptake in *Solea senegalensis*. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.*, **166**, 26–35.
- Brown, A.M., Castle, J., Hebbachi, A.M. & Gibbons, G.F. (1999) Administration of n-3 fatty acids in the diets of rats or directly to hepatocyte cultures results in different effects on hepatocellular ApoB metabolism and secretion. *Arterioscler. Thromb. Vasc. Biol.*, **19**, 106–114.
- Caballero, M.J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M. & Izquierdo, M.S. (2002) Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, **214**, 253–271.
- Caballero, M.J., Gallardo, G., Robaina, L., Montero, D., Fernandez, A. & Izquierdo, M. (2006) Vegetable lipid sources affect in vitro biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*). *Br. J. Nutr.*, **95**, 448–454.
- Chen, X.W., Jiang, S. & Shi, Z.Y. (2012) Identification and expression analysis of fabp2 gene from common carp *Cyprinus carpio*. *J. Fish Biol.*, **80**, 679–691.
- Cheung, M.C., Sibley, S.D., Palmer, J.P., Oram, J.F. & Brunzell, J.D. (2003) Lipoprotein lipase and hepatic lipase their relationship with HDL subspecies Lp(A-I) and Lp(A-I, A-II). *J. Lipid Res.*, **44**, 1552–1558.
- Cho, C.Y., Slinger, S.J. & Bayley, H.S. (1982) Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. *Comp. Biochem. Physiol.*, **73**, 25–41.
- Corraze, G. (2001) Lipid Nutrition. In: Nutrition and Feeding of Fish and Crustaceans (Guillaume, J., Kaushik, S., Bergot, P. & Métailler, K. eds), pp. 111–130. Springer Prarois, Chichester.
- Dias, J., Huelvan, C., Dinis, M.T. & Metailler, R. (1998) Influence of dietary bulk agents (silica, cellulose and a natural zeolite) on protein digestibility, growth, feed intake and feed transit time in European seabass (*Dicentrarchus labrax*) juveniles. *Aquat. Living Resour.*, **11**, 219–226.
- Dias, J., Alvarez, M.J., Arzel, J., Corraze, G., Diez, A., Bautista, J.M. & Kaushik, S.J. (2005) Dietary protein source affects lipid metabolism in the European seabass (*Dicentrarchus labrax*). *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.*, **142**, 19–31.
- Enes, P., Panserat, S., Kaushik, S. & Oliva-Teles, A. (2006) Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.*, **143**, 89–96.
- Enes, P., Peres, H., Sanchez-Gurmaches, J., Navarro, I., Gutiérrez, J. & Oliva-Teles, A. (2011) Insulin and IGF-I response to a glucose load in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture*, **315**, 321–326.
- Engelking, L.R. (2010) LDL receptors and HDL. In: Textbook of Veterinary Physiological Chemistry, updated 2nd edn (Engelking, L.R. ed.), pp. 371–376. Academic Press (Elsevier), Amsterdam.
- Figueiredo-Silva, A., Rocha, E., Dias, J., Silva, P., Rema, P., Gomes, E. & Valente, L.M.P. (2005) Partial replacement of fish oil by soybean oil on lipid distribution and liver histology in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquac. Nutr.*, **11**, 147–155.
- Figueiredo-Silva, A.C., Panserat, S., Kaushik, S., Geurden, I. & Polakof, S. (2012) High levels of dietary fat impair glucose homeostasis in rainbow trout. *J. Exp. Biol.*, **215**, 169–178.
- Folch, J., Lees, M. & Sloane-Stanley, G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.*, **226**, 497–509.
- Francis, D.S., Turchini, G.M., Jones, P.L. & De Silva, S.S. (2007) Effects of fish oil substitution with a mix blend vegetable oil on nutrient digestibility in Murray cod, *Maccullochella peelii peelii*. *Aquaculture*, **269**, 447–455.
- Frenais, R., Nazih, H., Ouguerram, K., Maugais, C., Zair, Y., Bard, J.M., Charbonnel, B., Magot, T. & Krempf, M. (2001) In vivo evidence for the role of lipoprotein lipase activity in the regulation of apolipoprotein A1 metabolism: a kinetic study in control subjects and patients with type 2 diabetes mellitus. *J. Clin. Endocrinol. Metab.*, **86**, 1962–1967.
- Furukawa, A. & Tsukahara, H. (1966) On the acid digestion method for the determination of chromic oxide as an indicator substance in the study of digestibility in fish. *Bull. Jpn. Soc. Sci. Fish.*, **32**, 502–506.
- Geay, F., Santigosa, I., Culi, E. et al. (2010) Regulation of FADS2 expression and activity in European sea bass (*Dicentrarchus labrax*, L.) fed a vegetable diet. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **156**, 237–243.

- Geay, F., Ferrarese, S., Zambonino-Infante, J., Bargelloni, L., Quentel, C., Vandepitte, M., Kaushik, S., Cahu, C. & Mazurais, D. (2011) Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. *BMC Genomics*, **12**, 522.
- González-Rovira, A., Mourente, G., Zheng, X., Tocher, D.R. & Pendon, C. (2009) Molecular and functional characterization and expression analysis of a delta-6 fatty acyl desaturase cDNA of European sea bass (*Dicentrarchus labrax* L.). *Aquaculture*, **298**, 90–100.
- van Greevenbroek, M.M. & de Bruin, T.W. (1998) Chylomicron synthesis by intestinal cells in vitro and in vivo. *Atherosclerosis*, **141**, S9–S16.
- Harris, W.S. (1989) Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. *J. Lipid Res.*, **30**, 785–807.
- Harris, W.S. (1997) n-3 Fatty acids and serum lipoproteins: human studies. *Am. J. Clin. Nutr.*, **65**, 1645S–1654S.
- Iqbal, J. & Hussain, M.M. (2009) Intestinal lipid absorption. *Am. J. Physiol. Endocrinol. Metab.*, **296**, E1183–E1194.
- Jordal, A.-E.O., Hordvik, I., Pelsers, M., Bernlohr, D.A. & Torstensen, B.E. (2006) FABP3 and FABP10 in Atlantic salmon (*Salmo salar* L.) – general effects of dietary fatty acid composition, and life cycle variations. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **145**, 147–158.
- Jordal, A.-E.O., Lie, Ø. & Torstensen, B.E. (2007) Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). *Aquac. Nutr.*, **13**, 114–130.
- Jump, D.B. (2008) N-3 polyunsaturated fatty acid regulation of hepatic gene transcription. *Curr. Opin. Lipidol.*, **19**, 242–247.
- Kamalam, B.S., Panserat, S., Aguirre, P., Geurden, I., Fontagné-Dicharry, S. & Médale, F. (2013) Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.*, **164**, 417–427.
- Kjaer, M.A., Vegusdal, A., Gjøen, T., Rustan, A.C., Todorcević, M. & Ruyter, B. (2008) Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochim. Biophys. Acta*, **1781**, 112–122.
- Kortner, T.M., Gu, J., Krogdahl, A. & Bakke, A.M. (2012) Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.). *Br. J. Nutr.*, **30**, 1–12.
- Koven, W.M., Henderson, R.J. & Sargent, J.R. (1994) Lipid digestion in turbot (*Scophthalmus maximus*): I. Lipid class and fatty acid composition of digesta from different segments of the digestive tract. *Fish Physiol. Biochem.*, **13**, 69–79.
- Liland, N.S., Espe, M., Rosenlund, G., Waagbo, R., Hjelle, J.I., Lie, Ø., Fontanillas, R. & Torstensen, B.E. (2013) High levels of dietary phytosterols affect lipid metabolism and increase liver and plasma TAG in Atlantic salmon (*Salmo salar* L.). *Br J Nutr.*, **110**, 1958–1967.
- López-Olmeda, J.F., Egea-Álvarez, M. & Sánchez-Vázquez, F.J. (2009) Glucose tolerance in fish: is the daily feeding time important? *Physiol. Behav.*, **96**, 631–636.
- López-Olmeda, J.F., López-García, I., Sánchez-Muros, M.J., Blanco-Vives, B., Aparicio, R. & Sánchez-Vázquez, F.J. (2012) Daily rhythms of digestive physiology, metabolism and behaviour in the European eel (*Anguilla anguilla*). *Aquacult. Int.*, **20**, 1085–1096.
- Madsen, L., Rustan, A.C., Vaagenes, H., Berge, K., Dyrøy, E. & Berge, R.K. (1999) Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. *Lipids*, **34**, 951–963.
- Martins, D.A., Gomes, E., Rema, P., Dias, J., Ozorio, R.O.A. & Valente, L.M.P. (2006) Growth, digestibility and nutrient utilization of rainbow trout (*Oncorhynchus mykiss*) and European sea bass (*Dicentrarchus labrax*) juveniles fed different dietary soybean oil levels. *Aquacult. Int.*, **14**, 285–295.
- Martins, D.A., Valente, L.M.P. & Lall, S.P. (2009) Apparent digestibility of lipid and fatty acids in fish oil, poultry fat and vegetable oil diets by Atlantic halibut, *Hippoglossus hippoglossus* L. *Aquaculture*, **294**, 132–137.
- Menoyo, D., Lopez-Bote, C.J., Bautista, J.M. & Obach, A. (2003) Growth, digestibility and fatty acid utilization in large Atlantic salmon (*Salmo salar*) fed varying levels of n-3 and saturated fatty acids. *Aquaculture*, **225**, 295–307.
- Menoyo, D., Diez, A., Lopez-Bote, C.J., Casado, S., Obach, A. & Bautista, J.M. (2006) Dietary fat type affects lipid metabolism in Atlantic salmon (*Salmo salar* L.) and differentially regulates glucose transporter GLUT4 expression in muscle. *Aquaculture*, **261**, 294–304.
- Moghadasian, M.H. & Frohlich, J.J. (1999) Effects of dietary phytoestrogens on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. *Am. J. Med.*, **107**, 588–594.
- Montoya, A., Lopez-Olmeda, J.F., Yufera, M., Sanchez-Muros, M.J. & Sanchez-Vazquez, F.J. (2010) Feeding time synchronises daily rhythms of behaviour and digestive physiology in gilthead seabream (*Sparus aurata*). *Aquaculture*, **306**, 315–321.
- Morais, S., Monroig, O., Zheng, X., Leaver, M.J. & Tocher, D.R. (2009) Highly unsaturated fatty acid synthesis in Atlantic salmon: isolation of genes of fatty acyl elongases and characterisation of ELOVL5- and ELOVL2-like elongase cDNAs. *Mar. Biotechnol.*, **11**, 627–639.
- Morais, S., Pratoomyot, J., Taggart, J., Bron, J., Guy, D., Bell, J.G. & Tocher, D. (2011a) Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genomics*, **12**, 255.
- Morais, S., Pratoomyot, J., Torstensen, B.E., Taggart, J.B., Guy, D.R., Bell, J.G. & Tocher, D.R. (2011b) Diet × genotype interactions in hepatic cholesterol and lipoprotein metabolism in Atlantic salmon (*Salmo salar*) in response to replacement of dietary fish oil with vegetable oil. *Br. J. Nutr.*, **106**, 1457–1469.
- Morais, S., Edvardsen, R.B., Tocher, D.R. & Bell, J.G. (2012) Transcriptomic analyses of intestinal gene expression of juvenile Atlantic cod (*Gadus morhua*) fed diets with Camelina oil as replacement for fish oil. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **161**, 283–293.
- Mourente, G. & Dick, J.R. (2002) Influence of partial substitution of dietary fish oil by vegetable oils on the metabolism of [^{14}C] 18:3n-3 in isolated hepatocytes of European sea bass (*Dicentrarchus labrax* L.). *Fish Physiol. Biochem.*, **26**, 297–308.
- Mourente, G., Dick, J.R., Bell, J.G. & Tocher, D.R. (2005a) Effect of partial substitution of dietary fish oil by vegetable oils on desaturation and oxidation of [^{14}C]18:3n-3 and [^{14}C] 20:5n-3 in hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.). *Aquaculture*, **248**, 173–186.
- Mourente, G., Good, J.E. & Bell, J.G. (2005b) Partial substitution of fish oil with rapeseed, linseed and olive oils in diet for European sea bass (*Dicentrarchus labrax* L.) effects on flesh fatty acid composition, plasma prostaglandins E₂ and F_{2a}, immune function and effectiveness of a fish oil finishing diet. *Aquac. Nutr.*, **11**, 25–40.
- Mu, H. & Hoy, C.E. (2004) The digestion of dietary triacylglycerols. *Prog. Lipid Res.*, **43**, 105–133.

- Mullen, E., Brown, R.M., Osborne, T.F. & Shay, N.F. (2004) Soy isoflavones affect sterol regulatory element binding proteins (SREBPs) and SREBP-regulated genes in HepG2 cells. *J. Nutr.*, **134**, 2942–2947.
- Nakamura, M.T. & Nara, T.Y. (2002) Gene regulation of mammalian desaturases. *Biochem. Soc. Trans.*, **30**, 1076–1079.
- Nestel, P.J. (1990) Effects of N-3 fatty acids on lipid metabolism. *Annu. Rev. Nutr.*, **10**, 149–167.
- Ng, W.-K. & Gibon, V. (2010) Palm oil and saturated fatty acid-rich vegetable oils. In: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (Turchini, G.M., Ng, W.-K. & Tocher, D. eds), pp. 99–132. CRC Press, New York, NY.
- Ng, W.-K., Campbell, P.J., Dick, J.R. & Bell, J.G. (2003) Interactive effects of dietary palm oil concentration and water temperature on lipid digestibility in rainbow trout, *Oncorhynchus mykiss*. *Lipids*, **38**, 1031–1038.
- Normén, L., Dutta, P., Lia, A. & Andersson, H. (2000) Soy sterol esters and beta-sitostanol ester as inhibitors of cholesterol absorption in human small bowel. *Am. J. Clin. Nutr.*, **71**, 908–913.
- Olsen, R.E. & Ringø, E. (1997) Lipid digestibility in fish: a review. *Recent Res. Dev. Lipid Res.*, **1**, 199–265.
- Oxley, A., Torstensen, B.E., Rustan, A.C. & Olsen, R.E. (2005) Enzyme activities of intestinal triacylglycerol and phosphatidylcholine biosynthesis in Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **141**, 77–87.
- Oxley, A., Jutfelt, F., Sundell, K. & Olsen, R.E. (2007) Sn-2-monoacylglycerol, not glycerol, is preferentially utilised for triacylglycerol and phosphatidylcholine biosynthesis in Atlantic salmon (*Salmo salar* L.) intestine. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **146**, 115–123.
- Pan, X. & Hussain, M.M. (2012) Gut triglyceride production. *Biochim. Biophys. Acta*, **1821**, 727–735.
- Panserat, S., Perrin, A. & Kaushik, S. (2002) High dietary lipids induce liver glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J. Nutr.*, **132**, 137–141.
- Peres, M.H. & Oliva-Teles, A. (1999) Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (*Dicentrarchus labrax*). *Aquaculture*, **179**, 325–334.
- Peres, H. & Oliva-Teles, A. (2002) Utilization of raw and gelatinized starch by European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture*, **205**, 287–299.
- Peres, M.H., Goncalves, P. & Oliva-Teles, A. (1999) Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). *Aquaculture*, **179**, 415–423.
- Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.*, **29**, E45.
- Ranheim, T., Gedde-Dahl, A., Rustan, A.C. & Drevon, C.A. (1994) Effect of chronic incubation of CaCo-2 cells with eicosapentaenoic acid (20:5, n-3) and oleic acid (18:1, n-9) on triacylglycerol production. *Biochem. J.*, **303**, 155–161.
- Richard, N., Kaushik, S., Larroquet, L., Panserat, S. & Corraze, G. (2006a) Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). *Br. J. Nutr.*, **96**, 299–309.
- Richard, N., Mourente, G., Kaushik, S. & Corraze, G. (2006b) Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture*, **261**, 1077–1087.
- Robaina, L., Corraze, G., Aguirre, P., Blanc, D., Melcion, J.P. & Kaushik, S. (1999) Digestibility, postprandial ammonia excretion and selected plasma metabolites in European sea bass (*Dicentrarchus labrax*) fed pelleted or extruded diets with or without wheat gluten. *Aquaculture*, **179**, 45–56.
- Rozen, S. & Skaletsky, H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology (Kratwitz, S. & Misener, S. eds), pp. 365–386. Humana Press Inc., Totowa, NJ.
- Santha, N.C. & Ackman, R.G. (1990) Nervonic acid versus tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *J. Chromatogr. B Biomed. Appl.*, **533**, 1–10.
- Santulli, A., Curatolo, A., Modica, A. & D'Amelio, V. (1988) Time-course changes of plasma lipid levels and lipoprotein pattern after feeding in cultured sea bass, *Dicentrarchus labrax*. *L. J. Fish Biol.*, **32**, 859–867.
- Seiliez, I., Panserat, S., Corraze, G., Kaushik, S. & Bergot, P. (2003) Cloning and nutritional regulation of a Δ6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus aurata*). *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.*, **135**, 449–460.
- Stadtman, T.C. (1957) Determination of cholesterol and ergosterol by Liebermann-Burchard reaction. *Methods Enzymol.*, **3**, 362–365.
- Sundaram, M. & Yao, Z. (2010) Recent progress in understanding protein and lipid factors affecting hepatic VLDL assembly and secretion. *Nutr. Metab.*, **7**, 35.
- Tocher, D.R. (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.*, **11**, 107–184.
- Tocher, D.R., Zheng, X., Schlechtriem, C., Hastings, N., Dick, J.R. & Teale, A.J. (2006) Highly unsaturated fatty acid synthesis in marine fish: cloning, functional characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). *Lipids*, **41**, 1003–1016.
- Torstensen, B.E., Lie, O. & Froyland, L. (2000) Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.) — effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids*, **35**, 653–664.
- Torstensen, B.E., Froyland, L. & Lie, O. (2004) Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil — effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquac. Nutr.*, **10**, 175–192.
- Torstensen, B.E., Nanton, D., Olsvik, P.A., Sundvold, H. & Stubhaug, I. (2009) Gene expression of fatty acid binding proteins (FABPs), fatty acid transport proteins (cd36 and FATP) and β-oxidation related genes in Atlantic salmon (*Salmo salar* L.) fed fish oil or vegetable oil. *Aquac. Nutr.*, **15**, 440–445.
- Vance, J.E. & Adeli, K. (2008) Assembly and secretion of triacylglycerol-rich lipoproteins. In: Biochemistry of Lipids, Lipoproteins and Membranes, 5th edn (Vance, D.E. & Vance, J.E. eds), pp. 507–532. Elsevier, Oxford.
- Venold, F.F., Penn, M.H., Thorsen, J., Gu, J., Kortner, T.M., Krogdahl, A. & Bakke, A.M. (2013) Intestinal fatty acid binding protein (fabp2) in Atlantic salmon (*Salmo salar*): localization and alteration of expression during development of diet induced enteritis. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.*, **164**, 229–240.
- Xiao, C., Hsieh, J., Adeli, K. & Lewis, G.F. (2011) Gut–liver interaction in triglyceride-rich lipoprotein metabolism. *Am. J. Physiol. Endocrinol. Metab.*, **301**, E429–E446.

Chapter 3-

Effects of fish oil replacement by vegetable oil blend on digestive enzymes and tissue histomorphology of European sea bass (*Dicentrarchus labrax*) juveniles

Fish Physiology and Biochemistry

Effects of fish oil replacement by vegetable oil blend on digestive enzymes and tissue histomorphology of European sea bass (*Dicentrarchus labrax*) juveniles

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Abstract The impact of replacing circa 70 % fish oil (FO) by a vegetable oil (VO) blend (rapeseed, linseed, palm oils; 20:50:30) in diets for European sea bass juveniles (IBW 96 ± 0.8 g) was evaluated in terms of activities of digestive enzymes (amylase, lipase, alkaline phosphatase, trypsin and total alkaline proteases) in the anterior (AI) and posterior (PI) intestine and tissue morphology (pyloric caeca—PC, AI, PI, distal intestine—DI and liver). For that purpose, fish were fed the experimental diets for 36 days and then liver and intestine were sampled at 2, 6 and 24 h after the last meal. Alkaline protease characterization was also done in AI and PI at 6 h post-feeding. Dietary VO promoted higher alkaline phosphatase activity at 2 h post-feeding

in the AI and at all sampling points in the PI. Total alkaline protease activity was higher at 6 h post-feeding in the PI of fish fed the FO diet. Identical number of bands was observed in zymograms of alkaline proteases of fish fed both diets. No alterations in the histomorphology of PC, AI, PI or DI were noticed in fish fed the VO diets, while in the liver a tendency towards increased hepatocyte vacuolization due to lipid accumulation was observed. Overall, and with the exception of a higher intestine alkaline phosphatase activity, 70 % FO replacement by a VO blend in diets for European sea bass resulted in no distinctive alterations on the postprandial pattern of digestive enzyme activities and intestine histomorphology.

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Introduction

In the aquaculture sector, constraints in fish oil (FO) availability and price dictate an urgent need to replace it by more readily available, cheaper and sustainable alternatives such as vegetable oils (VO). However, a drawback of using VO in aquafeeds is their fatty acid (FA) profile, which has a different n3:n6 ratio comparatively to FO, and lacks n-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly

eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which are essential FA for marine fish. Also, changes in dietary FA composition has been reported to affect FA composition of fish storage lipids and of cell membranes, including that of hepatic (Caballero et al. 2002; Kjaer et al. 2008; Fountoulaki et al. 2009; Castro et al. 2015b) and intestinal (Caballero et al. 2003; Ruyter et al. 2006) cells, the histomorphology of the liver (Caballero et al. 2002; Kjaer et al. 2008; Fountoulaki et al. 2009) and digestive tract (Olsen et al. 1999; Moldal et al. 2014). Dietary FO replacement by a single source or by blends of VOs was shown to induce accumulation of lipid droplets in: hepatocytes of rainbow trout (Caballero et al. 2002), Atlantic salmon (Kjaer et al. 2008) and gilthead sea bream (Fountoulaki et al. 2009), and enterocytes of Arctic char (Olsen et al. 1999), rainbow trout (Caballero et al. 2002; Olsen et al. 2003), gilthead sea bream (Caballero et al. 2003; Santigosa et al. 2011) and pikeperch (Kowalska et al. 2010), and to affect gut integrity and induce tissue damage in Arctic char (Olsen et al. 1999). Additionally, reduction of mucosal fold height in the mid-intestine and reduction of wall thickness in the distal intestine were also reported in Atlantic salmon fed diets with olive, rapeseed or soybean oils (Moldal et al. 2014). These alterations may interfere with nutrient digestion, transport, diffusion and absorption efficiency or function and metabolism (Olsen et al. 1999; Caballero et al. 2002, 2003; Jutfelt et al. 2007; Wassef et al. 2007; Geurden et al. 2009; Santigosa et al. 2011).

Studies assessing the effect of dietary FO substitution by VOs on digestive enzyme activities in fish are scarce (Santigosa et al. 2011; Bowyer et al. 2012; Ribeiro et al. 2015). In yellowtail kingfish, dietary coconut oil promoted a decrease in trypsin and lipase activities but had no effect on amylase activity (Bowyer et al. 2012). In meagre, the dietary incorporation of a VO blend had no marked effects on the intestinal alkaline phosphatase activity (Ribeiro et al. 2015). In gilthead sea bream fed diets including a VO blend, lipase and amylase were unaffected, while total alkaline protease (TAP) activity increased in pyloric caeca and decreased in proximal intestine (Santigosa et al. 2011). Furthermore, zymogram determination of TAP composition revealed higher intensity of trypsin- and chymotrypsin-like proteolytic bands in the pyloric caeca of gilthead sea bream fed VO diet, concurring with the higher TAP activity observed in that tissue (Santigosa et al. 2011).

In fish, digestive enzyme activities were shown to be modulated by diet composition (Munilla-Morán and Saborido-Rey 1996a, b; Hidalgo et al. 1999; Fernández et al. 2001; Fountoulaki et al. 2005; Furné et al. 2005; Santigosa et al. 2008; Pérez-Jiménez et al. 2009; Castro et al. 2013) and by intestinal transit time, which determines the digesta arrival to each intestinal section (Einarsson et al. 1996; Fountoulaki et al. 2005; Caruso et al. 2008; Santigosa et al. 2008; Rodiles et al. 2012). The digesta transit time along the gastrointestinal tract is itself also affected by diet composition (Santigosa et al. 2008; Rodiles et al. 2012). All these conditions may influence the digestive and absorptive processes (Castro et al. 2015a).

European sea bass (*Dicentrarchus labrax*) is an economically important carnivorous fish species of aquaculture in Europe. In this species, data obtained so far demonstrate that FO can be replaced by a single source (soybean oil) or a blend (rapeseed, linseed palm and/or olive oils at different proportions) of VO up to 60 % without compromising growth performance and survival (Figueiredo-Silva et al. 2005; Mourente et al. 2005; Richard et al. 2006b; Castro et al. 2015b). While the impact of such substitutions at metabolic level, mainly in liver, has been assessed (Richard et al. 2006b; Castro et al. 2015b), little information is available on the potential effects in physiological functions of intestine (Castro et al. 2015a). Thus, this study aimed to evaluate the effect of replacing circa 70 % fish oil (FO) by a VO blend on the activities of digestive enzymes of pancreatic (total alkaline proteases, trypsin, amylase and lipase) and brush border membrane (alkaline phosphatase) origin and on the histomorphology of different sections of the digestive tract and liver of European sea bass juveniles.

Materials and methods

Experimental diets

Two isoproteic (45 % crude protein; CP) and isolipidic (18 % crude lipids; CL) diets were formulated to contain 20 % gelatinized maize starch and FO or VO blend as lipid sources (Table 1). The VO blend was composed of rapeseed, linseed and palm oils (20:50:30) and replaced circa 70 % of dietary lipids provided by cod liver oil and fish meal. The diets were similar in saturated fatty acids (SFA) content, but,

comparatively to the VO diet, the FO diet had higher levels of eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and the monounsaturated fatty acids (MUFA) 20:1 and 22:1 and lower levels of linoleic acid (LA, 18:2n-6),

Table 1 Proximate composition of the experimental diets

Diets	FO	VO
<i>Ingredients (% dry weight)</i>		
Fish meal ^a	63.6	63.6
Gelatinized maize starch ^b	20	20
Cod liver oil ^c	12.4	0.4
Vegetable oil blend ^d	—	12.0
Vitamin premix ^e	1.0	1.0
Mineral premix ^f	1.0	1.0
Choline chloride (50 %)	0.5	0.5
Binder ^g	1.0	1.0
Chromium	0.5	0.5
<i>Proximate analyses (% dry matter)</i>		
Dry matter (DM)	87.5	87.2
Crude protein (CP)	46.2	47.1
Crude lipid (CL)	17.9	18.2
Ash	13.9	13.7
Starch	20.4	19.8
Gross energy (MJ kg ⁻¹)	21.4	21.0

^a Steam-dried low-temperature fish meal, Pesquera Diamante, Perú (CP: 70.2 % DM; CL: 8.08 % DM)

^b C-Gel Instant-12018, Cerestar, Mechelen, Belgium

^c Fagron Iberica S.A.U., Spain

^d 30 % palm oil (Colmi, Malaysia), 50 % linseed oil (Sociedade Portuense de Drogas, S.A., Portugal) and 20 % rapeseed oil (Huilerie Emile Noël S.A.S., France)

^e Vitamins (mg kg⁻¹ diet): retinol acetate, 18,000 (IU kg⁻¹ diet); cholecalciferol, 2000 (IU kg⁻¹ diet); alpha-tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamine-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400. Premix, Viana do Castelo, Portugal

^f Minerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.40 (g kg⁻¹ diet). Premix, Viana do Castelo, Portugal

^g Aquacube (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulphate). Agil, England

linolenic acid (LNA, 18:3n-3) and oleic acid (OA, 18:1n-9) (Table 2). Details on diet preparation and analysis are given in Castro et al. (2015a).

Animals, experimental conditions and sampling

The experiment was directed by accredited scientists (following FELASA category C recommendations) and conducted according to the European Union

Table 2 Fatty acid composition (% of total fatty acids) of the experimental diets

Diets	FO	VO
14:0	5.3	1.4
15:0	0.4	0.1
16:0	13.7	14.1
17:0	0.3	0.2
18:0	3.0	3.7
20:0	0.1	0.3
Sum saturates (Sat)	22.9	19.9
16:1	7.6	2.1
18:1	18.7	27.0
20:1	7.1	0.8
22:1	5.4	0.2
Sum monoenes	39.0	30.2
16:2 n-4	0.5	0.2
16:3 n-4	0.9	0.3
16:4 n-1	0.8	0.4
Sum other PUFA	2.4	1.0
18:2 n-6	2.1	10.6
18:3 n-6	0.2	0.1
20:2 n-6	0.2	0.1
20:4 n-6	0.7	0.5
Sum n-6 PUFA	3.3	11.4
18:3 n-3	0.8	21.1
18:4 n-3	2.0	0.6
20:4 n-3	0.7	0.2
20:5 n-3	10.2	5.3
21:5 n-3	—	0.2
22:5 n-3	0.8	0.7
22:6 n-3	12.3	7.6
Sum n-3 PUFA	26.9	35.6
Sat/PUFA	0.7	0.4
n3/n6	8.2	3.1

PUFA polyunsaturated fatty acids

Directive (2010/63/EU) on the protection of animals for scientific purposes, at the Marine Zoological Station, University of Porto, Portugal. European sea bass (*D. labrax*) juveniles with a mean initial body mass of 96 ± 0.8 g were placed in a thermoregulated recirculation water system equipped with a battery of twelve fibreglass tanks of 60 L capacity and supplied with continuous flow of filtered seawater. After 2 weeks of adaptation to the experimental conditions, 16 fish were randomly distributed to each tank and each diet was randomly assigned to triplicate groups of fish. The trial lasted 36 days, and fish were hand-fed to satiation twice a day. During the trial, water temperature averaged 24.1 ± 0.9 °C, salinity averaged 34.7 ± 0.8 g L⁻¹ and dissolved oxygen was kept near saturation. The photoperiod was the natural one for May to June. This study is part of a bigger study, and further information on nutrient digestibility, serum metabolites and hepatic and intestinal gene expression analysis of key genes involved in lipid transport and lipoprotein metabolism is presented elsewhere (Castro et al. 2015a).

At the end of the trial, two fish from each tank were randomly sampled at 2, 6 and 24 h after the last meal and euthanized by a sharp blow to the head. Fish were dissected on chilled trays, and the liver and digestive tract were excised and freed from adherent adipose and connective tissues. For digestive enzyme analyses, the intestine was divided in two sections: anterior intestine (AI, section between the last pyloric caeca and the mid-line of the intestinal length) and posterior intestine (PI, section from the mid-line of the intestinal to distal intestine). Each section with intestinal content was immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

For histological analyses, liver and intestine samples were collected from the fish euthanized at 6 h post-feeding. For intestinal evaluation, two pyloric caeca, and sections with approximately 0.5 cm of AI, PI and distal intestine (DI, collected from the last part of the posterior intestine, visually distinguished by a darker and thicker mucosa) were collected. Tissue samples were rinsed in phosphate-buffered saline (PBS), blotted dry with a paper towel, immediately fixed in phosphate-buffered formalin (4 %, pH 7.4) for 24 h and then transferred to ethanol (70 %) until further processing. An additional section of the liver was collected to cryotubes containing 10 % formal + 30 % saccharose + 1 % CaCl₂, left at 4 °C

for 48 h, then transferred to frozen embedding medium (Thermo Scientific™ Shandon Cryomatrix™) and stored at -20 °C until processing.

Digestive enzyme activities

Each section of the digestive tract was homogenized (dilution 1:9, w/v) in ice-cold buffer (100 mM Tris-HCl, 0.1 mM EDTA and 0.1 %Triton-X-100 (v/v), pH 7.8) and centrifuged at 30,000g for 30 min at 4 °C. The resultant supernatants were collected, and aliquots were stored at -80 °C until digestive enzyme analysis.

For each enzyme activity, assay dilution tests were previously done to ensure optimum ratio between enzyme and substrate. All enzyme activities were measured at 37 °C in a microplate reader (ELx808; Bio-Tek Instruments). The specific assay conditions for each enzyme were as follows:

α -Amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain), with modification in the proportion of supernatants and assay buffer (200 µL of the assay buffer with 10 µL of supernatants). The rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm.

Lipase (EC 3.1.1.3) activity was determined using a commercial kit (ref. 1001275, Spinreact, Girona, Spain) with modification in the proportion of supernatants and assay reactives (200 µL of the assay buffer, 40 µL substrate with 10 µL of supernatants). 1-2-O-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin)-ester was used as substrate, and the formation rate of methylresorufin was followed at 580 nm.

Trypsin (EC 3.4.21.4) activity was determined according to Faulk et al. (2007), using 1 mM $\text{N}\alpha$ -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) as substrate combined with 50 mM Tris-HCl, 20 mM CaCl₂, pH 8.2 buffer. Production of p-nitroaniline was monitored at 410 nm.

Alkaline phosphatase activity was determined using a commercial kit from Sigma (Sigma procedure no. 104) as previously described by Krogdahl et al. (2003). The reaction was measured at 420 nm using p-nitrophenyl phosphate as substrate and p-nitrophenol as standard.

Total alkaline protease activity was determined by the casein-hydrolysis method described by Walter (1984) and adapted by Hidalgo et al. (1999). A reaction mixture containing 0.25 mL casein at 1 % (w/

v), 0.25 mL buffer (0.1 M Tris HCl, pH 9) and supernatant from the homogenates (0.1 mL) was incubated for 1 h at 37 °C. A control blank for each sample was assayed adding the supernatant from the homogenates after the incubation time. The reaction was stopped by the addition of 0.6 mL 8 % (w/v) trichloroacetic acid solution in blanks and reaction samples. After being kept for 1 h at 4 °C, blanks and reaction samples were centrifuged at 1800g for 10 min and the absorbance of supernatants measured at 280 nm. Tyrosine solution was used as standard.

Unit (U) of enzyme activity was defined as μmol of product generated per minute under the measurement conditions described above and expressed per mg soluble protein (specific activity). Protein concentration was determined according to Bradford (1976) using Sigma protein assay kit and bovine serum albumin as standard.

Protease inhibition, SDS-PAGE and casein zymography

Alkaline protease zymograms were obtained after resolving by SDS-PAGE the homogenates of AI and PI of fish sampled at 6 h post-feeding (Moyano et al. 1996). Prior to electrophoresis, a range of specific inhibition solutions were used and selected according to Alarcón et al. (1998). Thus, 10 mM TLCK (N-alpha-tosyl-L-lysyl-chloromethylketone) was used to inhibit trypsin-like serine proteases; 10 mM ZPCK (N-carbobenzoxy-L-phenylalanine chloromethyl ketone) was used to inhibit chymotrypsin-like serine proteases, and 100 mM PMSF (phenylmethanesulfonyl fluoride) was used to inhibit serine proteases in general. Inhibition reactions were prepared by combining 40 μL of the intestinal homogenate containing 35 mg of soluble protein with 10 μL of each inhibitor (TLCK, ZPCK or PMSF) or with 10 μL of H₂O (control). Following 45-min incubation at room temperature, each sample mixture (with or without inhibitor) was mixed with sample loading buffer (2 % SDS, 0.125 M Tris, 5 % glycerol, 0.025 % bromophenol blue) and loaded on 12.5 % polyacrylamide gel for protein resolution by SDS-PAGE, according to Laemmli (1970) and adapted by García-Carreño et al. (1993). The full-range Amersham Rainbow Marker (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to estimate the molecular weight of proteins. Electrophoresis was performed on a Mini-PROTEAN® Tetra Cell (Bio-

Rad Laboratories, Inc.) at 25 mA per gel during 120 min. After electrophoresis, the gels were washed 3 × 10 min with 2.5 % Triton-X-100 to remove SDS and then incubated for 30 min at 4 °C, under agitation, with a 2 % casein substrate solution prepared in 50 mM TrisHCl, pH 8.2. The gels were then kept for additional 90 min at room temperature with shaking. Following substrate incubation, the gels were stained for 50 min with staining solution (0.3 % Coomassie blue R-250, 50 % ethanol, 10 % acetic acid) and de-stained using a mixture of 30 % ethanol and 10 % acetic acid, for protein visualization. The effect of each inhibitor on digestive proteases was visualized by partial or total disappearance of one or more hydrolysis bands, when compared to the profile of enzyme extract pre-incubated with distilled water.

Histology

Samples were processed and sectioned using standard histological techniques and stained with haematoxylin and eosin (H-E). Cryopreserved liver samples were sectioned with cryostat (CM3050 S, Leica, Deerfield, IL), placed on slides, air-dried for 30 min and stored at -20 °C until Sundan black staining (SB). SB was performed as follows: slides were washed in water, passed through ethanol 70 %, stained in SB (Merck 1387, Darmstadt, Germany; 3 % w/v in ethanol 70 %), washed in ethanol 70 %, passed through water and mounted with aqueous mounting media (S3025-Dako).

Liver and intestinal sections were subjected to a double-blinded evaluation using a semi-quantitative scoring system ranging from 1 to 5. Score 1 was considered the normal tissue appearance, and subsequent scores were accounted for increasing alterations of normal tissue histomorphology. Intestinal samples were evaluated according to the criteria suggested by Krogdahl et al. (2003): widening and shortening of the intestinal folds, loss of the supranuclear vacuolization in the absorptive cells (enterocytes) in the intestinal epithelium, widening of the lamina propria in the intestinal folds and infiltration of mixed leucocyte population in the lamina propria and submucosa.

Liver samples were evaluated for general histomorphology, giving particular attention to cytoplasm vacuolization of the hepatocytes and any signs of inflammation. The SB sections were used to confirm the nature of hepatocellular vacuolization observed in H-E-stained preparations.

Statistical analysis

Digestive enzyme activity data were checked for normal distribution and homogeneity of variances (Shapiro–Wilk and Levene tests, respectively) and normalized when appropriate. The effects of dietary lipid source (LS) sampling time (TIME) and intestinal section (SECTION) on digestive enzyme activities were analysed by 3×3 factorial analysis (three-way ANOVA). Significant differences between means were evaluated by the Tukey multiple range test. In case of significant interaction, a one-way ANOVA was performed to evaluate differences between time and section. Histological data were neither normal nor homogeneous; thus, the Kruskal–Wallis nonparametric test was used. The significance level of 0.05 was used for rejection of the null hypothesis. All statistical analyses were done using the IBM SPSS 21 software package (SPSS® Inc, Chicago, IL, USA) for Windows.

Results

Dietary VO inclusion had no effect on amylase, lipase, total alkaline proteases and trypsin activities ($\text{mU mg protein}^{-1}$) in the AI and PI. Contrarily, alkaline phosphatase activity was higher in both intestinal sections in fish fed the VO diet (Table 3).

Differences in digestive enzyme activities among AI and PI sections were mostly time-related (time \times section interaction) (Table 3). At 2 h post-feeding, amylase, lipase, alkaline phosphatase and trypsin activities were higher in AI than in PI, while at 6 and 24 h after feeding, lipase and total alkaline protease activities were lower in AI than in PI (Fig. 1). At 6 and 24 h post-feeding, alkaline phosphatase and trypsin activities were still higher in AI than in PI, respectively (Fig. 1). Differences among AI and PI sections on amylase (at 6 and 24 h post-feeding), total alkaline proteases (at 2 h post-feeding), alkaline phosphatase (at 24 h post-feeding) and trypsin (at 6 h post-feeding) activities were not observed (Fig. 1). Time \times LS confirmed that there were no significant differences in these enzymes among AI and PI.

In AI, digestive enzyme activities were similar at 2 and 6 h after feeding, and lowest values were observed at 24 h (Fig. 1). In PI, digestive enzyme activity tended to increase from 2 to 6 h post-feeding and lowest values were observed at 24 h (Fig. 1).

Zymogram analysis of alkaline proteases present in AI and PI sections of fish fed the two diets showed that the number of detectable active proteases was not affected by the dietary lipid source. Five bands with proteolytic activity against a casein substrate were detected in both intestinal sections, with molecular weights ranging between 17 and 52 kDa (band numbers 1–5, Fig. 2). All these proteins are predicted to be serine proteases, since their hydrolytic activity was reduced upon addition of PMSF, an inhibitor of serine protease activity. An additional band with an estimated molecular weight of less than 17 kDa (band number 6, Fig. 2) was visible in PI section. This band was apparently not inhibited by any of the inhibitors used (TLCK, ZPCK or PMSF), indicating that it probably corresponds to a nonserine protease (different catalytic residue). The two bands with estimated molecular weight between 17 and 24 kDa (bands 4 and 5, Fig. 2) were identified as chymotrypsin-like serine enzymes, as observed by a reduction in the band intensity when using the chymotrypsin-specific inhibitor ZPCK. On opposite, the band immediately above (band 3, Fig. 2), with an estimated molecular weight higher than 24 kDa, corresponds most probably to a trypsin-like serine protease, since a reduction in its intensity could be observed with TLCK (trypsin inhibitor). The two uppermost bands (bands 1 and 2, Fig. 2), with highest molecular weights, could putatively be identified as serine protease enzymes (other than trypsin-like or chymotrypsin-like), since their intensity was only reduced with PMSF treatment.

The inclusion of VO in the diets had no effects on the intestinal histomorphology (Table 4; Fig. 3). Fish from both dietary treatments showed similar histological features, with long, well-defined villus without fusion. Lamina propria and submucosa presented normal thickness without increased cellularity. The frequency of eosinophilic granulocytes and intraepithelial leucocytes was similar in fish from both dietary groups.

Both dietary treatments resulted in great hepatocyte vacuolization, and the group fed VO diets exhibited a tendency ($p = 0.071$) towards higher degree of vacuolization than the FO group (Fig. 4). The cytoplasmic vacuoles appeared clear and optically empty, often pushing the nucleus to the cell periphery without altering the shape of the nuclei. The Sudan black staining (Fig. 4c, d) confirmed the lipidic nature of the material contained in the cytoplasmatic vacuoles,

Table 3 Postprandial specific digestive enzyme activities (mU mg^{-1} protein) in the anterior and posterior intestine of European sea bass fed the experimental diets

Enzymes		Amylase			Lipase			Alkaline phosphatase		
Section	LS	2 h	6 h	24 h	2 h	6 h	24 h	2 h	6 h	24 h
AI	FO	42 ± 25	34 ± 12	3.3 ± 1.7	3.9 ± 2.3	3.5 ± 1.4	0.4 ± 0.1	199 ± 100	242 ± 46	46 ± 12
	VO	36 ± 8	35 ± 16	3.9 ± 1.8	3.8 ± 0.7	2.8 ± 1.0	0.4 ± 0.1	311 ± 102	227 ± 104	73 ± 37
PI	FO	26 ± 15	28 ± 19	3.1 ± 1.6	2.4 ± 1.6	5.7 ± 2.8	0.5 ± 0.1	117 ± 30	150 ± 55	57 ± 12
	VO	11 ± 6	28 ± 17	4.1 ± 1.9	1.1 ± 0.7	6.5 ± 4.5	0.5 ± 0.1	121 ± 45	214 ± 65	68 ± 21
<i>p value</i> *										
LS		0.470			0.174			0.020		
TIME		<0.001			<0.001			<0.001		
SECTION		0.007			0.867			<0.001		
LS × TIME		0.164			0.428			0.509		
LS × SECTION		0.477			0.269			0.607		
TIME × SECTION		0.043			<0.001			0.002		
LS × TIME × SECTION		0.368			0.180			0.019		
Enzymes		Total alkaline proteases			Trypsin					
Section	LS	2 h	6 h	24 h	2 h	6 h	24 h	2 h	6 h	24 h
AI	FO	193 ± 49	188 ± 30	31 ± 4	133 ± 138	162 ± 98	5.8 ± 1.0			
	VO	158 ± 29	178 ± 57	37 ± 14	106 ± 41	140 ± 67	5.8 ± 0.6			
PI	FO	167 ± 47	341 ± 132	80 ± 27	72 ± 94	312 ± 177	3.9 ± 1.0			
	VO	126 ± 20	252 ± 67	79 ± 19	18 ± 22	294 ± 207	4.2 ± 0.5			
<i>p value</i> *										
LS		0.100				0.182				
TIME		<0.001				<0.001				
SECTION		<0.001				0.022				
LS × TIME		0.230				0.384				
LS × SECTION		0.519				0.297				
TIME × SECTION		<0.001				<0.001				
LS × TIME × SECTION		0.991				0.255				

Values represent mean ± SD ($n = 6$)

LS dietary lipid source, FO fish oil, VO vegetable oil blend, AI anterior intestine, PI posterior intestine, TIME sampling time, SECTION intestinal section

* Significant differences ($p < 0.05$). Results of one-way ANOVA performed to evaluate differences between sampling time and intestinal section are given in the “Results” section and Fig. 1

showing that fish fed the VO diet had a tendency to higher hepatic lipid accumulation than fish fed FO diet.

Discussion

A few studies have already evaluated the effect of dietary protein source on the profile of digestive enzyme activities over time in fish (Santigosa et al. 2008; Rodiles et al. 2012). But, as far as we are aware,

this is the first study addressing the effect of dietary lipid source on digestive enzyme activities over time carried out in fish. Accordingly, partial or total replacement of fish meal (FM) by plant protein sources in the diets for rainbow trout induced a drastic decrease in postprandial alkaline protease activity and a lack of enzyme activity peak when dietary FM was totally replaced by plant protein (Santigosa et al. 2008). On the contrary, the same authors observed in gilthead sea bream similar postprandial protease activity profiles, independently of diet composition,

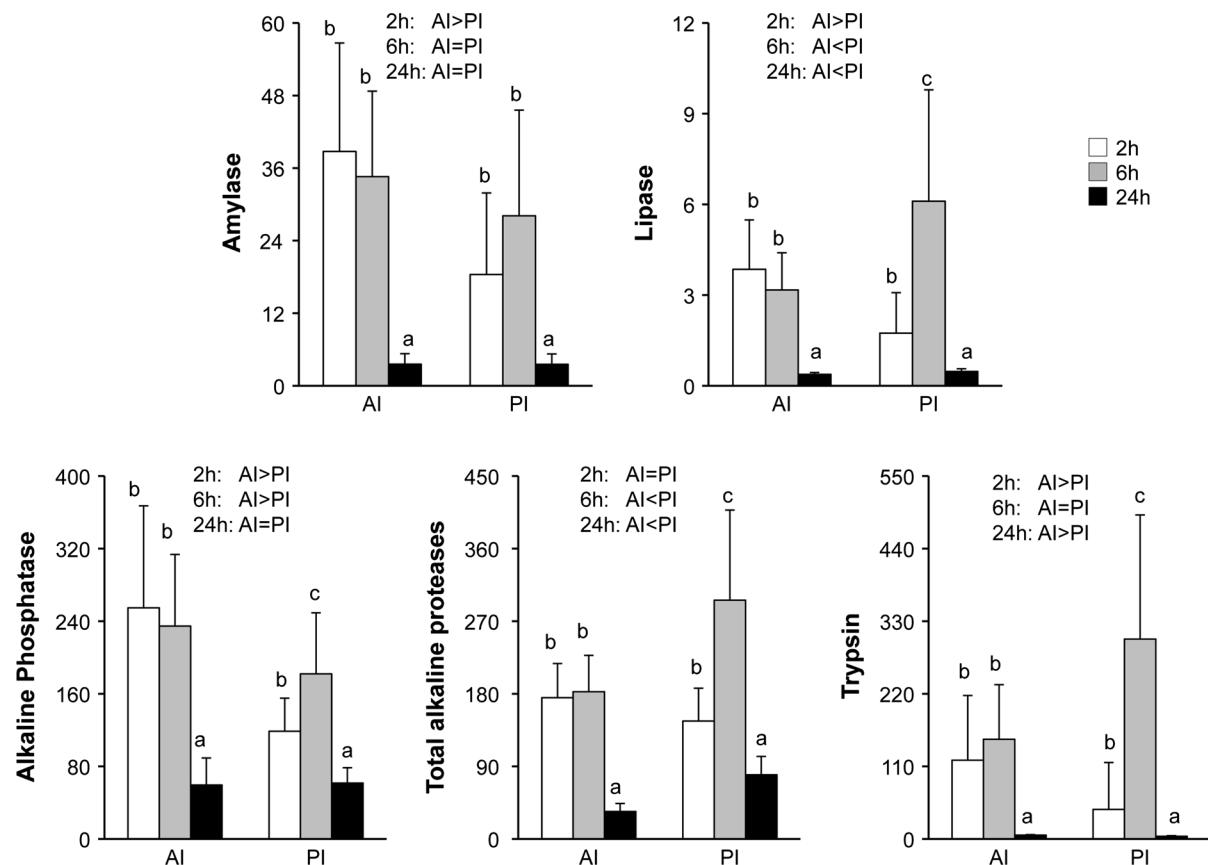


Fig. 1 Summary of the statistical analyses of the specific digestive enzymes (mU mg^{-1} protein) in European sea bass whose activities showed a significant sampling time (TIME) \times intestinal section (SECTION) interaction* (means of the fish fed two experimental diets, $n = 12$). Anterior (AI) and posterior (PI) intestine. *Identified by three-way ANOVA.

Values are mean \pm SD. Significant differences ($p < 0.05$) among sampling times are indicated by different lower-case letters, while within each sampling times, significant differences ($p < 0.05$) between AI and PI are indicated by symbols ($>$ or $<$), and the lack of differences ($p > 0.05$) between AI and IP is indicated by symbol (=)

though a gradual decrease in alkaline protease activity peak was observed as dietary plant protein increased (Santigosa et al. 2008). The authors also observed a postprandial peak in amylase activity in both species when fed FM-based diets, but no clear peak in amylase activity was observed when fed plant-protein-based diets. In Senegalese sole, different postprandial profiles of total proteases, trypsin and chymotrypsin activities in fish fed FM-based diets or diets with FM partially replaced by plant protein sources were also reported (Rodiles et al. 2012).

It is reasonable to assume that differences in dietary fatty acid (FA) composition may induce changes in digesta residence time along the digestive tract and, consequently, distinctive digestive enzyme activity profiles over time. However, present results indicate a

lack of time-related changes in digestive enzyme activities in response to dietary lipid source. On the other hand, the postprandial digestive enzyme activity profiles evidenced that enzyme secretion was stimulated by the presence of nutrients in the digestive tract. The similar digestive enzyme activities in the AI at 2 and 6 h post-feeding suggest that digesta was still arriving to the AI at 6 h post-feeding, while the higher enzyme activity in the PI at 6 h post-feeding suggests that at 2 h only a small amount of digesta was present in that region, while at 6 h post-feeding PI was full of digesta. The very low activity values of digestive enzymes at 24 h post-feeding in both sections of the intestine indicate that, by then, digestive processes were already accomplished and that pre-feeding enzyme activity values were achieved.

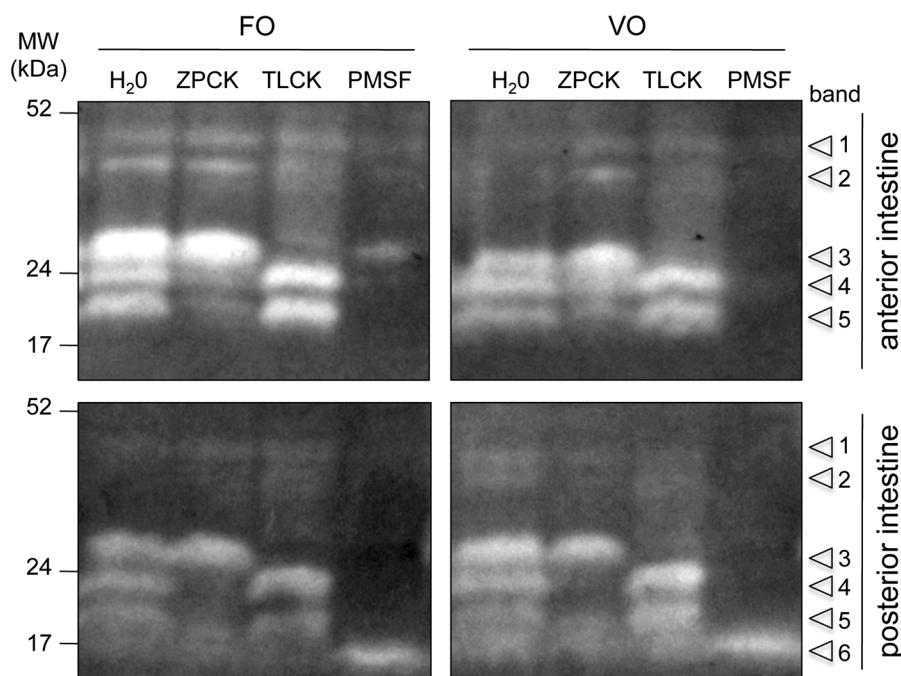


Fig. 2 Zymograms of alkaline proteases in anterior (AI) and posterior (PI) intestine homogenates of European sea bass fed the fish oil (FO) and the vegetable oil blend (VO) diets at 6 h post-feeding and treated with different specific inhibitors:

chymotrypsin inhibitor (ZPCK), trypsin inhibitor (TLCK), general serine protease inhibitor (PMSF) or water (H_2O) used as control. MW molecular weight in kDa

Table 4 Mean values of histomorphological semi-quantitative evaluation of the pyloric ceca (PC), anterior intestine (AI), posterior intestine (PI), distal intestine (DI) and liver (Li) of European sea bass fed the experimental diets

	PC	AI	PI	DI	Li
FO	1.7 ± 0.2	1.5 ± 0.2	1.9 ± 0.2	1.6 ± 0.4	3.0 ± 0.9
VO	1.5 ± 0.2	1.5 ± 0.3	1.7 ± 0.2	1.6 ± 0.3	3.8 ± 0.4
p value*					
LS	0.163	0.329	0.411	0.936	0.071

Values represent mean ± SD ($n = 6$)

LS dietary lipid source, FO fish oil, VO vegetable oil blend

* Significant differences ($p < 0.05$)

Digestive enzymes that are synthesized in fish exocrine pancreas are secreted into the anterior section of the digestive tract (Kuz'mina 2008; Bakke et al. 2010). Accordingly, this section seems to contribute more for nutrient digestion and absorption than more posterior sections, as demonstrated by a number of histomorphological, enzymatic and nutrient digestibility studies along the digestive tract of fish (Olsen et al. 1999; Nordrum et al. 2000; Chikwati et al. 2013; Hartviksen et al. 2014). In the present study, activities

of digestive enzymes in both intestinal sections were in general high and differences among intestinal sections were mostly time-related (time × section interaction), reinforcing both the stimulation of pancreatic secretions by the feed ingestion and the evolution of digesta along the digestive tract. Activity of the selected digestive enzymes of pancreatic (total alkaline proteases, trypsin, amylase and lipase) and enterocyte brush border membrane (BBM) (alkaline phosphatase) origin in the present study has also been

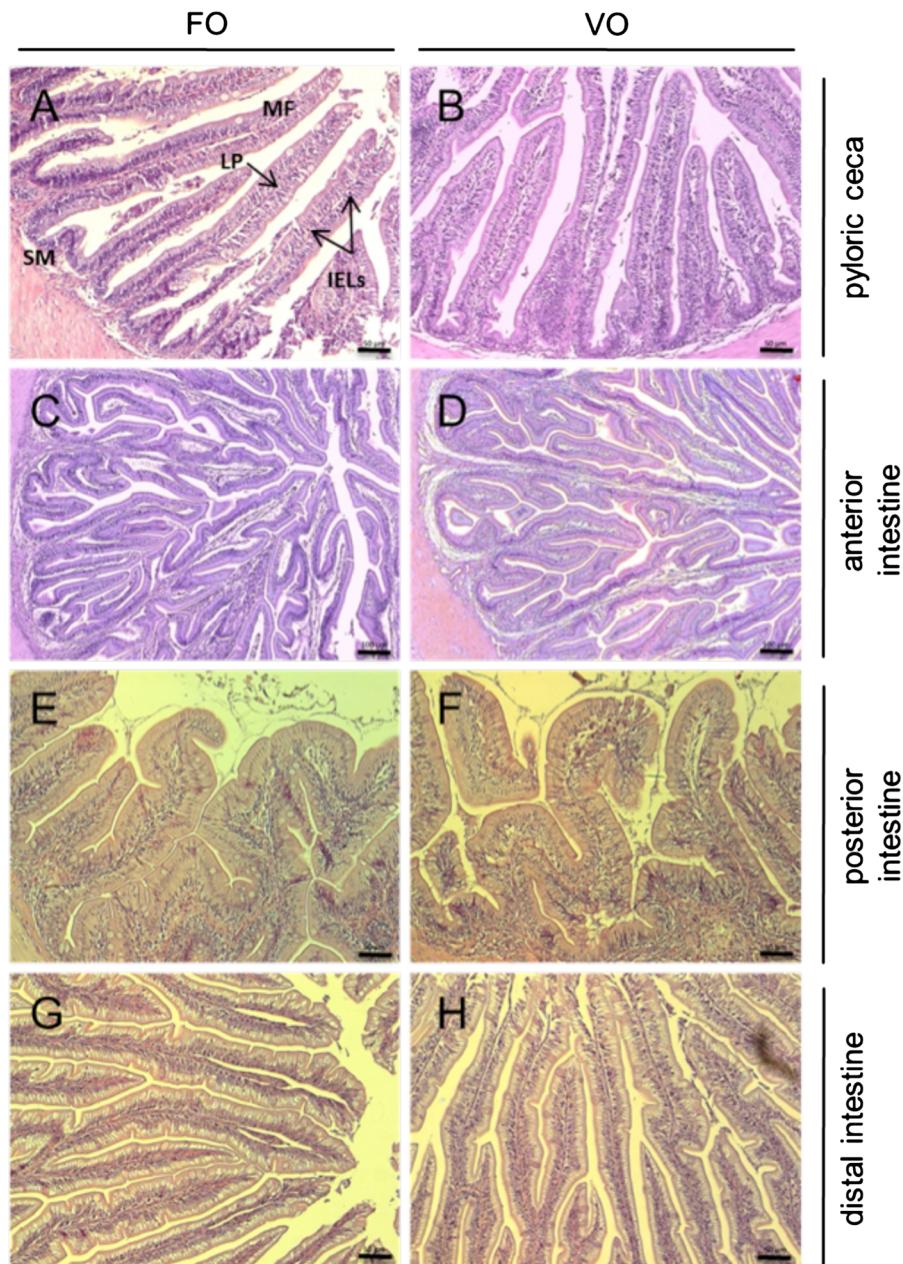


Fig. 3 Histomorphological features of the pyloric ceca (**a**, **b**), anterior intestine (**c**, **d**), posterior intestine (**e**, **f**) and distal intestine (**g**, **h**) of European sea bass fed the fish oil—FO (**a**, **c**, **e**,

g) or the vegetable oil—VO (**b**, **d**, **f**, **h**) diets. *MF* mucosal fold, *SM* submucosa, *LP* lamina propria, *IELs* intraepithelial leucocytes. Scale **a**, **b**, **e**, **f**, **g**, **h** 50 μ m; **c**, **d** 100 μ m; H-E staining

detected throughout the digestive tract in several fish species (Krogdahl et al. 2005; Pérez-Jiménez et al. 2009).

The digestive enzyme activity profiles in response to a single meal fit well with the postprandial profiles of serum metabolites (reported in Castro et al. 2015a). However, present results do not allow linking the

distinctive postprandial profile of serum lipids observed in fish fed the two experimental diets to different specificities of lipases in relation to FA profile of the tested diets. Lipase specificity is known to change in function of the unsaturation degree and of the chain length of dietary FA (Koven et al. 1994; Olsen and Ringø 1997; Tocher 2003). However,

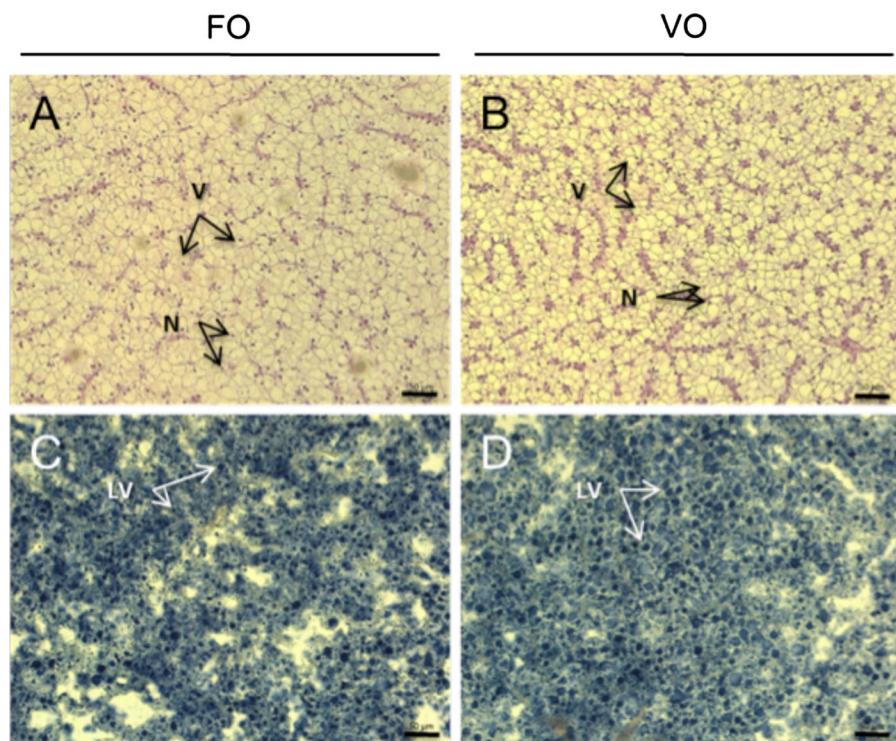


Fig. 4 Histomorphological features of the liver of European sea bass fed fish oil—FO (**a, c**) or vegetable oil—VO (**b, d**). V cytoplasmatic vacuoles, N nuclei, LV lipid-filled vacuoles. Scale bar 50 μ m; H-E (**a, b**) and Sudan Black (**c, d**) stainings

present results did not evidence a lipase activity modulation in relation to dietary lipids. This was not unexpected, as FA chain length and unsaturation degree of the dietary lipids were not much different. These results are also in accordance with the small differences recorded in apparent lipid digestibility of the tested diets (see Castro et al. 2015a). Similarly, no difference on lipase activity was observed in gilthead sea bream juveniles fed diets including FO or a blend of VO (Santigosa et al. 2011). On the other hand, in yellowtail kingfish, lower lipase activity was observed in fish fed diets with canola oil than with FO (Bowyer et al. 2012). On the contrary, in European sea bass larvae, an increment in lipase activity was observed with the ingestion of coconut oil (Morais et al. 2004).

In the present study, no differences in the number of alkaline proteases present in AI or PI sections of intestine were observed between dietary treatments. Also, and consistent with zymogram results, no effect on trypsin activity was detected with the dietary replacement of FO by VO. On the contrary, a decrease in trypsin activity was observed in yellowtail kingfish juveniles fed a diet with canola oil (Bowyer et al.

2012) and in Senegalese sole larvae fed a diet with soybean oil (Morais et al. 2006). The presence of anti-nutritional factors (ANFs), in particular trypsin inhibitors, which may be fat soluble in some types of oils, could explain the differences between the two previous studies and the present results. However, in gilthead sea bream, Santigosa et al. (2011) reported higher total alkaline protease activity in the pyloric caeca of fish fed VO than FO diets, while the opposite was observed in the proximal intestine. Inhibition of luminal proteases due to the presence of ANFs in VO diets was at first hypothesized as the possible cause for the observed differences. However, the authors concluded that the increment in proteolytic activity in pyloric caeca and the higher intensity of trypsin- and chymotrypsin-like proteolytic bands in zymograms promoted by the VO were mainly linked to a slower release of proteases into the intestinal lumen in the VO group due to a decrease in digesta transit rate.

Due to its close nature with the hydrophobic core of intestinal microvillus membrane, the activity of alkaline phosphatase is often used as indicator of intestinal membrane integrity. Available data on the effect of

dietary plant protein (Krogdahl et al. 2003) or lipid level (Cahu et al. 2000) on alkaline phosphatase indicate a decline in activity as indicator of malnutrition. According to Cahu et al. (2000), the decline in alkaline phosphatase activity in fish fed high-lipid diets was due to modifications of FA composition of brush border membrane and, consequently, modification in membrane fluidity. In rainbow trout, Ducasse-Cabanot et al. (2007) found that alkaline phosphatase activity in the AI decreased with the removal of FO from the diet. The authors pointed that the lower digestive capacity in the enterocyte membrane of fish fed low-lipid diets was related to the lower levels of dietary lipids rather than to a modification in the enterocyte brush border membrane FA composition, which remained unchanged. Contrarily to the present data, a recent study from Ribeiro et al. (2015) reported that alkaline phosphatase activity in intestine of meagre was not affected when dietary FO was replaced by a VO blend (rapeseed, linseed and palm oils). Although increased alkaline phosphatase activity in our study may suggest some modification at the enterocyte level in response to the presence of VO in the diet, no histopathological alterations were observed (see below). Even so, and considering that alkaline phosphatase is involved in several other cellular functions, further studies are required to understand potential mechanisms of action of dietary VO.

Although accumulation of lipid droplets in the enterocytes due to dietary VO has been reported in previous studies in other fish species (Caballero et al. 2003; Kowalska et al. 2010), in the present study, dietary VO did not affect intestinal histomorphology of European sea bass. A previous study in this species (Mourente et al. 2007) using identical VO blend and dietary inclusion levels also found no major histomorphological alterations on mid- or distal intestine, just increased absorptive vacuolization in the proximal intestine. Apart from species differences, the degree of lipid droplet accumulation in the enterocytes was also shown to vary with the level and type of dietary FA composition (Caballero et al. 2002, 2003; Santigosa et al. 2011). Accordingly, Caballero et al. (2003) found high accumulation of lipid droplets in gilthead sea bream fed diets containing linseed or soybean oils replacing 60 % FO, and even higher accumulation when fish were fed the same VO replacing 80 % FO, or with rapeseed oil at 60 %. Also in gilthead sea

bream, Santigosa et al. (2011) found increased lipid droplet content in proximal intestine as the percentage of dietary VO blend increased, replacing from 33 to 100 % of dietary FO. These histomorphological alterations are thought to occur due to modifications in lipid transport mechanisms, more precisely with interferences of dietary lipid source on the intestinal lipoprotein assembly, namely in the reacylation mechanisms of the absorbed lipids (Caballero et al. 2003, 2006). The lack of alterations on tissue histomorphology due to dietary treatments in the present study is in accordance with the lack of transcriptional modifications of proteins related to lipid transport and lipoprotein metabolism (FABP, DGAT, ApoB, ApoA4, ApoA1, MTP) (see Castro et al. 2015a).

The effects of dietary VO on fish liver histomorphology are very diverse. In European sea bass fed diets with dietary soybean oil replacing 50 % of FO (Figueiredo-Silva et al. 2005) or a blend of VO replacing 60 % of FO (Mourente et al. 2007), no histomorphological effects were observed. Similarly, Ribeiro et al. (2015) observed in meagre that histological structure of liver was little affected by the 60 % replacement of FO by a VO blend. Contrarily, occurrence of variable size vacuoles and large amounts of lipid droplets in the liver of European sea bass fed rapeseed and linseed oils replacing 60 % of FO was described by Mourente et al. (2005). In the liver of rainbow trout (Caballero et al. 2002), gilthead sea bream (Caballero et al. 2004; Wassef et al. 2007; Fountoulaki et al. 2009) and Atlantic salmon (Kjaer et al. 2008), increased lipid accumulation was detected when fish were fed VO-rich diets. In the present study, the tendency towards higher hepatocellular vacuolization caused by an accumulation of lipid droplets in the VO group may be related to an induction of lipogenesis. In several fish species, VO administration promoted an increase (Jordal et al. 2007; Morais et al. 2011; Peng et al. 2014) in the transcript or enzymatic activity of lipogenic-related enzymes. In addition, and even though no changes were reported on the hepatic gene expression level in our fish (Castro et al. 2015a), it is also possible that factors involved in the assembly and secretion of the lipoprotein particles may also be involved. However, these hypotheses need further investigation, as available literature on the effects of FO replacement by VO on lipogenesis (Regost et al. 2003; Menoyo et al. 2004; Torstensen et al. 2004; Richard et al. 2006a, b; Castro et al. 2015b)

and on the mechanisms of lipoprotein assembly and secretion (Richard et al. 2006a, b; Jordal et al. 2007; Kjaer et al. 2008; Leaver et al. 2008) is not clear and contradictory. Nevertheless, this kind of tissue histomorphological alteration at the hepatic level seems to be nonpathological and reversible when fish are re-fed with a balanced diet (Caballero et al. 2004).

Overall, FO replacement by circa 70 % of a balanced VO blend in European sea bass juveniles did not substantially modulate digestive enzyme activities, suggesting that luminal digestive processes were not affected by dietary lipids tested. The kinetics of digestive enzyme activity response to a single meal reflected feed ingestion and was not affected by dietary oil source. VO blend promoted no hepatic or intestinal histomorphological alterations. Nevertheless, a trend towards higher lipid accumulation in the hepatocytes was observed in the fish fed the VO diet, which may result in effects at long term, and requires to be further investigated.

In the context of the development of more sustainable and economic aquafeeds, the present data seem promising as it suggests that FO can be replaced up to 70 % with VO in diets for European sea bass without major alterations in the digestive function and intestinal histomorphology.

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References

- Alarcón FJ, Díaz M, Moyano FJ, Abellán E (1998) Characterization and functional properties of digestive proteases in two sparids; gilthead sea bream (*Sparus aurata*) and common dentex (*Dentex dentex*). Fish Physiol Biochem 19:257–267
- Bakke AM, Glover C, Krogdahl Å (2010) Feeding, digestion and absorption of nutrients. In: Grosell M, Farrell AP, Brauner CJ (eds) Fish physiology. Academic Press, London, pp 57–110
- Bowyer JN, Qin JG, Adams LR, Thomson MJS, Stone DAJ (2012) The response of digestive enzyme activities and gut histology in yellowtail kingfish (*Seriola lalandi*) to dietary fish oil substitution at different temperatures. Aquaculture 368–369:19–28
- Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye-binding. Anal Biochem 72:248–254
- Caballero MJ, Obach A, Rosenlund G, Montero D, Gisvold M, Izquierdo MS (2002) Impact of different lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. Aquaculture 214:253–271
- Caballero MJ, Izquierdo MS, Kjørsvik E, Montero D, Socorro J, Fernández AJ, Rosenlund G (2003) Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225:325–340
- Caballero MJ, Izquierdo MS, Kjørsvik E, Fernández E, Rosenlund G (2004) Histological alterations in the liver of sea bream, *Sparus aurata* L., caused by short- or long-term feeding with vegetable oils. Recovery of normal morphology after feeding fish oil as the sole lipid source. J Fish Dis 27:531–541
- Caballero MJ, Gallardo G, Robaina L, Montero D, Fernández A, Izquierdo MS (2006) Vegetable lipid sources affect in vitro biosynthesis of triacylglycerols and phospholipids in the intestine of seabream (*Sparus aurata*). Br J Nutr 95:448–454
- Cahu CL, Infante JLZ, Corraze G, Coves D (2000) Dietary lipid level affects fatty acid composition and hydrolase activities of intestinal brush border membrane in sea bass. Fish Physiol Biochem 23:165–172
- Caruso G, Denaro MG, Genovese L (2008) Temporal changes in digestive enzyme activities in the gastrointestinal tract of European eel (*Anguilla anguilla*) (Linneo 1758) following feeding. Mar Freshw Behav Phys 41:215–228
- Castro C, Pérez-Jiménez A, Coutinho F, Pousão-Ferreira P, Brandão TM, Oliva-Teles A, Peres H (2013) Digestive enzymes of meagre (*Argyrosomus regius*) and white seabream (*Diplodus sargus*). Effects of dietary brewer's spent yeast supplementation. Aquaculture 416–417:322–327
- Castro C, Corraze G, Panserat S, Oliva-Teles A (2015a) Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. Nutr, Aquac. doi:10.1111/anu.12184
- Castro C, Corraze G, Pérez-Jiménez A, Larroquet L, Cluzeaud M, Panserat S, Oliva-Teles A (2015b) Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. Br J Nutr 1–14. doi:10.1017/S0007114515002731
- Chikwati EM, Sahlmann C, Holm H, Penn MH, Krogdahl Å, Bakke AM (2013) Alterations in digestive enzyme activities during the development of diet-induced enteritis in Atlantic salmon *Salmo salar* L. Aquaculture 402–403:28–37

- Ducasse-Cabanot S, Zambonino-Infante J, Richard N, Médale F, Corraze G, Mambriani M, Robin J, Cahu C, Kaushik S, Panserat S (2007) Reduced lipid intake leads to changes in lipid digestive enzymes in the intestine but has minor effects on key enzymes of hepatic intermediary metabolism in rainbow trout (*Oncorhynchus mykiss*). *Animal* 1:1272–1282
- Einarsson S, Davies PS, Talbot C (1996) The effect of feeding on the secretion of pepsin, trypsin and chymotrypsin in the Atlantic salmon, *Salmo salar* L. *Fish Physiol Biochem* 15:439–446
- Faulk CK, Benninghoff AD, Holt GJ (2007) Ontogeny of the gastrointestinal tract and selected digestive enzymes in cobia *Rachycentron canadum* (L.). *J Fish Biol* 70:567–583
- Fernández I, Moyano FJ, Díaz M, Martínez TF (2001) Characterization of α -amylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei). *J Exp Mar Biol Ecol* 262:1–12
- Figueiredo-Silva A, Rocha E, Dias J, Silva P, Rema P, Gomes E, Valente LMP (2005) Partial replacement of fish oil by soybean oil on lipid distribution and liver histology in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquac Nutr* 11:147–155
- Fountoulaki E, Alexis MN, Nengas I, Venou B (2005) Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (*Sparus aurata* L.). *Aquac Res* 36:1243–1251
- Fountoulaki E, Vasilaki A, Hurtado R, Grigorakis K, Karacostas I, Nengas I, Rigos G, Kotzamanis Y, Venou B, Alexis MN (2009) Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. *Aquaculture* 289:317–326
- Furné M, Hidalgo MC, López A, García-Gallego M, Morales AE, Domenzain A, Domezain J, Sanz A (2005) Digestive enzyme activities in Adriatic sturgeon *Acipenser naccarii* and rainbow trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture* 250:391–398
- García-Carreño FL, Dimes LE, Haard NF (1993) Substrate gel-electrophoresis for composition and molecular-weight of proteinases of proteinaceous proteinase-inhibitors. *Anal Biochem* 214:65–69
- Geurden I, Jutfelt F, Olsen R, Sundell K (2009) A vegetable oil feeding history affects digestibility and intestinal fatty acid uptake in juvenile rainbow trout *Oncorhynchus mykiss*. *Comp Biochem Physiol A* 152:552–559
- Hartviksen M, Bakke AM, Vecino JG, Ringo E, Krogdahl A (2014) Evaluation of the effect of commercially available plant and animal protein sources in diets for Atlantic salmon (*Salmo salar* L.): digestive and metabolic investigations. *Fish Physiol Biochem* 40:1621–1637
- Hidalgo MC, Urea E, Sanz A (1999) Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture* 170:267–283
- Jordal AEO, Lie O, Torstensen BE (2007) Complete replacement of dietary fish oil with a vegetable oil blend effects liver and plasma lipoprotein levels in Atlantic salmon (*Salmo salar*). *Aquac Nutr* 13:114–130
- Jutfelt F, Olsen RE, Björnsson BTh, Sundell K (2007) Parr-smolt transformation and dietary vegetable lipids affect intestinal nutrient uptake, barrier function and plasma cortisol levels in Atlantic salmon. *Aquaculture* 273:298–311
- Kjaer MA, Vegusdal A, Gjøen T, Rustan AC, Todorčević M, Ruyter B (2008) Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochim Biophys Acta* 1781:112–122
- Koven WM, Henderson RJ, Sargent JR (1994) Lipid digestion in turbot (*Scophthalmus maximus*): I. Lipid class and fatty acid composition of digesta from different segments of the digestive tract. *Fish Physiol Biochem* 13:69–79
- Kowalska A, Zakés Z, Jankowska B, Siwicki A (2010) Impact of diets with vegetable oils on the growth, histological structure of internal organs, biochemical blood parameters, and proximate composition of pikeperch *Sander lucioperca* (L.). *Aquaculture* 301:69–77
- Krogdahl Å, Bakke-McKellep AM, Baeverfjord G (2003) Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquac Nutr* 9:361–371
- Krogdahl Å, Hemre GI, Mommsen TP (2005) Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquac Nutr* 11:103–122
- Kuz'mina VV (2008) Classical and modern concepts in fish digestion. In: Cyrino JEP, Bureau DP, Kapoor BG (eds) Feeding and digestive functions of fishes. Science Publishers, Enfield, pp 85–154
- Laemmli UK (1970) Cleavage of structural proteins during assembly of head of bacteriophage-T4. *Nature* 227:680–688
- Leaver MJ, Villeneuve LAN, Obach A, Jensen L, Bron JE, Tocher DR, Taggart JB (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genom* 9:299
- Menoyo D, Izquierdo MS, Robaina L, Ginés R, Lopez-Bote CJ, Bautista JM (2004) Adaptation of lipid metabolism, tissue composition, and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soybean oils. *Br J Nutr* 92:41–52
- Moldal T, Løkka G, Wiik-Nielsen J, Austbø L, Torstensen BE, Rosenlund G, Dale OB, Kaldhusdal M, Koppang EO (2014) Substitution of dietary fish oil with plant oils is associated with shortened mid intestinal folds in Atlantic salmon (*Salmo salar*). *BMC Vet Res* 10:60
- Morais S, Cahu C, Zambonino-Infante J, Robin J, Rønnestad I, Dinis M, Conceição L (2004) Dietary TAG source and level affect performance and lipase expression in larval sea bass (*Dicentrarchus labrax*). *Lipids* 39:449–458
- Morais S, Caballero MJ, Conceição LEC, Izquierdo MS, Dinis MT (2006) Dietary neutral lipid level and source in Senegalese sole (*Solea senegalensis*) larvae: effect on growth, lipid metabolism and digestive capacity. *Comp Biochem Physiol B* 144:57–69
- Morais S, Pratooyot J, Taggart JB, Bron JE, Guy DR, Bell JG, Tocher DR (2011) Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement

- by vegetable oil: a liver transcriptomic analysis. *BMC Genom* 12:255
- Mourente G, Good JE, Bell JG (2005) Partial substitution of fish oil with rapeseed, linseed and olive oils in diet for European sea bass (*Dicentrarchus labrax* L.) effects on flesh fatty acid composition, plasma prostaglandins E2 and F2a, immune function and effectiveness of a fish oil finishing diet. *Aquac Nutr* 11:25–40
- Mourente G, Good JE, Thompson KD, Bell JG (2007) Effects of partial substitution of dietary fish oil with blends of vegetable oils, on blood leucocyte fatty acid compositions, immune function and histology in European sea bass (*Dicentrarchus labrax* L.). *Br J Nutr* 98:770–779
- Moyano FJ, Díaz M, Alarcón FJ, Sarasquete MC (1996) Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). *Fish Physiol Biochem* 15:121–130
- Munilla-Morán R, Saborido-Rey F (1996a) Digestive enzymes in marine species. I. Proteinase activities in gut from redfish (*Sebastes mentella*), seabream (*Sparus aurata*) and turbot (*Scophthalmus maximus*). *Comp Biochem Physiol B* 113:395–402
- Munilla-Morán R, Saborido-Rey F (1996b) Digestive enzymes in marine species. II. Amylase activities in gut from seabream (*Sparus aurata*), turbot (*Scophthalmus maximus*) and redfish (*Sebastes mentella*). *Comp Biochem Physiol B* 113:827–834
- Nordrum S, Krogdahl Å, Røsjø C, Olli JJ, Holm H (2000) Effects of methionine, cysteine and medium chain triglycerides on nutrient digestibility, absorption of amino acids along the intestinal tract and nutrient retention in Atlantic salmon (*Salmo salar* L.) under pair-feeding regime. *Aquaculture* 186:341–360
- Olsen RE, Ringø E (1997) Lipid digestibility in fish: a review. *Recent Res Dev Lipid Res* 1:199–265
- Olsen RE, Myklebust R, Kaino T, Ringø E (1999) Lipid digestibility and ultrastructural changes in the enterocytes of Arctic charr (*Salvelinus alpinus* L.) fed linseed oil and soybean lecithin. *Fish Physiol Biochem* 21:35–44
- Olsen RE, Dragnes BT, Myklebust R, Ringø E (2003) Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *Oncorhynchus mykiss* Walbaum. *Fish Physiol Biochem* 29:327–329
- Peng M, Xu W, Mai K, Zhou H, Zhang Y, Liufu Z, Zhang K, Ai Q (2014) Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. *Aquaculture* 433:442–449
- Pérez-Jiménez A, Cardenete G, Morales AE, García-Alcázar A, Abellán E, Hidalgo MC (2009) Digestive enzymatic profile of *Dentex dentex* and response to different dietary formulations. *Comp Biochem Physiol A* 154:157–164
- Rogest C, Arzel J, Robin J, Rosenlund G, Kaushik SJ (2003) Total replacement of fish oil by soybean or linseed oil with a return to fish oil in turbot (*Psetta maxima*). I. Growth performance, flesh fatty acid profile, and lipid metabolism. *Aquaculture* 217:465–482
- Ribeiro L, Moura J, Santos M, Colen R, Rodrigues V, Bandarra N, Soares F, Ramalho P, Barata M, Moura P, Pousão-Ferreira P, Dias J (2015) Effect of vegetable based diets on growth, intestinal morphology, activity of intestinal enzymes and haematological stress indicators in meagre (*Argyrosomus regius*). *Aquaculture*. doi:10.1016/j.aquaculture.2014.12.017
- Richard N, Kaushik S, Larroquet L, Panserat S, Corraze G (2006a) Replacing dietary fish oil by vegetable oils has little effects on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). *Br J Nutr* 96:299–309
- Richard N, Mourente G, Kaushik S, Corraze G (2006b) Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture* 261:1077–1087
- Rodiles A, Santigosa E, Herrera M, Hachero-Cruzado I, Cordero ML, Martínez-Llorens S, Lall SP, Alarcón FJ (2012) Effect of dietary protein level and source on digestive proteolytic enzyme activity in juvenile Senegalese sole, *Solea senegalensis* Kaup 1850. *Aquacult Int* 20:1053–1070
- Ruyter B, Moya-Falcón C, Rosemund G, Vegusdal A (2006) Fat content and morphology of liver and intestine of Atlantic salmon (*Salmo salar*): effects of temperature and soybean oil. *Aquaculture* 252:441–452
- Santigosa E, Sánchez J, Médale F, Kaushik S, Pérez-Sánchez J, Gallardo MA (2008) Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and seabream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. *Aquaculture* 282:68–74
- Santigosa E, García-Meilán I, Valentín JM, Navarro I, Pérez-Sánchez J, Gallardo MÁ (2011) Plant oils' inclusion in high fish meal-substituted diets: effect on digestion and nutrient absorption in gilthead sea bream (*Sparus aurata* L.). *Aquac Res* 42:962–974
- Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* 11:107–184
- Torstensen BE, Froyland L, Lie O (2004) Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil—effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquac Nutr* 10:175–192
- Walter HE (1984) Proteinases: methods with hemoglobin, casein and azocoll as substrates. In: Bergmeyer HJ (ed) *Methods of enzymatic analysis*, vol V. Verlag Chemie, Weinham, pp 270–277
- Wassef EA, Wahby OM, Sakr EM (2007) Effect of dietary vegetable oils on health and liver histology of gilthead seabream (*Sparus aurata*) growers. *Aquac Res* 38:852–861

Chapter 4-

**Dietary lipid and carbohydrate interactions:
implications on lipid and glucose absorption,
transport in gilthead sea bream (*Sparus
aurata*) juveniles**

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Dietary lipid and carbohydrate interactions: implications on lipid and glucose absorption, transport in gilthead sea bream (*Sparus aurata*) juveniles

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Abstract

A digestibility trial was performed with gilthead sea bream juveniles (IBW=72g) fed four diets differing in lipid source (fish oil, FO; or a blend of vegetable oil, VO) and starch content (0%, CH-; or 20%, CH+) to evaluate the potential interactive effects between carbohydrates and VO on the processes involved in digestion, absorption and transport of lipids and glucose. In fish fed VO diets a decrease on lipid digestibility and on cholesterol (CHOL), High Density Lipoprotein (HDL)-CHOL and Low Density Lipoprotein (LDL)-CHOL (only in CH+ group) were recorded. Contrarily, dietary starch induced postprandial hyperglycaemia and time related alterations on serum triacylglycerol (TAG), phospholipid (PL) and CHOL concentrations. Fish fed CH+ diet presented lower serum TAG than CH- group at 6h post-feeding, and the reverse was observed at 12h post-feeding for TAG and PL. Lower serum CHOL and PL at 6h post-feeding were recorded only in VOCH+ group. No differences between groups were observed in hepatic and intestinal transcript levels of proteins involved in lipid transport and hydrolysis (FABP, DGAT, GPAT, MTP, LPL, LCAT). Lower transcript levels of proteins related to lipid transport (ApoB, ApoA1, FABP2) were observed in the intestine of fish fed CH+ diet, but remained unchanged in the liver. Overall, transcriptional mechanisms involved in lipid transport and absorption were not linked to changes in lipid serum and digestibility. Dietary starch affected lipid absorption and transport, probably due to a delay of lipid

absorption. This study suggests that combination of dietary VO and starch may negatively affect cholesterol absorption or transport.

Key words: alternative ingredients; digestibility; gene expression; nutrient absorption and transport; serum metabolites.

1. Introduction

Fish gastrointestinal tract is involved in nutrient assimilation, digestion and absorption, while liver is the central organ involved in nutrient metabolism. Apart other functions, both organs constitute critical interfaces in the control of glucose and lipid homeostasis [1,2]. Fish in general digest dietary lipids efficiently [3,4]. Despite the poor utilization of dietary carbohydrates by carnivorous fish, high carbohydrate (gelatinized starch) digestibility has been reported in gilthead sea bream (*Sparus aurata*) and other species [5-8]. The general mechanisms involved in digestion and absorption of dietary lipid and carbohydrates have been verified in all fish species investigated to date, namely the enzymatic machinery for lipid and carbohydrate hydrolysis and a system of specialized protein transporters, such as fatty acid binding proteins and glucose transporters [1,3,4,9]. Most of the processes involved in the digestion and absorption of lipids and carbohydrates occur predominantly in the proximal and middle intestine [1,4,10,11]. As in mammals, lipid digestion products mostly accumulate in the enterocytes as lipid droplets. These need to be resynthesized into lipoprotein particles, as triacylglycerol (TAG)-rich lipoproteins (chylomicrons), to be exported through the vascular system to the liver which is the interface between the exogenous pathway (dietary lipids) and the endogenous pathway. The endogenous pathway mediates the transport of lipids from the liver, where both dietary lipids and *de novo* synthesized lipids are incorporated into TAG-rich lipoproteins (VLDLs), to the sites of conversion, storage or energy utilization [12,13]. Lipoproteins are lipid complexes with a neutral lipid core consisting of TAG and cholesteryl esters surrounded by a surface monolayer of phospholipids, small amounts of unesterified cholesterol and specialized proteins - apolipoproteins (Apo) [2]. The association of lipids and protein during lipoprotein synthesis is a complex multistep process that involves several proteins, such as ApoB, ApoA1, microsomal transfer protein, MTP (for details see Gu *et al.* [14] and Mansbach and Gorelick [15]). These proteins were shown to be highly expressed both in the liver and in the intestine of different fish species [5,8,14,16,17]. Once in blood circulation lipoproteins, especially TAG-rich VLDL and chylomicrons, suffer intra-vascular transformations that lead to modifications in the lipoprotein composition (TAG, phospholipids, cholesterol,

apolipoproteins) resulting in the formation of low-density (LDL) and high-density (HDL) lipoproteins by the action of lipoprotein lipase (LPL) and lecithin-cholesterol-acyltransferase (LCAT) [12].

Dietary carbohydrate and lipid source have been demonstrated to induce changes in nutrient digestibility [7,18-20] and in serum metabolites and lipoprotein profiles [5,20-26]. Most of these changes are thought to be linked to intracellular events involved in lipid and glucose absorption and transport (as described above) and that were shown in different fish studies to be under nutritional control, at least at the transcriptional level [8,17,27-29]. For instance, carbohydrate intake induced an increase in midgut ApoA1 and of hindgut ApoA1 and ApoB transcript levels in rainbow trout (*Oncorhynchus mykiss*) fed FO-based diets [8]. Furthermore, carbohydrate intake increased intestinal transcript levels of MTP, ApoA1 and ApoA4, along with higher serum cholesterol concentrations in rainbow trout fed VO-based diets [17]. In Atlantic salmon (*Salmo salar*), replacement of fish oil (FO) by rapeseed oil (RO) promoted a decrease in the hepatic expression of MTP2 and ApoA1 genes [28], while replacement of FO with RO or linseed oil (LO) induced an up-regulation of hepatic transcript levels of ApoB [29]. Similarly, up-regulation of hepatic transcript levels of ApoB and ApoA1 was reported in European sea bass (*Dicentrarchus labrax*) fed plant-based diets [27].

Fish meal (FM) and FO have traditionally been used as reliable sources of protein and lipids in carnivorous fish feeds. However, the limited availability of fisheries by-products together with the escalating cost and demand of these commodities made it critical the development of aquafeeds rich in more sustainable ingredients such as vegetable protein and oil sources. Vegetable feedstuffs are usually rich in carbohydrates and, contrarily to FO, do not have n-3 long-chain polyunsaturated fatty acids (LC-PUFA) [30,31]. Although a number of studies demonstrated the potential of vegetable feedstuffs as alternative ingredients in aquafeeds there still are problems related to their use by carnivorous fish, particularly due to limitations in carbohydrate metabolism [32-34] and of LC-PUFA biosynthesis [31,35]. As dietary inclusion of VO and carbohydrate are particularly challenging in diets for carnivorous fish, such as gilthead sea bream, more knowledge is needed on the potential implications of dietary carbohydrates and VO, and of the potential interaction between them, on the processes involved in lipid and glucose digestion, absorption and transport. Thus, this study aimed to evaluate the effects of dietary carbohydrate content and lipid source on digestibility, serum metabolites, hepatic and intestinal expression of key proteins involved in lipid and glucose absorption and

transport in juveniles of an economically important carnivorous fish species of aquaculture in Europe, gilthead sea bream.

2. Material and methods

Experimental diets

Four diets differing in carbohydrate content (0% and 20% gelatinized maize starch, CH- and CH+, respectively) and lipid source (fish oil or a vegetable oil blend, FO and VO, respectively) were formulated (Table 1). The diets were isolipidic and the dietary carbohydrate content was increased at the expense of dietary protein, which was always above the 45-46% protein requirement of this species [36]. Fish meal was added as a major dietary protein source to isolate the impacts of dietary VO and to avoid the interference of dietary plant protein on lipid metabolism. The major lipid source of FO diets was cod liver oil. In VO diets, 100% of the cod liver oil was replaced by a VO blend composed of 20% rapeseed, 50% linseed and 30% palm oils.

Table 1- Ingredient and chemical composition of the experimental diets

Lipid source Carbohydrates	Experimental Diets			
	FO		VO	
	CH-	CH+	CH-	CH+
<i>Ingredients (% dry weight)</i>				
Fish meal ^a	86.8	64.5	86.8	64.5
Gelatinized maize starch ^b	0	20	0	20
Cod liver oil ^c	9.2	11.5	0	0
Vegetable oil blend ^d	0	0	9.2	11.5
Vitamins ^e	1.5	1.5	1.5	1.5
Minerals ^f	1.0	1.0	1.0	1.0
Binder ^g	1.0	1.0	1.0	1.0
Cr ₂ O ₃	0.5	0.5	0.5	0.5
<i>Proximate analyses (% dry matter)</i>				
Dry matter (DM)	84.1	84.7	83.9	84.8
Crude protein (CP)	66.2	51.3	65.6	50.2
Crude fat (CF)	17.9	17.9	17.2	17.8
Starch	-	17.5	-	17.3
Gross energy (MJ kg ⁻¹)	23.0	22.6	23.2	22.7
Ash	14.6	11.8	15.1	11.9
Cholesterol	0.7	0.4	0.5	0.4

Fish oil (FO), blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch.

^a Steam dried LT fish meal, (Superprime). Inproquisa, Madrid, Spain/Pesquera Diamante, Perú (CP:74.6% DM; CL: 10.1% DM)

^b C-Gel Instant-12018, Cerestar, Mechelen, Belgium.

^c Labchem, Laborspirit Lda, Lisboa, Portugal.

^d 30% palm oil (Colmi, Malasia), 50% linseed oil (Sociedade Portuense de Drogas, S.A.,Portugal) and 20% rapeseed oil (Huilerie Emile Noël S.A.S., France)

^eVitamins (mg kg⁻¹ diet): retinol acetate, 18000 (IU kg⁻¹ diet); cholecalciferol, 2000 (IU kg⁻¹ diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400. Premix, Viana do Castelo, Portugal.

^fMinerals (mg kg⁻¹ diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg⁻¹ diet); potassium chloride, 1.15 (g kg⁻¹ diet); sodium chloride, 0.40 (g kg⁻¹ diet). Premix, Viana do Castelo, Portugal.

^gAquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate). Agil, England.

The diets presented small differences in the proportions of total saturated fatty acids (SFA), which were slightly higher in VO diets, and of monounsaturated fatty acids (MUFA) which were higher in FO diets (Table 2). Within MUFA, levels of oleic acid (18:1 n-9) were higher in the VO diets while the opposite occurred for palmitoleic acid (16:1 n-7), eicosenoic acid (20:1 n-9) and erucic acid (22:1 n-9). Linoleic acid (18:2 n-6) was considerably higher in VO diets than in FO diets. Compared to the FO diets, the VO diets were rich in linolenic acid (18:3 n-3) and poor in eicosapentaenoic acid (20:5 n-3) and docosahexaenoic acid DHA (22:6 n-3). Dietary cholesterol content was higher in the

diets without starch supplementation, particularly in the FOCH- diet. Cr₂O₃ was incorporated in the diets as an indigestible marker for digestibility measurement of dietary components.

Table 2- Fatty acid composition (expressed as %mol) of the experimental diets

Lipid source	Experimental Diets				
	FO		VO		
	Carbohydrates	CH-	CH+	CH-	CH+
14:0		7.3	7.3	3.5	2.7
15:0		0.8	0.7	0.5	0.4
16:0		21.6	19.8	26.8	24.8
17:0		0.6	0.5	0.5	0.4
18:0		4.2	3.6	5.5	4.9
20:0		0.2	0.1	0.3	0.3
ΣSFA		34.7	32.1	37.4	33.5
16:1 n-7		8.2	8.4	2.9	2.1
18:1 n-9		19.0	20.6	29.4	29.7
20:1 n-9		4.7	5.3	0.9	0.7
22:1 n-9		3.3	3.7	0.5	0.4
ΣMUFA		35.1	37.9	33.7	32.9
18:2 n-6		2.3	2.8	10.6	11.5
18:3 n-6		0.1	0.2	0.1	0.1
20:2 n-6		0.2	0.3	0.1	0.1
20:4 n-6		1.1	0.9	0.9	0.7
Σn-6 PUFA		4.1	4.3	11.9	12.4
18:3 n-3		1.2	2.0	18.6	20.8
18:4 n-3		2.0	2.2	0.6	0.4
20:4 n-3		0.5	0.6	0.2	0.1
20:5 n-3		7.5	7.2	3.5	2.4
21:5 n-3		0.3	0.3	0.1	0.1
22:5 n-3		1.1	1.0	0.6	0.4
22:6 n-3		10.5	9.5	6.6	4.6
Σn-3 PUFA		23.3	23.0	30.3	28.9
Sat/PUFA		1.2	1.1	0.9	0.8
n3 / n6		5.7	5.3	2.6	2.3

Fish oil (FO), blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch.
SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; n-3 LC-PUFA= n-3 long chain polyunsaturated fatty acids

All ingredients were finely ground, well mixed, and dry extruded in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA), through a 3-mm die. The pellets were air dried for 24h and stored in a refrigerator (4°C) until used.

Digestibility trial

The digestibility trial was performed at the Marine Zoology Station, Porto University, in a thermo-regulated recirculation water system equipped with a battery of 12 fiberglass tanks of 60 L capacity designed according Cho *et al.* [37] and with a feces settling column connected to the outlet of each tank.

The trial was performed with gilthead sea bream juveniles with a mean initial body mass of 72 g. Before the trial the fish were acclimatized for 15 days to the tanks and rearing conditions and were fed a commercial diet. Thereafter, 22 fish were randomly distributed to each tank and each diet was randomly assigned to triplicate groups of these fish. During the trial, water temperature averaged $24.0 \pm 0.5^{\circ}\text{C}$, salinity averaged $34.7 \pm 0.8 \text{ g L}^{-1}$ and dissolved oxygen was kept near saturation. Fish were hand fed to satiation twice a day. The first 15 days of the trial were used for fish to adapt to the diets; then, feces were collected once a day before the morning meal during the following 17 days. Immediately after collection feces from each tank were centrifuged, pooled for the whole period, and stored at -20°C until analysis.

Tissues and blood sampling

After 36 days of being fed with the experimental diets, blood samples were collected at 2, 4, 6, 8, 12 and 24 h after feeding for analysis of serum metabolites. Blood was collected from the caudal vein by puncture with a heparinized syringe and immediately centrifuged at 1800 g for 12 min, and serum aliquots were stored at 4°C until analysis. In order to minimize stress due to sampling, at each sampling point blood was collected from 9 fish from one tank and that tank was only sampled again after at least 6 h.

The remaining fish continued to be fed for two more days and then two fish from each tank were randomly sampled 6 h after the last meal to collect liver and intestine, just after the last pyloric caecum, for gene expression analysis. Immediately after collection, tissue samples were frozen in liquid nitrogen and then stored at -80°C until analysis.

Analytical methods

Details on diet and feces analysis are given in Castro *et al.* [5]. Apparent digestibility coefficients (ADC) were calculated according to the formula:

$$\text{ADC (\%)} = 100 - [100 \times (\text{Cr}_2\text{O}_7\text{diet} / \text{Cr}_2\text{O}_7\text{faeces}) \times (\text{N}_{\text{faeces}}/\text{N}_{\text{diet}})]$$

where N is nutrient (protein, lipid), starch or dry matter

Serum metabolites

Serum total cholesterol, high-density lipoprotein-cholesterol (HDL-CHOL), low-density lipoprotein-cholesterol (LDL-CHOL), triacylglycerol (TAG), phospholipid (PL), non-esterified fatty acids (NEFA) and glucose were performed on a Clinical Laboratory certified NP EN ISO 9001- 2000 by Bureau Veritas, following standard clinical methods and using an auto-analyzer (Architect ci8200; Abbot Diagnostics, Canada).

Gene expression

Analyses of mRNA levels were performed on liver and intestine samples (2 fish/tank). Tissues for RNA analyses were homogenized in 2mL tubes containing Trizol reagent (Invitrogen, Carlsbad, CA, USA) using rapid vibration (liver: 2 x 10s, with an interval of 10s, at 5000 rpm; intestine: 3 x 10s, with an interval of 10s, at 6500 rpm) in a Precellys®24 (Bertin Technologies, Montigny-le- Bretonneux, France). The extraction of total RNA was then performed according to manufacturer recommendations. RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Nanodrop Labtech, Palaiseau, France). Complementary DNA (cDNA) synthesis was performed with 1µg of the resulting total RNA using SuperScript III RNaseH- Reverse Transcriptase kit (Invitrogen) and random primers (Promega, Charbonnières, France). Gene expression levels were determined by real-time quantitative PCR (q-PCR) using LightCycler® 480 II apparatus (Roche Diagnostics, Neuilly sur Seine, France). Analyses were performed using 2 µL of the diluted cDNA (1:76) mixed with 0.24 µL of each primer (10µM), 3 µL LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) and 0.52 µL DNase/RNase/Protease-free water (5 prime GmbH, Hamburg, Germany) in a total volume of 6 µL. Primers were either obtained in the literature or designed from gilthead

sea beam expressed sequence tag (EST) sequences available on SIGENAE database (<http://www.sigenae.org>) using Primer3 software [38] (Table 3). For gene targets that had not been previously validated, primers were tested on a pool of cDNA and amplified products were systematically sequenced.

Table 3- Sequences of the primer pairs used for real-time quantitative PCR determination of the transcript level of several gilthead sea bream genes.

Gene	5'-3'Forward primer	5'-3'Reverse primer	EL	EI	Accession number
ApoA1	GAATACAAGGAGCAGATGAA GCAGATG	TGGTGACGGAGGCAGCG ATG	1.97	1.96	Varó <i>et al.</i> [39]
ApoB	ACTCTGAAGGCTTGTGCA	GCTGCAGGAAACCAGAA ACA	1.90	1.97	CV133385*
DGAT	GAGCCAATCACTGTGCCAAA	TGAGGAGGGTCTGAAGA TGC	1.93	1.97	AM957740*
ef1	CATGCTGGAGACCAGTGAAA	CGGGTACAGTTCCAATAC CG	1.95	1.92	Enes <i>et al.</i> [40]
FABP	AAATGGTTGAGGCTTCTGT GCTAC	ATCGCTACTGTCGGCTTG GTG	1.84	1.87	Varó <i>et al.</i> [39]
FABP2	CGAGCACATTCCGCACCAAA G	CCCACGCACCCGAGACT TC	**	1.99	Pérez-Sánchez <i>et al.</i> [41]
GPAT	GATCCAGTACGGAGTTCTCT ACG	AAAGGGCTCGGGAAC T	2.00	1.98	Mininni <i>et al.</i> [42]
LCAT	CCAACATCAAGATCCGCGAG	ACACCAACACCTCTACACC AG	1.94	1.96	AM950576*
LPL	CGTTGCCAAGTTGTGACCT G	AGGGTGTCTGGTTGTCT GC	1.79	1.85	Pérez-Sánchez <i>et al.</i> [43]
MTP	AGCCATCGACATCTCTGGAG	TTGTCCATCTGCATGCAC AC	2.00	1.80	CB184347*
PLA2	CCAGACCATCTCACCATCC	CACCCAATCCACAGGAG TTC	1.90	2.00	AM972037*

Primer efficiency in liver (EL) and intestine (EI), respectively. *Sigenae accession no. ApoA1, apolipoprotein A-1; EF1α, elongation factor 1α; FABP, fatty acid-binding protein; GPAT, Glycerol-3-phosphateacyl transferase; LCAT, Lecithin-cholesterol acyltransferase; LPL, Lipoprotein lipase; MTP, microsomal triglyceride transfer protein; PLA2, phospholipase A2 ; - no expression; ** not assessed.

Thermal cycling was initiated with incubation at 95°C for 10 min for hot-start iTaq™DNA polymerase activation. 45 steps of PCR were then performed, each one consisting of heating at 95 °C for 15 s for denaturing, at 60 °C for 10s for annealing, and at 72°C for 15 s for extension. Following the PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C 5 s⁻¹ from 55 to 94°C) to ensure that only one fragment was amplified. Each PCR run included duplicates of reverse transcription for each sample and negative controls (reverse transcriptase-free samples, RNA-free samples). The PCR run for reference gene included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and negative controls. Quantification of the target gene transcripts in the liver was done using the elongation factor 1α (EF1α) gene expression as reference, as previously used in gilthead

sea bream by Enes *et al.* [40], and that was stably expressed in the present study (data not shown). Relative quantification of the target gene transcript with the EF1 α reference gene transcript was performed using the mathematical model described by Pfaffl [44]. The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and the CT deviation (Δ CT) of the unknown sample compared with a control sample and expressed in comparison with the EF1 α reference gene:

$$R = [(E_{\text{target gene}}) \Delta\text{CT}_{\text{target gene (mean control-mean sample)}}]/[(E_{\text{EF1}\alpha}) \Delta\text{CT}_{\text{EF1}\alpha} (\text{mean control-mean sample})].$$

Efficiency of q-PCR was measured by the slope of a standard curve using serial dilutions of cDNA.

Statistical analysis

Data are presented as means \pm standard deviation. Data were checked for normal distribution and homogeneity of variances and when appropriate normalized. The apparent digestibility coefficients and gene expression data were analyzed by two-way ANOVA, with lipid source and carbohydrate level as fixed factors. The effects of diet and sampling time on serum metabolites level were analyzed by three-way ANOVA, with lipid source, carbohydrate level and sampling time as fixed factors. In case of significant interactions one-way ANOVA was performed for each fixed factor. Significant differences between sampling times were evaluated by the Tukey multiple range test. The significance level of 0.05 was used for rejection of the null hypothesis. All statistical analyses were done using the SPSS 22.0 software package (IBM Corp., New York, NY, USA) for Windows.

3. Results

Mortality was lower than 6% and it was negatively affected by dietary VO administration in fish fed carbohydrate rich diets (data not shown).

The ADC of nutrients was high, irrespective of dietary treatments (Table 4). The ADC of dry matter, protein and starch were not affected by diet composition, but the ADC of lipids was lower in the VO diets.

Table 4- Apparent digestibility coefficients (%) of the experimental diets¹

Lipid source (LS)	Experimental diets				p value*		
	FO		VO		CH	LS	CH x LS
Carbohydrates (CH)	CH-	CH+	CH-	CH+			
Dry matter	73.5±6.0	81.0±3.0	69.1±7.2	74.1±8.2	0.126	0.173	0.726
Protein	85.8±3.6	85.6±3.4	82.1±7.5	81.2±7.8	0.890	0.308	0.947
Lipid	96.7±1.1	97.4±0.2	94.7±1.3	94.7±1.1	0.512	0.003	0.465
Starch	-	92.1±0.9	-	90.0±1.5	-	0.109†	-

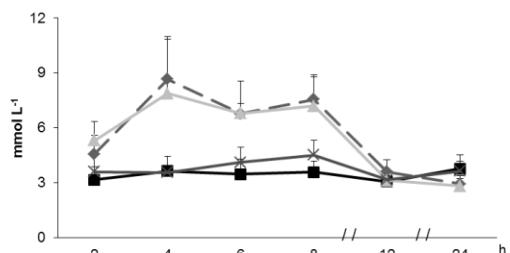
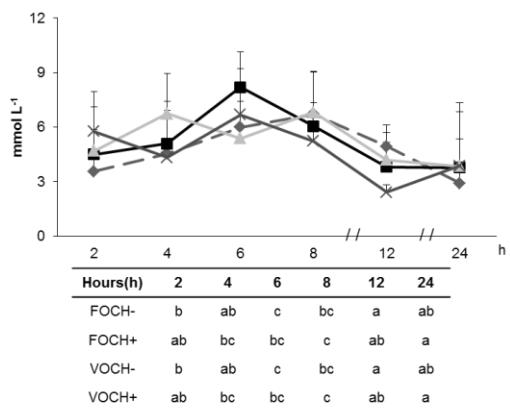
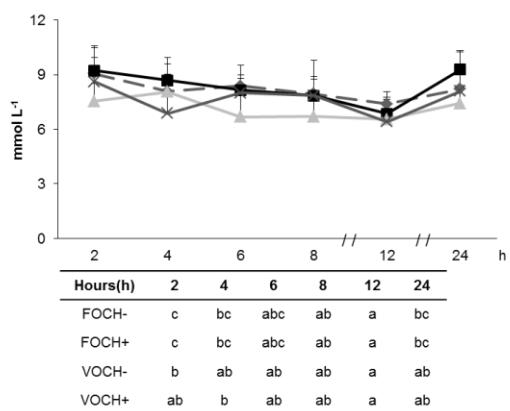
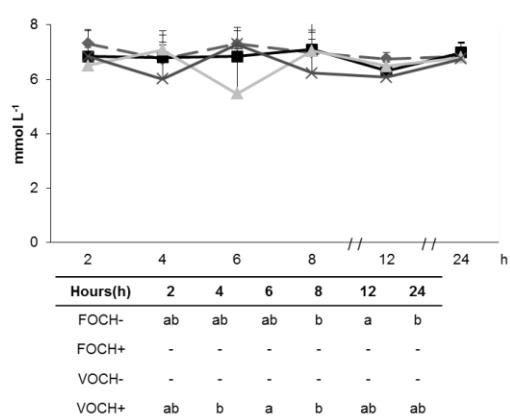
Fish oil (FO), blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch.

¹ Values are presented as means (n = 3) and standard deviation (SD) and were analyzed by two-way ANOVA.

† p-value assessed by Student's t-test: effect of lipid source p=0.109. values not showing an asterisk are non-statistically significant (p >0.05);

* Differences were considered statistically significant at: p < 0.05.

Postprandial serum glucose (GLU), triacylglycerol (TAG), cholesterol (CHOL), phospholipids (PL), non-esterified fatty acids (NEFA), low-density lipoprotein-cholesterol (LDL-CHOL) and high-density lipoprotein-cholesterol (HDL-CHOL) levels at 2, 4, 6, 8, 12 and 24 h after feeding are presented in Figure 1 and the summary of statistical analysis is presented in Table 5.

GLU**TAG****CHOL****PL****Fig. 1 (continued)**

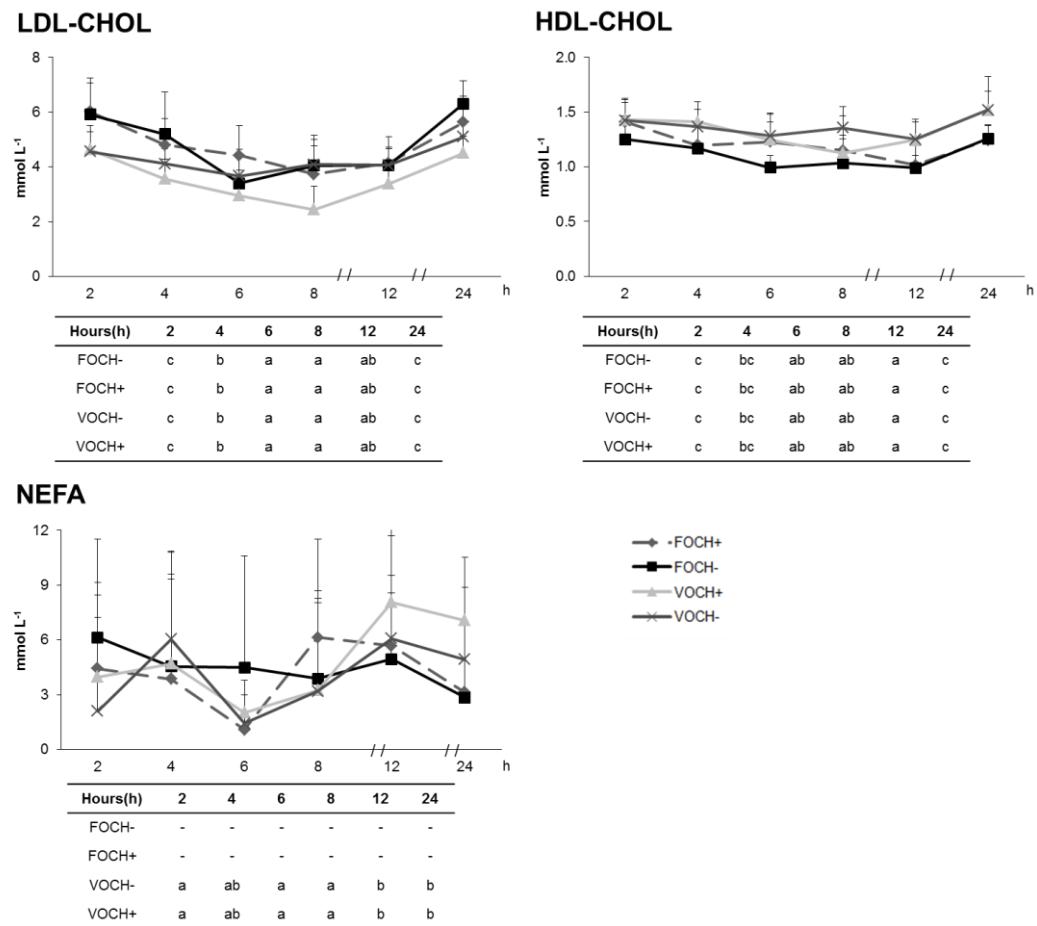


Fig. 1. Postprandial serum glucose (GLU), triacylglycerol (TAG), total cholesterol (CHOL), phospholipid (PL), high density lipoprotein cholesterol (HDL-CHOL), low density lipoprotein cholesterol (LDL-CHOL) and non-esterified fatty acids (NEFA) concentrations in gilthead sea bream juveniles after feeding the diets. Values are means \pm SD (n=9). Results of statistical differences in postprandial serum metabolite profiles related to the effect of diet (lipid source and carbohydrate content), time point and interaction among the factors analysed by three-way analysis of variance-ANOVA ($P < 0.05$) are given in the Results section and Table 5. In the figure, within each diet, significant differences (three-way ANOVA followed by one-way ANOVA, in case of significant interaction, and Tukey's Post Hoc, $p < 0.05$) among sampling times are indicated by different lowercase letters.

Postprandial serum GLU levels in CH+ groups increased to a peak at 4h post-feeding and remained higher until 8h post-feeding; thereafter GLU decreased to basal values which was attained 12h post-feeding. In CH- group GLU levels remained relatively constant, though a small peak was observed at 8h post-feeding. While during the first 8h after feeding CH+ groups exhibited higher serum GLU concentrations than CH- groups, at 12h and 24h post-feeding no differences or the reverse pattern was observed, respectively (CH x time interaction). In CH- groups, increased levels of GLU were found in fish fed VO based diet (CH x LS interaction).

Table 5- Summary of the statistical analyses (3-way ANOVA) of postprandial serum glucose (GLU), triacylglycerol (TAG), total cholesterol (CHOL), phospholipid (PL), high density lipoprotein cholesterol (HDL-CHOL), low density lipoprotein cholesterol (LDL-CHOL) and non-esterified fatty acids (NEFA) in gilthead sea bream fed the experimental diets.

	CH	L	Time	CH x L	CH x time	L x time	CH x L x time
p-value*							
GLU	<0.001	0.325	<0.001	0.007	<0.001	0.123	0.564
TAG	0.852	0.970	<0.001	0.066	0.003	0.081	0.723
PL	0.444	0.005	0.669	0.887	0.065	0.790	0.004
CHOL	0.050	<0.001	<0.001	0.331	0.169	0.962	0.039
HDL-CHOL	0.335	<0.001	<0.001	0.018	0.577	0.454	0.254
LDL-CHOL	0.008	<0.001	<0.001	0.015	0.117	0.193	0.251
NEFA	0.534	0.982	0.028	0.153	0.304	<0.001	0.382

*p-value assessed by 3-way ANOVA. Differences were considered statistically significant at: p< 0.05.

Serum TAG in CH+ groups attained a peak at 8h post-feeding and decreased to the basal level at 24h post-feeding. In CH- groups serum TAG peaked at 6h post-feeding and decreased to basal levels 12h post-feeding. Dietary carbohydrate affected TAG concentrations only at 6h and 12h post-feeding (CH x time interaction). Increased serum TAG was observed in CH- groups at 6h after feeding, while the reverse was observed at 12h post-feeding. TAG concentration was not affected by dietary lipid source.

All diets followed a similar postprandial CHOL profile. Accordingly, serum CHOL decreased to basal values 12h after feeding, and 24h post-feeding increased to values similar to those observed 2h after feeding. The VOCH+ group followed a similar CHOL pattern, except that a peak of CHOL was observed at 4h post-feeding. Serum CHOL concentration was lower in VO groups than FO groups, except at 8 and 24h post-feeding.

Serum CHOL was also affected by dietary carbohydrate but only in VO groups. Thus, 4h post-feeding higher CHOL concentration was observed in VOCH+ group than in VOCH- group while the opposite was observed 6h post-feeding(CH x LS x time interaction).

Postprandial differences in PL concentration were observed in FOCH- and VOCH+ groups. In VOCH+ group, two peaks of PL concentration were observed, the first one at 4h and the second one at 8 h post-feeding, while in FOCH- group PL peaks were also observed at 8h and at 24h post-feeding. Serum PL concentration was affected by dietary carbohydrate only at 6h and 12h post-feeding. Lower PL levels were observed in VOCH+ group 6h post-feeding, while 12h post-feeding higher PL was found in CH+ groups than in CH- groups (CH x LS x time interaction). Dietary VO promoted an effect on serum PL concentration only at 6h post-feeding (CH x LS x time interaction). Accordingly, in CH+ group lower serum PL concentrations was observed, while the reverse was observed in the CH- group.

Postprandial differences in NEFA were only observed in VO groups, with NEFA levels being lower at 2, 6 and 8h post-feeding than at 12 and 24h post-feeding. Dietary carbohydrate did not affect serum NEFA concentration. However, NEFA concentration was lower at 2h and higher at 24h post-feeding in VO than in FO groups (LS x time interaction).

HDL-CHOL and LDL-CHOL exhibited similar postprandial profiles with the higher values being observed at 2h and at 24h after feeding. Within VO groups, carbohydrate intake induced lower serum LDL-CHOL. In fish fed CH+ diets, VO intake also promoted a decrease in LDL-CHOL. On the contrary, dietary carbohydrate promoted an increase in serum HDL-CHOL but only in the FO group. Dietary VO induced a decrease of serum HDL-CHOL (CH x LS interaction).

Data on the expression of key genes encoding proteins involved in lipid digestion (phospholipase A2,PLA2), lipid transport (fatty acid-binding protein, FABP and FABP2), TAG and PL resynthesis (glycerol-3-phosphateacyl transferase, GPAT; diacylglycerol acyltransferase, DGAT), lipoprotein assembly (apolipoproteins B, A1: ApoB, ApoA1; microsomal triglyceride transfer protein, MTP) and lipolysis (lecithin-cholesterol acyltransferase, LCAT; lipoprotein lipase, LPL) in liver and intestine are presented in Tables 6 and 7, respectively. In the liver, only LCAT was affected by diet composition (CH x LS interaction).

Table 6- Relative mRNA abundance of selected proteins involved in lipid digestion (PLA2), lipid transport (FABP), TAG and PL resynthesis (DGAT, GPAT), lipoprotein assembly (ApoB, ApoA1, MTP) and lipolysis (LCAT, LPL) in the liver of gilthead sea bream fed the experimental diets.

Lipid source (LS)	Experimental diets						p-value*	
	FO		VO		CH	LS		
	CH-	CH+	CH-	CH+				
I-ApoA1	1.10±0.4	1.38±0.6	1.13±0.3	1.30±0.3	0.222	0.89	0.747	
I-ApoB	1.08±0.5	1.07±0.3	1.18±0.3	1.00±0.2	0.652	0.715	0.513	
I-DGAT	1.20±1.0	1.42±0.8	1.45±0.5	1.30±0.5	0.910	0.821	0.535	
I-FABP	1.07±0.5	0.83±0.3	1.28±0.4	0.90±0.3	0.068	0.387	0.644	
I-GPAT	1.13±0.6	1.18±0.5	1.05±0.5	1.38±1.6	0.443	0.321	0.206	
I-LCAT	1.08±0.5	1.45±0.5	1.73±0.5	1.13±0.5	0.55	0.395	0.02	
I-LPL	1.08±0.5	1.38±1.0	1.00±0.2	0.97±0.6	0.881	0.434	0.488	
I-MTP	1.05±0.4	1.37±0.7	1.48±0.6	1.35±0.4	0.674	0.343	0.307	
I-PLA2	1.28±0.9	1.33±0.8	1.13±0.3	1.17±0.3	0.874	0.549	0.975	

Fish oil (FO), blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; DGAT, diacylglycerol acyltransferase; FABP, fatty acid-binding protein; GPAT, Glycerol-3-phosphateacyl transferase; LCAT, Lecithin-cholesterol acyltransferase, LPL, Lipoprotein lipase; MTP, microsomal triglyceride transfer protein; PLA2, phospholipase A2. Considering FOCH- group as control, relative fold difference between treatments are presented as means ± SD (n = 6) and were analyzed using two-way ANOVA. * Differences were considered statistically significant at p< 0.05.

Hepatic LCAT transcript levels were higher in VO group than in FO group when fish were fed the diet with no starch. On the other hand, in fish fed the VO diets higher hepatic transcript levels were recorded in CH- than in CH+ groups (CH x LS interaction).

Table 7- Relative mRNA abundance of selected proteins involved in lipid digestion (PLA2), lipid transport (FABP), TAG and PL resynthesis (DGAT, GPAT), lipoprotein assembly (ApoB, ApoA1, MTP) and lipolysis (LCAT, LPL) in the intestine of gilthead sea bream fed the experimental diets.

Lipid source (LS)	Experimental diets						p-value*	
	FO		VO		CH	LS		
	CH-	CH+	CH-	CH+				
i-ApoA1	1.03±0.2	0.80±0.2	1.20±0.2	0.93±0.2	0.007	0.086	0.843	
i-ApoB	1.12±0.5	1.23±0.6	1.70±1.0	1.43±0.9	0.814	0.227	0.548	
i-DGAT	1.02±0.3	0.82±0.5	1.48±0.5	0.93±0.4	0.033	0.091	0.300	
i-FABP	1.42±1.0	1.08±1.0	0.72±1.0	1.57±1.2	0.573	0.814	0.204	
i-FABP2	1.08±0.4	0.53±0.2	1.16±0.5	0.63±0.1	0.001	0.528	0.933	
i-GPAT	1.08±0.6	1.08±0.4	1.58±0.7	1.52±0.7	0.896	0.068	0.896	
i-LCAT	1.42±1.4	1.33±1.5	2.68±2.1	2.38±1.8	0.792	0.12	0.882	
i-LPL	1.12±0.5	0.85±0.3	0.82±0.3	0.72±0.3	0.247	0.181	0.604	
i-MTP	1.05±0.4	0.98±0.5	1.40±0.2	1.15±0.5	0.365	0.146	0.597	
i-PLA2	2.55±4.0	1.50±0.6	1.36±1.5	1.82±1.6	0.764	0.658	0.448	

Fish oil (FO), blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch. ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; DGAT, diacylglycerol acyltransferase; FABP, fatty acid-binding protein; GPAT, Glycerol-3-phosphateacyl transferase; LCAT, Lecithin-cholesterol acyltransferase, LPL, Lipoprotein lipase; MTP, microsomal triglyceride transfer protein; PLA2, phospholipase A2. Considering FOCH- group as control, relative fold difference between treatments are presented as means ± SD (n = 6) and were analyzed using two-way ANOVA. * Differences were considered statistically significant at p<0.05.

In the intestine, APOA1, FABP2 and DGAT transcripts were modulated by dietary carbohydrate but not by lipid source. Intestinal transcript levels of APOA1, FABP2 and DGAT were lower in fish fed the carbohydrate diets.

4. Discussion

The effect of dietary lipid source and carbohydrate content are discussed separately below as very few interactions between both factors were recorded.

Replacement of FO by a VO blend in the diets induced a slight but significant decrease in apparent digestibility of lipids, which could be related to differences in dietary FA profile. Lipid digestibility is modulated by FA chain length and degree of unsaturation [45], with digestibility decreasing with FA chain length and increasing with FA unsaturation. This is in accordance with the FA composition of diets in the present study, as FO replacement by VO blend led to an increase of n-6 PUFA and a decrease of the unsaturation index. Further, FA digestibility decreases when the position of the first double bond moves from the methyl end of the carbon chain [18] and the n-3/n-6PUFA ratio was lower in the VO diets, which may also contribute to explain the lower lipid digestibility of these diets. As stated by Francis *et al.* [18] the lower lipid digestibility of VO diets can be related not to just a single factor but to several dependent factors that

are known to affect FA digestibility such as the ones linked to emulsification, enzymatic hydrolysis, lipase specificities and micellar incorporation of the FA.

Although dietary FA profile affected lipid digestibility, post-prandial changes in serum TAG levels, which are the main lipid class in diets, followed a similar kinetic in fish fed the different diets. Postprandial TAG peaked within 6 and 8h post-feeding, which is in the range of peak times reported for other fish species such as common carp (*Cyprinus carpio*) [46], Senegalese sole (*Solea senegalensis*) [16] and European sea bass [5]. This more pronounced phase in serum TAG concentrations is possibly indicative of appearance of TAG-rich chylomicrons into circulation. PL along with TAG are two important lipid components in lipoprotein assembly and export of the cell, the former being crucial for lipoprotein export [47]. Contrary to previous observations in European sea bass [5], serum PL profile over time differed from that of serum TAG but, as for TAG, serum PL concentration was also little affected by dietary lipid source.

Resynthesis of TAG and PL, a key step in lipoprotein assembly, is thought to be a selective process in fish based mainly on the FAs unsaturation degree [4]. Indeed, higher specificities for C18 PUFA to be resynthesized into TAG, and of LC-PUFA to be resynthesized into PL were reported in rainbow trout and in gilthead sea bream [48]. Previously, it was shown in vitro with gilthead sea bream intestinal microsomes that 60 % substitution of FO by rapeseed oil had no effects on PL resynthesis but induced a reduction in TAG resynthesis in both glycerol-3-phosphate (G3P) and monoacylglycerol pathways, the two pathways involved in TAG and PL resynthesis [49,50]. Even though, the lack of alterations of serum PL and TAG concentrations in the present study is in accordance with an absence of intestinal and hepatic regulation of DGAT and GPAT, two enzymes involved in TAG and PL resynthesis. Similarly, lack of effects on DGAT activity [28,51] or gene expression [5] were reported in other studies in fish with dietary replacement of FO by VO.

Dietary FA profile was demonstrated to affect serum CHOL and LDL- CHOL in humans [52,53]. In the present study, lower serum total CHOL, LDL- CHOL (only in the CH+ diet) and HDL- CHOL were observed in fish fed the VO diets. Similarly, replacement of FO by VO was reported to decrease serum total CHOL or LDL and LDL- CHOL in rainbow trout [25] and in Atlantic salmon [22,23]. Low dietary CHOL levels and the presence of phytosterols in VO diets were linked to these effects. In this study, in the CH- groups CHOL content was slightly higher in fish fed the FO than the VO diet (0.7% against 0.5%)

but such difference was not observed in the CH+ diets. Thus, we assume that difference in dietary CHOL was not the main cause for the present results.

Additionally, in previous studies where FO was substituted by VO but diets had the same CHOL levels, plasma CHOL concentration did not change [5, 54]. In this sense, presence of phytosterols in VO diets (not measured in the present study) might explain our results. Phytosterols are abundant in VO and are structurally and functionally similar to CHOL in animals. Their ability to lower plasma cholesterol and LDL-CHOL has been documented both in humans [55-58] and in fish [59]. The hypocholesterolaemic effect of phytosterols is thought to be promoted by an impairment of intestinal CHOL absorption through modulation of CHOL transporters uptake-capacity, competition for CHOL transporters, and decreased incorporation of dietary and biliary CHOL into micelles [55,56,60].

Capacity of CHOL biosynthesis at hepatic level was demonstrated in several fish species, such as Atlantic salmon [29] rainbow trout [61] and European sea bass [21]. However, under VO challenge, both negative [61] and positive [21,29] regulation of the transcript levels of proteins involved in CHOL biosynthesis were reported. The up-regulation of CHOL biosynthesis induced no major changes of hepatic CHOL content [21,29] or serum CHOL levels [21] in fish fed VO or FO based diets. This suggests that hepatic CHOL upregulation might be enough to counteract a deficit in absorbed CHOL.

CHOL, either diet derived or synthesized by the liver, is transported in the serum from the liver to extra-hepatic tissues by LDL lipoproteins, which in turn are derived of TAG-rich VLDL by sequential lipolysis mediated by LPL [4,12]. Thus, increased uptake of plasma LDL-CHOL by peripheral tissue cannot be excluded as the cause of reduced serum LDL-CHOL concentration in VOCH+ group. Indeed, increased uptake of serum LDL by peripheral tissues due to up-regulation of LDL receptors (LDL-r) or increased expression and activity of LPL in fish fed VO based diets was advanced as cause for the lower serum lipid and LDL observed in Atlantic salmon fed VO diets compared to FO diets [22]. In other study with the same species fed VO diet, no dietary regulation of hepatic LDL-r transcripts was reported; however, one of the three transcripts for LPL were up-regulated in the fat line of Atlantic salmon [23]. In the present study, LPL expression was not nutritionally regulated either in the liver or the intestine. Contrarily, up-regulation of LPL transcripts were reported in turbot (*Scophthalmus maximus*) fed VO based diets [62].

In the present study, despite the decreased serum HDL-CHOL concentration in fish fed the VO diets no dietary regulation of hepatic and intestinal transcript levels of ApoA1 were observed. VO diets induced an up-regulation of liver LCAT transcript levels, but only in the CH- group. ApoA1 and LCAT are two important components involved in HDL metabolism, especially in the reverse cholesterol transport pathway [63]. ApoA1 is the main structural protein of HDL and the most potent activator of LCAT. LCAT is synthesized and secreted primarily by the liver and reversibly binds to lipoproteins, mainly on HDL, and catalyzes the esterification of free cholesterol into cholesteryl ester (CE) [64,65]. LCAT also catalyzes the transfer of FA from phosphatidylcholine in the reesterification of free cholesterol to CE. Thus, transcript levels of LCAT may also be regulated by the nature of phospholipids and FA available for esterification [64,65].

Changes in HDL-CHOL are expected to be in part mediated by changes in LCAT and ApoA1 concentrations. However, the underlying mechanisms responsible for regulation of serum HDL by apoA1 and LCAT are not yet completely elucidated in mammals [63, 66] and unknown in fish. Indeed, hepatic overexpression of ApoA1 was reported in Atlantic salmon fed VO-based diets, although differences on serum HDL-CHOL were not observed [23]. In European sea bass fed plant-based diets hepatic overexpression of ApoA1 was also reported [27]. Although the authors suggested that reverse transport of cholesterol was induced, no data on serum HDL or HDL-CHOL concentrations was provided to support that assumption. Even though our data may suggest an induction of cholesterol transport from tissues to the liver in VOCH- group, no positive variation of serum HDL-CHOL concentration was observed. So, as for LDL-CHOL, the involvement of other mechanisms to explain the decreased serum HDL-CHOL concentration in fish fed the VO diets, namely those related to HDL catabolism (HDL uptake/clearance), cannot be discarded.

Lipid metabolism is reported to be highly modulated by dietary carbohydrates or glucose load [8,17,33,67-69]. Accordingly, changes on postprandial triacylglycerolemia [8], phospholipidemia [21] and cholesterolemia [17,21] promoted by dietary carbohydrates were described in carnivorous fish.

Hypertriacylglycerolemia is normally associated with intake of carbohydrate-rich diets in humans [70,71] and in fish it was reported in rainbow trout [17]. Several mechanisms may be involved in the induction of hypertriacylglycerolemia by dietary carbohydrates, such as mobilization and utilization of stored TAG, increase of lipid synthesis or decrease of TAG clearance. In a previous study with gilthead sea bream, serum TAG peaked 1 h

after an intramuscular load of glucose, and that peak was associated to increased catabolism of stored body reserves [69]. On the contrary, in the present study lower serum TAG was observed 6h post-feeding in fish fed the CH+ diets. A large body of evidence points that TAG production, and consequently lipoprotein formation and secretion, are driven to a large extent by lipid substrate availability [2,4]. Thus, the lower serum TAG concentration observed 6h after feeding (absorptive phase) may be related to a reduced intestinal lipid uptake in the CH+ groups, as corroborated by the decreased transcript levels of key chylomicron assembly proteins (FABP2, ApoA1, DGAT) in the intestine but not in the liver. As the experimental diets were isolipidic, it may be assumed that changes in serum TAG and of intestinal transcript of proteins involved in chylomicron assembly are an effect of dietary carbohydrates on lipid digestibility, as described in several fish species, including gilthead sea bream [19,72,73]. However, as in the present study no differences on lipid digestibility were observed, the delay of TAG peaks in the CH+ groups (8h post-feeding) as compared to the CH- groups (6h post-feeding) could be related to a delay in lipid digestion or absorption induced by dietary carbohydrates. On the contrary, higher serum TAG concentrations were observed at 12h post-feeding in the CH+ groups than in the CH- groups. At that time, most of the TAG in circulation are of endogenous origin, suggesting that dietary carbohydrate intake might have induced *de novo* lipid synthesis and mobilization of stored TAG. It would be of interest to have data on gene expression of key proteins involved in lipogenesis, lipolysis and lipid uptake at 12h post-feeding, to confirm this hypothesis.

In European sea bass, in a trial similar to the present one, increased serum CHOL at 18h after feeding in CH+ groups matched the hepatic up-regulation of transcript levels of HMGCR (3-hydroxy-3-methyl-glutaryl-coenzyme reductase), a key protein involved in CHOL biosynthesis. However, such induction apparently did not occur in the present study.

Contrary to TAG, which are exclusively transported via the chylomicron pathway, CHOL across the intestinal epithelial cells may occur via the chylomicron pathway or the apoB-independent pathway. Although ApoA1 is needed in both pathways, it is a key driver in the apoB-independent pathway [74,75]. Thus, though the decreased transcript levels of intestinal ApoA1 in CH+ group may be linked to slower lipid absorption, mainly in the form of TAG, it is also possible that intestinal uptake or transport of CHOL might also have been affected, particularly in fish fed the VO diets, as serum total CHOL was only lower in fish feeding VOCH+ at 6h after feeding. This may be explained by an increased

CHOL-lowering effect of VO in the VOCH+ group but further investigation is required to confirm this hypothesis.

Serum PL concentration was reported to be induced by carbohydrate rich diets in European sea bass [21]. Similar results were observed in the present study with gilthead sea bream, but only at 12h post-feeding. Glucose is an important carbon source for FA synthesis but also for G3P synthesis [76]. G3P is a precursor for the synthesis of both TAG and PL in mammals but the relative contribution of G3P pathway for PL synthesis in fish is still a matter of debate [50,51]. Although changes in the transcript levels of hepatic and intestinal GPAT, an enzyme involved in the first committed step of the G3P pathway, were not observed in the present study, it is possible that the rate of G3P synthesis from glucose metabolism in the fish fed the CH+ diets was adequate for PL synthesis.

Conclusions

Lipid digestibility, serum total CHOL and HDL-CHOL were negatively affected by the inclusion of VO in diets for gilthead seam bream juveniles, probably due to differences in dietary FA profile and the presence of phystosterols in VO, but no changes were observed at transcriptional level on lipid absorption and transport mechanisms. On the other hand, dietary carbohydrates seem to modulate transcriptional mechanisms involved in lipid absorption and transport at intestinal level, probably by interfering with TAG availability and by delaying lipid absorption. Considering the reduction in dietary CHOL content with the progressive substitution of FM and FO with vegetable feedstuffs, attention should be paid in the regulation mechanisms of CHOL metabolism, as present data suggest that an interaction between dietary VO and carbohydrates exist that may affect CHOL absorption and transport.

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References

- 1- Bakke AM, Glover C, Krogdahl Å (2010) In: Grosell M, Farrell AP, Brauner CJ (eds) Feeding, digestion and absorption of nutrients, Fish Physiology, Academic Press, USA, pp. 57–110.
- 2- Xiao C, Hsieh J, Adeli K, Lewis GF (2011) Gut–liver interaction in triglyceride-rich lipoprotein metabolism. *Am J Physiol Endocrinol Metab* 301: E429–E446.
- 3- Olsen RE, Ringø E (1997) Lipid digestibility in fish: a review. *Recent Res Dev Lipid Res* 1:199–265.
- 4- Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* 11: 107–184.
- 5- Castro C, Corraze G, Panserat S, Oliva-Teles A (2015) Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquac Nutr* 21: 592-603.
- 6- Couto A, Enes P, Peres H, Oliva-Teles A (2012) Temperature and dietary starch level affected protein but not starch digestibility in gilthead sea bream juveniles. *Fish Physiol Biochem* 38:595–601.
- 7- Enes P, Panserat S, Kaushik S, Oliva-Teles A (2006) Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. *Comp Biochem Physiol A* 143: 89–96.
- 8- Kamalam BS, Panserat S, Aguirre P, Geurden I, Fontagné-Dicharry S, Médale F (2013) Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp Biochem Physiol A* 164: 417–427.
- 9- Krogdahl Å, Hemre GI, Mommsen TP (2005) Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. *Aquac Nutr* 11:103–122.
- 10- Castro C, Pérez-Jiménez A, Coutinho F, Pousão-Ferreira P, Brandão TM, Oliva-Teles A, Peres H (2013) Digestive enzymes of meagre (*Argyrosomus regius*) and white seabream (*Diplodus sargus*). Effects of dietary brewer's spent yeast supplementation. *Aquaculture* 416–417: 322–327.
- 11- Olsen RE, Myklebust R, Kaino T, Ringø E (1999) Lipid digestibility and ultrastructural changes in the enterocytes of Arctic charr (*Salvelinus alpinus* L.) fed linseed oil and soybean lecithin *Fish Physiol Biochem* 21: 35–44.

- 12- Corraze G (2001) In: Guillaume J, Kaushik S, Bergot P, Metailler K (eds) Lipid Nutrition- Nutrition and Feeding of Fish and Crustaceans, Springer, Chichester UK, pp. 111–130.
- 13- Sheridan MA (1988) Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. *Comp Biochem Physiol B* 90: 679 –690.
- 14- Gu M, Kortner TM, Penn M, Hansen AK, Krogdahl Å (2014) Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* 111: 432-444.
- 15- Mansbach CM 2nd, Gorelick F (2007) Development and Physiological Regulation of Intestinal Lipid Absorption. II. Dietary lipid absorption, complex lipid synthesis, and the intracellular packaging and secretion of chylomicrons. *Am J Physiol Gastrointest Liver Physiol* 293: G645–G650.
- 16- Borges P, Médale F, Véron V, Pires M dos A, Dias J, Valente LMP (2013) Lipid digestion, absorption and uptake in *Solea senegalensis*. *Comp Biochem Physiol A* 166: 26–35.
- 17- Kamalam BS, Médale F, Larroquet L, Corraze G, Panserat S (2013) Metabolism and fatty acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. *PLoS one* 8: e76570.
- 18- Francis DS, Turchini GM, Jones PL, De Silva SS (2007) Effects of fish oil substitution with a mix blend vegetable oil on nutrient digestibility in Murray cod, *Maccullochella peelii* peelii. *Aquaculture* 269: 447–455.
- 19- Storebakken T, Shearer KD, Refstie S, Lagocki S, McCool J (1998) Interactions between salinity, dietary carbohydrate concentration on the digestibility of macronutrients and energy in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 163: 347-359.
- 20- Torstensen BE, Lie O, Froyland L (2000) Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.)— effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids* 35: 653–664.
- 21- Castro C, Corraze G, Pérez-Jiménez A, Larroquet L, Cluzeaud M, Panserat S, Oliva-Teles A (2015) Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles *Br J Nutr* 114: 1143-1156.

- 22- Jordal AEO, Lie Ø, Torstensen BE (2007) Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). *Aquac Nutr* 13: 114–130.
- 23- Morais S, Pratoomyot J, Torstensen BE, Taggart JB, Guy DR, Bell JG, Tocher DR (2011) Diet x genotype interactions in hepatic cholesterol and lipoprotein metabolism in Atlantic salmon (*Salmo salar*) in response to replacement of dietary fish oil with vegetable oil. *Br J Nutr* 106: 1457–1469.
- 24- Luo L, Xue M, Vachot C, Geurden I, Kaushik S (2014) Dietary medium chain fatty acids from coconut oil have little effects on postprandial plasma metabolite profiles in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 420-421: 24-31.
- 25- Richard N, Kaushik S, Larroquet L, Panserat S, Corraze G (2006) Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). *Br J Nutr* 96: 299–309.
- 26- Richard N, Mourente G, Kaushik S, Corraze G (2006) Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture* 261: 1077–1087.
- 27- Geay D, Ferraresto S, Zambonino-Infante JL, Bargelloni L, Quentel C, Vandepitte M, Kaushik S, Cahu CL, Mazurais D (2011) Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rate with the plant-based diet. *BMC Genomics* 12: 522 – 539.
- 28- Kjaer MA, Vegusdal A, Gjøen T, Rustan AC, Todorcevic M, Ruyter B (2008) Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochi Biophys Acta* 1781: 112–122.
- 29- Leaver MJ, Villeneuve LA, Obach A, Jensen L, Bron JE, Tocher DR, Taggart JB (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* 9: 299 – 313.
- 30- Gatlin DM, Barrows FT, Brown P, Dabrowski K, Gibson GT, Hardy RW, Elliot H, Hu G, Krogdahl A, Nelson R, Overturf K, Rust M, Sealey W, Skonberg D, Souza EJ, Stone D, Wilson R, Wurtele E (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquacult Res* 38: 551–579.

- 31- Tocher DR (2015) Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture*. doi:10.1016/j.aquaculture.2015.01.010
- 32- Enes P, Panserat S, Kaushik S, Oliva-Teles A (2009) Nutritional regulation of hepatic glucose metabolism. *Fish Physiol Biochem* 35: 519–539.
- 33- Enes P, Panserat S, Kaushik S, Oliva-Teles A (2011) Dietary carbohydrate utilization by European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.) juveniles. *Rev Fish Sci* 19: 201 – 215.
- 34- Polakof S, Panserat S, Soengas JL, Moon TW (2012) Glucose metabolism in fish: a review. *J Comp Physiol B* 182: 1015-1045.
- 35- Tocher DR (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquacult Res* 41: 717-732.
- 36- Oliva-Teles A (2000) Recent advances in European sea bass and gilthead sea bream nutrition. *Aquacult Int* 8: 477–492.
- 37- Cho CY, Slinger SJ, Bayley HS (1982) Bioenergetics of salmonid fishes: energy intake, expenditure and productivity. *Comp Biochem Physiol* 73: 25–41.
- 38- Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132: 365–386.
- 39- Varó I, Navarro J, Rigos G, Del Ramo J, Caldúch-Giner J, Hernández A, Pertusa J, Torreblanca A (2013) Proteomic evaluation of potentiated sulfa treatment on gilthead sea bream (*Sparus aurata* L.) liver. *Aquaculture* 376–379: 36-44.
- 40- Enes P, Panserat S, Kaushik S, Oliva-Teles A (2008) Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (*Sparus aurata*) juveniles reared at two temperatures. *Comp Biochem Physiol A* 149: 80–86.
- 41- Pérez-Sánchez J, Benedito-Palos L, Estensoro I, Petropoulos Y, Caldúch-Giner JA, Browdy CL, Sitjà-Bobadilla A (2015) Effects of dietary NEXT ENHANCE®150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). *Fish Shellfish Immunol* 44: 117–128.
- 42- Mininni AN, Milan M, Ferrarese S, Petochi T, Di Marco P, Marino G, Livi S, Romualdi C, Bargelloni L, Patarnello T (2014) Liver transcriptome analysis in gilthead sea bream upon exposure to low temperature. *BMC Genomics* 15: 765.
- 43- Pérez-Sánchez J, Borrel M, Bermejo-Nogales A, Benedito-Palos L, Saera-Vila A, Caldúch-Giner JA, Kaushik S (2013) Dietary oils mediate cortisol kinetics

- and the hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream (*Sparus aurata*) exposed to crowding stress. Implications on energy homeostasis and stress susceptibility. *Comp Biochem Physiol D* 8: 123–130.
- 44- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45.
- 45- Bell JG, Koppe W (2011) Lipids in Aquafeeds. In: Turchini GM, Ng WK, Tocher DR (eds), *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, Taylor & Francis, Boca Raton, pp. 21–59.
- 46- Geurden I, Kaushik S, Corraze G (2008) Dietary phosphatidylcholine affects postprandial plasma levels and digestibility of lipid in common carp (*Cyprinus carpio*). *Br J Nutr* 100: 512–517.
- 47- Tocher DR, Bendiksen EA, Campbell PJ, Bell JG (2008) The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* 280: 21–34.
- 48- Caballero MJ, Izquierdo MS, Kjørsvik E, Montero D, Socorro J, Fernández AJ, Rosenlund G (2003) Morphological aspects of intestinal cells from gilthead sea bream (*Sparus aurata*) fed diets containing different lipid sources. *Aquaculture* 225: 325–340.
- 49- Pérez JA, Rodríguez C, Henderson RJ (1999) The uptake and esterification of radiolabelled fatty acids by enterocytes isolated from rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol Biochem* 20: 125–134.
- 50- Caballero MJ, Gallardo G, Robaina L, Montero D, Fernández A, Izquierdo M (2006) Vegetable lipid sources affect in vitro biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*). *Br J Nutr* 95: 448–454.
- 51- Oxley A, Torstensen BE, Rustan AC, Olsen RE (2005) Enzyme activities of intestinal triacylglycerol and phosphatidylcholine biosynthesis in Atlantic salmon (*Salmo salar* L.), *Comp Biochem Physiol B* 141: 77–87.
- 52- Fernandez ML, West KL (2005) Mechanisms by which dietary fatty acids modulate plasma lipids. *J Nutr* 135: 2075 –2078.
- 53- Katan M, Zock P, Mensink R (1994) Effects of fats and fatty acids on blood lipids in humans: an overview. *Am J Clin Nutr* 60: 1017S–1022S.
- 54- Torstensen BE, Froyland L, Lie O (2004) Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil—effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquacult Nutr* 10: 175–192.

- 55- Brufau G, Canela MA, Rafecas M (2008) Phytosterols: physiologic and metabolic aspects related to cholesterolowering properties. *Nutr Res* 28: 217–225.
- 56- Moghadasi MH, Frohlich, JJ (1999) Effects of dietary phytosterols on cholesterol metabolism and atherosclerosis: clinical and experimental evidence. *Am J Med* 107: 588–594.
- 57- Ostlund RE (2002) Phytosterols in human nutrition. *Annu Rev Nutr* 22: 533–549.
- 58- Ostlund RE (2004) Phytosterols and cholesterol metabolism. *Curr Opin Lipidol* 15: 37–41.
- 59- Gilman CI, Leusch FD, Breckenridge WC, MacLatchy DL (2003) Effects of a phytosterol mixture on male fish plasma lipoprotein fractions and testis P450scc activity. *Gen Comp Endocrinol* 130: 172–184.
- 60- de Jong A, Plat J, Mensink RP (2003) Metabolic effects of plant sterols and stanols (Review). *J Nutr Biochem* 14: 362-369.
- 61- Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerré, D., Médale F, Corraze G, Kaushik S (2008) Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. *Br J Nutr* 100: 953–967.
- 62- Peng M, Xu W, Mai K, Zhou H, Zhang Y, Liufu Z, Zhang K, Ai Q (2014) Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. *Aquaculture* 433: 442-449.
- 63- Dorfman SE, Wang S, Vega-López S, Jauhainen M, Lichtenstein AH (2005) Dietary Fatty Acids and Cholesterol Differentially Modulate HDL Cholesterol Metabolism in Golden-Syrian Hamsters. *J Nutr* 135: 492-498.
- 64- Jonas A (2000) Lecithin cholesterol acyltransferase. *Biochim Biophys Acta* 1529: 245 – 256.
- 65- Kunnen S, Van Eck M (2012) Lecithin: cholesterol acyltransferase: old friend or foe in atherosclerosis? *J Lipid Res* 53: 1783–1799.
- 66- Hatahet W, Cole L, Kudchodkar BJ, Fungwe TV (2003) Dietary fats differentially modulate the expression of lecithin: cholesterol acyltransferase, apoprotein-A1 and scavenger receptor B1 in rats. *J Nutr* 133: 689 – 694.
- 67- Dias J, Alvarez MJ, Diez A, Arzel J, Corraze G, Bautista JM, Kaushik SJ (1998) Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (*Dicentrarchus labrax*). *Aquaculture* 161: 169–186.

- 68- Kamalam BS, Médale F, Kaushik S, Polakof S, Skiba-Cassy S, Panserat S (2012) Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J Exp Biol* 215: 2567–2578.
- 69- Peres MH, Gonçalves P, Oliva-Teles A (1999) Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). *Aquaculture* 179: 415-423.
- 70- Fried SK, Rao SP (2003) Sugars, hypertriglyceridemia, and cardiovascular disease. *Am J Clin Nutr* 78: 873S-880S.
- 71- Parks EJ, Hellerstein MK (2000) Carbohydrate-induced hypertriacylglycerolemia: historical perspective and review of biological mechanisms. *Am J Clin Nutr* 71: 412-433.
- 72- Fountoulaki E, Alexis MN, Nengas I, Venou B (2005) Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (*Sparus aurata* L.). *Aquacult Res* 36: 1243–1251.
- 73- Hemre GI, Sandnes K, Lie ø, Torrisen O, Waagboe R (1995) Carbohydrate nutrition in Atlantic salmon, *Salmo salar* L.: growth and feed utilization. *Aquacult Res* 26: 149-154.
- 74- Iqbal J, Hussain MM (2005) Evidence for multiple complementary pathways for efficient cholesterol absorption in mice. *J Lipid Res* 46: 1491–1501.
- 75- Pan X, Hussain MM (2012) Gut triglyceride production. *Biochim Biophys Acta* 1821: 727–735.
- 76- Engelking LR (2010) In: Engelking LR (ed) Triglycerides and Glycerophospholipids, Textbook of Veterinary Physiological Chemistry, updated 2nd edn., Academic Press (Elsevier), Amsterdam Netherlands, pp. 315-326.

Chapter 5-

Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles

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Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles

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Abstract

Plant feedstuffs (PF) are rich in carbohydrates, which may interact with lipid metabolism. Thus, when considering dietary replacement of fishery by-products with PF, knowledge is needed on how dietary lipid source (LS) and carbohydrates affect lipid metabolism and other metabolic pathways. For that purpose, a 73-d growth trial was performed with European sea bass juveniles (IBW 74 g) fed four diets differing in LS (fish oil (FO) or a blend of vegetable oils (VO)) and carbohydrate content (0 % (CH-) or 20 % (CH+) gelatinised starch). At the end of the trial no differences among diets were observed on growth and feed utilisation. Protein efficiency ratio was, however, higher in the CH+ groups. Muscle and liver fatty acid profiles reflected the dietary LS. Dietary carbohydrate promoted higher plasma cholesterol and phospholipids (PL), whole-body and hepatic (mainly 16 : 0) lipids and increased muscular and hepatic glycogen. Except for PL, which were higher in the FO groups, no major alterations between FO and VO groups were observed on plasma metabolites (glucose, TAG, cholesterol, PL), liver and muscle glycogen, and lipid and cholesterol contents. Activities of glucose-6-phosphate dehydrogenase and malic enzyme – lipogenesis-related enzymes – increased with carbohydrate intake. Hepatic expression of genes involved in cholesterol metabolism was up-regulated with carbohydrate (HMGCR and CYP3A27) and VO (HMGCR and CYP51A1) intake. No dietary regulation of long-chain PUFA biosynthesis at the transcriptional level was observed. Overall, very few interactions between dietary carbohydrates and LS were observed. However, important insights on the direct relation between dietary carbohydrate and the cholesterol biosynthetic pathway in European sea bass were demonstrated.

Key words: Carbohydrates; Lipid source; Cholesterol; Metabolism; Gene expression

Fish are an important source of *n*-3 long-chain PUFA (LC-PUFA) – namely, of EPA (20 : 5*n*-3) and DHA (22 : 6*n*-3) – which are considered of relevance to the health of human consumers. The *n*-3 LC-PUFA content of farmed fish has been guaranteed by the high incorporation levels of fish oil (FO) and fishmeal (FM) in aquafeeds, which are non-sustainable feed resources^(1,2). Thus, both from economic and environmental perspectives, a sustainable growth of the aquaculture sector demands that alternatives to these marine fishery-derived feedstuffs be used in aquafeeds. Plant feedstuffs (PF) and vegetable oils (VO) are among the most suitable potential alternatives to FM and FO. However, VO are devoid of *n*-3 LC-PUFA, which are characteristics of FO. In addition, increasing of VO levels in aquafeeds usually reduces dietary cholesterol content and increases phytosterol levels^(1,3).

Over the years, a large body of information has been obtained concerning the effects of such ingredients on growth

performance, feed efficiency, metabolic responses, health and nutritional quality of several fish species^(4–7). Data obtained so far indicate that FO in aquafeeds can be substantially replaced with VO without any major effects on fish performance^(7–11). However, VO are reported to affect fish body composition and lipid metabolism^(4,6,7). The effects of VO on lipogenesis are not consistent^(9,10,12–16), but obvious changes in other lipid metabolic processes such as LC-PUFA synthesis and cholesterol metabolism were reported^(4,16).

Although salmonids and freshwater species are capable of bioconversion of C18 fatty acids (FA) to *n*-3 LC-PUFA, this endogenous synthesis seems to be inefficient to counteract the dietary *n*-3 LC-PUFA deficit in fish fed VO-based diets, as reduction of *n*-3 LC-PUFA levels in the flesh and a decrease in the final product nutritional quality are usually observed⁽¹⁾. Nonetheless, in freshwater fish and salmonids fed with VO a

Abbreviations: FA, fatty acid; FAS, fatty acid synthase; FM, fishmeal; FO, fish oil; G6PDH, glucose-6-phosphate dehydrogenase; GK, glucokinase; LC-PUFA, long-chain PUFA; LS, lipid source; PEPCK, phosphoenolpyruvate carboxykinase; PF, plant feedstuff; PL, phospholipid; VO, vegetable oil.

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clear up-regulation of desaturase gene expression and increased activity of the enzymes involved in the conversion of C18 FA to *n*-3 LC-PUFA are observed^(1,17,18). Such responses are not so obvious in marine fish^(7,19,20).

Replacement of FM by PF will increase dietary carbohydrate content, as carbohydrates are present in high quantities in most PF. Carbohydrates are, however, almost absent in the natural food of most fish species and are not well utilised by fish, particularly by carnivorous species. Previous studies suggest that carbohydrates have an important role in the modulation of lipid metabolism in fish^(17,21–25). For instance, carbohydrate administration alters plasma TAG^(22,23), induces hepatic lipogenesis and lipid deposition^(21,24), and stimulates FA bioconversion in salmonids^(17,25). The latter aspect is of particular interest, as expression or increased activity of elongation and desaturation enzymes may contribute to increasing the amounts of *n*-3 LC-PUFA in fish tissues and hence the nutritional quality of fillets. Conversely, regulation of carbohydrate metabolism by dietary lipids was also reported in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar* L.)^(26,27).

To increase the incorporation of PF in aquafeeds, particularly in marine species, further knowledge is needed on how dietary FA composition influences lipid metabolism. It is also required to understand the potential interactions between dietary carbohydrate and lipid source (LS), and how it may affect the regulation of processes involved in lipid metabolism.

Regardless of the lack of a dietary requirement of cholesterol in fish, it is an essential molecule with important cell membrane functions, and it is also the precursor of many physiologically active compounds (such as bile acids, vitamin D, adrenal corticoids and sex hormones)⁽²⁾.

The metabolic pathway of cholesterol biosynthesis, particularly in the liver, was transcriptionally up-regulated by VO feeding in salmonids⁽⁴⁾. In contrast to *n*-3 LC-PUFA content, tissue cholesterol levels were unaffected, meaning that a lower intake of dietary cholesterol in fish fed VO diets was fully compensated by increased cholesterol synthesis⁽⁴⁾.

The aim of this study was to evaluate whether an interaction between dietary LS and dietary carbohydrate level induced liver enzymatic activity and expression of genes related to lipid metabolism, particularly LC-PUFA and cholesterol pathways, in juveniles of a marine fish species, the European seabass.

Methods

Experimental diets

Four diets meeting the nutrient requirements of European sea bass⁽²⁾ but differing in carbohydrate content (0 and 20% gelatinised starch, CH- and CH+, respectively) and LS (FO or VO) were formulated (Table 1). The dietary carbohydrate content was increased at the expense of dietary protein. In the CH- diets protein content was increased to replace carbohydrates. The VO was a blend of rapeseed (20%), linseed (50%) and palm (30%) oils, and replaced circa 70% of dietary lipids of the FO diets, which were provided by cod liver oil and FM.

All ingredients were finely ground, well mixed and dry pelleted in a laboratory pellet mill (California Pellet Mill),

Table 1. Ingredient and chemical composition of the experimental diets

Lipid source...	Experimental diets			
	FO	VO	CH-	CH+
Carbohydrates...	CH-	CH+	CH-	CH+
Ingredients (% dry weight)				
Fishmeal*	86.5	64.5	86.5	64.5
Gelatinised maize starch†	0	20	0	20
Cod liver oil‡	10	12	0	0
Vegetable oil blend§	0	0	10	12
Vitamins	1.5	1.5	1.5	1.5
Minerals¶	1.0	1.0	1.0	1.0
Binder††	1.0	1.0	1.0	1.0
Proximate analyses (% DM)				
DM	89.5	90.4	91.3	91.0
Crude protein (CP)	62.4	46.6	62.4	47.1
Crude lipid (CL)	18.4	18.4	18.2	18.3
Starch	0.8	18.7	0.8	17.5
Ash	17.6	14.0	17.6	13.8
Cholesterol	0.5	0.4	0.4	0.3

FO, fish oil; VO, blend of vegetable oils; carbohydrate content, 0% (CH-) or 20% (CH+) gelatinised maize starch.

* Steam-dried low temperature fishmeal; Pesquera Diamante (CP: 71.1% DM; CL: 8.8% DM).

† C-Gel Instant-12018; Cerestar.

‡ Labchem; Laborspirit Lda.

§ 30% palm oil (Colmi), 50% linseed oil (Sociedade Portuense de Drogas) and 20% rapeseed oil (Huilerie Emile Noël SAS).

|| Vitamins (mg/kg diet): retinyl acetate, 18 000 IU (6.19 mg)/kg diet; cholecalciferol, 2000 IU (0.04 mg)/kg diet; *a*-tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid, 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400 (Premix).

¶ Minerals (mg/kg diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.40 (g/kg diet) (Premix).

†† Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate) (Agil).

through a 3-mm die. The pellets were air-dried for 24 h and stored in a refrigerator (4°C) until use.

Animals, experimental conditions and sampling

This experiment was directed by trained scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was performed at the experimental facilities of the Marine Zoological Station, University of Porto, Portugal, in a thermo-regulated recirculation water system equipped with twelve fibreglass cylindrical tanks of 300 litre water capacity and supplied with continuous flow of filtered seawater. After 2 weeks of adaptation to the experimental conditions, twelve groups of twenty European sea bass (*Dicentrarchus labrax*) juveniles (initial body weight: 74.0 (SEM 1.5) g) were established and randomly distributed into the tanks. At the beginning of the trial, fifteen fish from the stock population were sampled and pooled for whole-body composition analysis. The experimental diets were randomly assigned to triplicate groups of fish. During the trial, salinity averaged 35 (SEM 1.0) g/l, dissolved oxygen was kept near saturation, and water temperature was regulated to

25.4 (SEM 0.5) °C. The growth trial lasted 73 d, and during this period fish were hand-fed twice a day, 6 d a week, to apparent visual satiety. At the end of the trial, fish were unfed for 1 d to empty gut content and then bulk-weighed after mild anaesthesia with 0.3 ml/l methylethanol. To eliminate handling stress, fish continued to be fed for one more week and then, 18 h after the last meal (the previous day's afternoon meal), nine fish from each tank were randomly sampled for blood, liver and muscle collection. Blood was collected without anaesthesia from the caudal vein using heparinised syringes and centrifuged at 2500 **g** for 10 min, and the recovered plasma was kept at -20°C until analysis. Thereafter, the fish were killed with a sharp blow to the head, and whole body, viscera and liver were weighed for determination of hepatosomatic index and viscerosomatic index (VSI). Liver and muscle sections were frozen in liquid N₂ and then stored at -80°C until biochemical, enzymatic and molecular analyses.

Diets, whole fish, liver, muscle and plasma analysis

Chemical analysis of experimental diets, whole fish, liver and muscle was conducted according to the following procedures: DM after drying at 105°C until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein content ($N \times 6.25$) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit (models 1015 and 1026, Tecator Systems; Höganäs); starch according to Beutler⁽²⁸⁾; and lipid by petroleum ether extraction (Soxtec HT System; Höganäs). The hepatic and muscular glycogen contents were determined as described by Roehrig & Allred⁽²⁹⁾. Lipids in liver and muscle were determined according to the method used by Folch *et al.*⁽³⁰⁾. FA methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride (BF₃) in methanol (14%) according to Santha & Ackman⁽³¹⁾ and analysed by GC (Varian 3900; Varian) as described in the study by Castro *et al.*⁽³²⁾. Total cholesterol in the diets and tissues (liver and muscle) was assayed on total lipid extract by means of the Liebermann-Burchard method⁽³³⁾.

Plasma metabolites were analysed using commercial kits from Spinreact: glucose (ref: 1001191), TAG (ref: 1001312), total cholesterol (ref: 1001090) and phospholipids (PL; ref: 1001140).

Enzymatic activity assays

The activity of key lipogenesis enzymes was determined in the liver. Liver was homogenised (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0.1 mM-EDTA and 0.1% triton X-100 (v/v), pH 7.8). All procedures were performed on ice. Homogenates were centrifuged at 30 000 **g** for 30 min at 4°C. After centrifugation, the resultant supernatant was collected and aliquots were stored at -80°C until analysis. All enzyme activities were measured at 37°C, monitoring the changes in absorbance of NADPH at 340 nm in a microplate reader (ELx808™, BioTek Instruments), using 6.22 mM/cm as the millimolar extinction coefficient used for NADPH. The optimal substrate and protein concentrations for measurement of each enzyme activity were established by preliminary assays. Assay conditions were as follows.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) activity was assayed as previously described by Morales *et al.*⁽³⁴⁾, using a reaction mixture containing 50 mM-imidazole-HCl buffer (pH 7.4), 5 mM-MgCl₂, 2 mM-NADP and 1 mM-glucose-6-phosphate.

Malic enzyme (ME; EC 1.1.1.40) activity was assayed using a reaction mixture containing 50 mM-imidazole-HCl buffer (pH 7.4), 5 mM-MgCl₂, 0.4 mM-NADP and 2 mM-L-malate⁽³⁵⁾.

Fatty acid syntase (FAS; EC 2.3.1.38) activity was assayed as previously described by Chang *et al.*⁽³⁶⁾, modified by Chakrabarty & Leveille⁽³⁷⁾. Samples were incubated with solution A (100 mM-potassium phosphate buffer pH 6.5, 0.1 mM-NADPH and 25 µM-acetyl-CoA) for 10 min. Then solution B (100 mM-potassium phosphate buffer pH 6.5 and 600 µM-malonyl-CoA) was added to this mixture.

Enzyme activities were expressed as milliunits per milligram of hepatic soluble protein (specific activity). Protein concentration was determined according to Bradford⁽³⁸⁾ using the Sigma protein assay kit and bovine serum albumin as standard. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 µmol of substrate/min under the above assay conditions.

Gene expression analysis

Analyses of mRNA levels were performed on liver samples (two fish per tank). Total RNA was extracted using TRIzol reagent (Invitrogen) according to manufacturer recommendations, and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000; Nanodrop Labtech). Complementary DNA (cDNA) synthesis was performed with 1 µg of the resulting total RNA using the SuperScript III RNaseH-Reverse Transcriptase kit (Invitrogen) and random primers (Promega). Gene expression levels were determined by real-time quantitative PCR (q-PCR) using LightCycler® 480 II apparatus (Roche Diagnostics). Analyses were carried out using 2 µl of the diluted cDNA (1:76) mixed with 0.24 µl of each primer (10 µM), 3 µl LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH) and 0.52 µl DNase/RNase/Protease-free water (5 prime GmbH) in a total volume of 6 µl. Primers were either found in the literature or designed to overlap an intron using Primer3 software⁽³⁹⁾ and known sequences of European sea bass nucleotides in databases (Public Sigenae Contig Browser, Ensembl; <http://public-contigbrowser.sigenae.org:9090/index.html>) (Table 2).

Thermal cycling was initiated with incubation at 95°C for 10 min for hot-start iTaq™DNA polymerase activation. A total of forty-five steps of PCR were then performed, each one consisting of heating at 95°C for 15 s for denaturing, at 60°C for 10 s for annealing and at 72°C for 15 s for extension. Following the PCR cycle, melting curves were systematically monitored (55°C temperature gradient at 0.5°C/5 s from 55 to 94°C) to ensure that only one fragment was amplified. Each PCR run included duplicates of reverse transcription for each sample and negative controls (RT-free samples, RNA-free samples). The PCR run for the reference gene included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and negative controls.

Table 2. Sequences of the primer pairs used for real-time quantitative PCR determination of the transcript level of several European sea bass genes involved in hepatic and intestinal lipid and glucose metabolism

Genes	5'-3' Forward primer	5'-3' Reverse primer	Primer efficiency	Accession number
CYP3A27	AGCCAACCAACCTATGTC	CACAAATCAGGGTCACGATG	1.97	FM015979
CYP51A1	GACTGACCCTCCGGTGT	TCCGGTCTTCAGCATTTCT	1.86	FM002086
EF1 α	GCTTCGAGGAATACCAAG	CAACCTTCATCCCTTGAC	1.87	(40)
elovl5	GCACGGTGGCTACAACCT	TGTCCATGAACCTCGATGAGC	—	*
FADS2	CCTTCACTGCTTCATCCCCA	CCCAGGTGGAGGCAGAAGAA	1.98	(40)
GLUT2	GAGCCCACGGTACCTTACA	CGGATCAAAGAAAGGATGGA	1.94	EF014277
GK	ATCGTCAGGGAACTCACCC	GAGTCAGGCTTGCTTCACC	1.92	AM986860
G6Pase	TGAGACCCGGTTTATGGAG	CATGCAGACCACAGCTCA	1.90	AM987970
HMGCR	CCAGCTTCGTATTTCAGCACA	GCTTGAGAGGTCGATGAG	2.00	(6)
LXR	CCCCGTATGCTGATGAAACT	AAGCCACAGAACATGGGACATC	1.97	FM016781
PEPCK	GCGCCATCAACACTAAAGGT	TTGTGCACTCTGCTCCAG	2.00	DV217087
PK	CTGTTTCCGTGGAGGCAGT	CAGCACAGCATTGAGGAG	1.93	AM981422
SREBP1	CTGGAGCCAAACAGAGGAG	GACAGGAAGGAGGGAGGAAG	1.95	(40)

CYP3A27, cytochrome P450 3A27; CYP51A1, cytochrome P450 51 (lanosterol 14- α -demethylase); EF1 α , elongation factor-1 α ; elovl5, elongase 5; —, no expression; FADS2, Δ 6 fatty acyl desaturase; GLUT2, GLUT type 2; GK, glucokinase; G6Pase, glucose-6-phosphatase; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; LXR, liver X receptor; PEPCK, phosphoenolpyruvate carboxykinase; PK, pyruvate kinase; SREBP1, sterol response element binding protein-1.

* D Mazurais (unpublished results).

Quantification of the target gene transcripts in the liver was done using the elongation factor-1 α (EF1 α) gene expression as reference, as previously used in European sea bass by Geay *et al.*⁽⁴⁰⁾ and that was stably expressed in the present study (data not shown). Relative quantification of the target gene transcript with the EF1 α reference gene transcript was performed using the mathematical model described by Pfaffl⁽³⁹⁾. The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and the CT deviation (ΔCT) of the unknown sample compared with a control sample and expressed in comparison with the EF1 α reference gene:

$$R = \frac{[(E_{\text{target gene}})^{\Delta CT \text{ target gene (mean control - mean sample)}}]}{[(E_{\text{EF1}\alpha})^{\Delta CT \text{ EF1}\alpha \text{ (mean control - mean sample)}}]}.$$

Efficiency of q-PCR was measured by the slope of a standard curve using serial dilutions of cDNA.

Statistical analysis

Data were checked for normal distribution and homogeneity of variances and when appropriate were normalised. Statistical evaluation of data was carried out by a 2×2 factorial arrangement of treatments in a completely randomised experimental design (two-way ANOVA) with carbohydrate level and LS as fixed factors. The significance level of 0.05 was used for rejection of the null hypothesis. In cases where interaction was significant, one-way ANOVA was performed for each factor. All statistical analyses were conducted using the SPSS 21.0 software package (IBM Corp.) for Windows.

Results

Dietary fatty acid composition

The four diets presented small differences in the proportions of total SFA (slightly higher in VO diets) and MUFA (higher in FO diets) (Table 3). Within MUFA, high levels of oleic acid

Table 3. Fatty acid composition (% of total fatty acids) of the experimental diets

Lipid source...	Experimental diets			
	FO		VO	
Carbohydrates...	CH-	CH+	CH-	CH+
14 : 0	5.2	5.0	2.7	2.0
15 : 0	0.4	0.4	0.2	0.2
16 : 0	14.5	13.6	17.8	17.2
17 : 0	0.3	0.3	0.3	0.2
18 : 0	2.9	2.7	3.7	3.5
20 : 0	0.2	0.1	0.3	0.3
Σ SFA	23.7	22.2	25.3	23.8
16 : 1n-7	7.5	7.5	3.3	2.4
18 : 1n-9	16.7	17.7	24.6	27.1
20 : 1n-9	5.9	7.0	0.5	0.5
22 : 1n-9	4.6	5.5	0.1	0.1
Σ MUFA	34.9	37.8	28.5	30.0
18 : 2n-6	1.5	1.7	9.2	10.8
18 : 3n-6	0.2	0.2	0.1	0.1
20 : 2n-6	0.3	0.3	0.1	0.1
20 : 4n-6	0.8	0.7	0.5	0.4
Σ n-6 PUFA	5.9	5.7	11.7	12.5
18 : 3n-3	0.9	1.0	17.4	21.3
18 : 4n-3	2.3	2.4	0.7	0.5
20 : 4n-3	0.7	0.7	0.3	0.2
20 : 5n-3	10.9	10.5	5.6	4.2
21 : 5n-3	0.5	0.5	0.2	0.2
22 : 5n-3	1.6	1.5	0.8	0.6
22 : 6n-3	13.9	13.1	7.3	5.4
Σ n-3 PUFA	30.8	29.7	32.4	32.4
SFA:PUFA	0.6	0.6	0.6	0.5
n3:n6	10.5	10.1	3.2	2.9

FO, fish oil, VO, blend of vegetable oils; carbohydrate content, 0% (CH-) or 20% (CH+) gelatinised maize starch.

(18 : 1n-9) were found in VO diets, whereas the opposite was seen for palmitoleic acid (16 : 1n-7), eicosenoic acid (20 : 1n-9) and erucic acid (22 : 1n-9). Linoleic acid (18 : 2n-6) was strongly higher in VO diets than in FO diets. Regarding n-3 PUFA, VO diets were particularly rich in linolenic acid (18 : 3n-3) and poor in EPA and DHA.

Table 4. Growth performance and feed utilisation of European seabass fed the experimental diets†
(Mean values with their standard errors; n 3)

Lipid source (LS)...	Experimental diets							
	FO		VO		SEM	P*		
	CH-	CH+	CH-	CH+		CH	LS	CH × LS
IBW (g)	74.3	74.3	74.3	74.3	0.007	1.000	0.370	0.094
FBW (g)	223	219	221	224	1.59	0.780	0.701	0.369
Daily growth index (DGI)‡	2.6	2.5	2.5	2.6	0.020	0.757	0.708	0.379
Feed intake (g/kg ABW/d)	16.7	16.6	16.2	17.0	0.210	0.408	0.876	0.299
Feed efficiency (FE)§	0.82	0.81	0.84	0.81	0.007	0.172	0.580	0.442
Protein efficiency ratio (PER)	1.3	1.8	1.4	1.7	0.061	<0.001	0.949	0.246
N intake (g/kg ABW/d)¶	1.7	1.2	1.6	1.3	0.060	<0.001	0.875	0.258
N retention (g/kg ABW/d)¶¶	0.39	0.38	0.40	0.39	0.005	0.231	0.359	1.000
N retention (% N intake)††	23.6	30.6	24.9	30.2	0.962	<0.001	0.341	0.128
Lipid intake (g/kg ABW/d)‡‡	3.1	3.1	2.9	3.1	0.039	0.371	0.641	0.250
Lipid retention (g/kg ABW/d)§§	2.4	3.0	2.5	2.9	0.086	<0.001	0.791	0.570
Lipid retention (% lipid intake)	78.3	97.3	84.1	94.7	2.62	0.001	0.582	0.175

Fish oil (FO), blend of vegetable oils (VO); CH content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch; IBW, initial body weight; FBW, final body weight; ABW, average body weight.

* Significant differences at P < 0.05 (two-way ANOVA).

† ABW: IBW + FBW/2.

‡ DGI: ((FBW^{1/3} – IBW^{1/3})/time in d) × 100.

§ FE, wet weight gain/dry feed intake.

|| PER, wet weight gain/crude protein intake.

¶ N retention = ((FBW × carcass N content) – (IBW × carcass N content))/(ABW × number of d).

¶¶ N retention = N retention (g/kg ABW/d)/N intake.

‡‡ Lipid retention = ((FBW × carcass lipid content) – (IBW × carcass lipid content))/(ABW × number of d).

§§ Lipid retention = lipid retention (g/kg ABW/d)/lipid intake.

Growth performance and feed utilisation

No mortality was observed during the trial. At the end of the feeding trial, there were no differences among dietary treatments on growth performance or feed utilisation (Table 4). Feed intake (gram per kilogram average body weight per d) was not affected by experimental diets, and therefore N intake was lower in the CH+ diet. N retention, expressed per unit weight gain, was not affected by diet composition, but when its retention was expressed as percentage of intake it was higher in the CH+ groups. Lipid retention, expressed as either percentage of lipid intake or per unit weight gain, increased with dietary carbohydrate intake (Table 4). No significant effect of LS or of interaction between carbohydrate and LS was observed for any of these parameters.

Whole-body, liver and muscle composition

Whole-body protein and ash contents were not affected by dietary treatments, but higher lipid content and hepatosomatic index and VSI were observed in the CH+ groups (Table 5). Glycogen content increased in the liver and muscle with the CH+ diet (Table 5). An increase in lipid content in the liver was also observed, but no differences were detected in muscle lipids, nor in cholesterol content, in either tissue.

Liver and muscle lipid, cholesterol and glycogen content were not affected by dietary LS (Table 5).

Liver and muscle fatty acid profiles

Muscle and liver FA composition was affected by diet composition and mirrored the FA profile of the dietary LS (Tables 6 and 7).

Accordingly, the muscle FA profile of fish fed the VO diet was characterised by lower levels of SFA, higher levels of n-6 PUFA (particularly high 18 : 2n-6 levels) and n-3 PUFA (particularly high 18 : 3n-3 levels), and low arachidonic acid (20 : 4n-6), EPA and DHA. The CH+ diet also increased muscle SFA and promoted a decrease of n-3 PUFA (Table 7).

Hepatic n-6 PUFA was higher in the VO groups than in the FO groups (particularly 18 : 2n-6, 18 : 3n-6 and 20 : 4n-6) and lower in groups fed the CH+ diet than in those fed the CH- diet (particularly 20 : 2n-6 and 20 : 4n-6 (Table 6)). Fish fed the CH+ diet also presented increased MUFA levels. In the liver, an interaction between dietary carbohydrate and LS was noticed in SFA and n-3 PUFA content. Thus, n-3 PUFA content in fish fed the CH+ diet slightly increased when FO was replaced by VO, whereas it considerably decreased in fish fed the CH- diet. Opposite results were observed for the SFA content, and this was essentially because of differences in palmitic acid levels.

Plasma metabolites

Dietary carbohydrate intake promoted an increase in plasma cholesterol and PL levels (Table 8), whereas dietary VO decreased plasma PL levels. No differences among dietary treatments were observed on plasma glucose and TAG levels.

Enzymes activity

Replacement of FO by VO did not affect the activity of hepatic FAS, ME or G6PDH, which are key hepatic enzymes involved in

Table 5. Whole-body, liver and muscle composition (wet-weight basis), hepatosomatic and viscerosomatic indices of European sea bass fed the experimental diets†
 (Mean values with their standard errors)

Lipid source (LS)...	Experimental diets							
	FO		VO		SEM	P*		
	CH-	CH+	CH-	CH+		CH	LS	CH × LS
Whole-body composition‡								
Protein (%)	17.0	16.7	17.4	16.9	0.110	0.089	0.303	0.597
Lipids (%)	15.3	18.2	15.6	17.9	0.420	<0.001	0.866	0.374
DM (%)	36.3	38.9	36.9	39.0	0.402	<0.001	0.430	0.558
Ash (%)	4.1	3.8	4.1	4.0	0.072	0.187	0.360	0.455
HSI§	1.5	2.6	1.5	2.7	0.075	<0.001	0.958	0.575
VSI	10.3	13.6	10.0	13.4	0.265	<0.001	0.481	0.889
Liver composition								
Lipids (%)	23.8	27.7	24.5	30.6	0.885	0.003	0.240	0.482
Cholesterol (%)	0.28	0.26	0.25	0.23	0.009	0.286	0.074	0.871
Glycogen (mg/g liver)	71.3	119	78.3	118	4.42	<0.001	0.349	0.286
Muscle composition								
Lipids (%)	3.7	3.9	4.1	3.8	0.271	0.942	0.752	0.685
Cholesterol (%)	0.08	0.06	0.08	0.06	0.004	0.119	0.981	0.859
Glycogen (μg/g muscle)	14.0	19.8	13.4	15.8	1.03	0.027	0.376	0.638

FO, fish oil; VO, blend of vegetable oils; CH content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch.

* Significant differences at $P < 0.05$ (two-way ANOVA).

† $n = 3$, for whole body composition; $n = 6$ for lipids and cholesterol; $n = 9$ for glycogen; $n = 18$ for hepatosomatic index (HSI) and viscerosomatic index (VSI).

‡ Initial body composition on the fish: DM, 27.47%; protein, 15.26%; lipid, 10.82%; ash, 4.87%.

§ HSI: (liver weight/body weight) $\times 100$.

|| VSI: (viscera weight/body weight) $\times 100$.

lipogenesis (Table 9). On the other hand, dietary carbohydrate induced an increase in ME and G6PDH activities but not in FAS activity.

Gene expression

In the liver, expression of glucokinase (*GK*), the first key glycolytic enzyme, and of phosphoenolpyruvate carboxykinase (*PEPCK*), the first key gluconeogenic enzyme, responded to diet composition, whereas pyruvate kinase (*PK*) and glucose 6-phosphatase (*G6Pase*), enzymes involved in the last step of glycolysis and gluconeogenesis, respectively, were not nutritionally regulated (Fig. 1). *PEPCK* mRNA levels were higher in fish fed the CH- diet and were not affected by dietary LS, whereas an interaction between carbohydrate level and LS was observed for *GK* transcripts. Accordingly, *GK* transcription was strongly induced by dietary carbohydrate (starch) intake, and it was also induced by VO intake, but only in fish fed the CH- diet. Liver GLUT type 2 (*GLUT2*), the protein involved in glucose transport, transcript levels were not affected by diet composition (Fig. 1).

Data on the expression marker genes encoding the proteins involved in cholesterol biosynthesis in the liver are shown in Fig. 2. Feeding VO diets up-regulated *CYP51A* (cytochrome P450 51) (lanosterol 14- α -demethylase) mRNA levels in the liver. Hepatic *HMGCR* (3-hydroxy-3-methylglutaryl-coenzyme A reductase) transcript pattern showed significant interaction between dietary carbohydrate level and LS in the liver. Induction of *HMGCR* mRNA levels was observed in fish fed the VOCH- diet. *HMGCR* transcript levels were also induced by

dietary carbohydrate, but only in fish fed the FO diet. In the liver, dietary carbohydrate also promoted an increase of *CYP3A27* (cytochrome P450 3A27) mRNA levels and a down-regulation of *LXR* (liver X receptor) transcription.

Liver expression of *FADS2* ($\Delta 6$ fatty acyl desaturase), a protein involved in the LC-PUFA biosynthesis pathway, and of *SREBP1* (sterol regulatory element binding protein 1) were not affected by diet composition (Fig. 3). We were unable to detect expression of hepatic elongase 5 (*elovl5*) in the liver.

Discussion

Although an effect of dietary protein level on the parameters analysed in this study cannot be completely discarded, protein requirements of European sea bass were fulfilled in all dietary treatments, and we assume that dietary carbohydrate level and LS were the main factors responsible for the observed effects.

Effect of dietary carbohydrate

As previously observed by Peres & Oliva-Teles⁽⁴²⁾, in the present study reduction of dietary protein from 60 to 45% by increasing gelatinised starch content did not affect growth performance. N retention (% N intake) was also higher in the CH+ groups, further supporting the protein-sparing effect of dietary starch.

The HSI in the CH+ groups is in agreement with the higher glycogen and lipid levels, and it is also in accordance with previous data on this species⁽⁴²⁾ and other fish species^(24,43,44).

Table 6. Liver fatty acid profile (% of total fatty acids; FA) of European seabass fed the experimental diets†
(Mean values with their standard errors; n 6)

Lipid source (LS)...	Experimental diets							
	FO		VO		SEM	P*		
	CH-	CH+	CH-	CH+		CH	LS	CH × LS
FA								
14 : 0	2.7	3.0	2.3	2.3	0.07	0.287	<0.001	0.142
15 : 0	0.21	0.13	0.11	0.07	0.013	0.001	<0.001	0.251
16 : 0	22.3	30.0	24.6	27.9	0.699	<0.001	0.898	0.005
17 : 0	0.30	0.18	0.22	0.13	0.015	<0.001	<0.001	0.262
18 : 0	6.3	7.6	9.6	7.4	0.334	0.368	0.004	0.002
20 : 0	0.11	0.11	0.14	0.13	0.004	0.056	<0.001	0.105
ΣSFA	31.9	41.0	37.0	37.9	0.822	<0.001	0.297	<0.001
16 : 1n-7	5.1	5.7	2.9	4.7	0.241	<0.001	<0.001	0.018
18 : 1n-9	34.7	36.6	39.3	41.6	0.804	0.115	0.001	0.909
20 : 1n-9	2.46	1.76	0.78	0.59	0.167	<0.001	<0.001	0.061
22 : 1n-9	1.10	0.93	0.05	—	0.107	0.005	<0.001	0.878
ΣMUFA	43.4	45.1	43.1	47.0	0.620	0.024	0.501	0.378
18 : 2n-6	1.32	0.88	3.47	3.09	0.264	0.153	<0.001	0.922
18 : 3n-6	0.13	0.10	0.19	0.20	0.013	0.700	0.001	0.376
20 : 2n-6	0.17	0.10	0.18	0.13	0.009	<0.001	0.244	0.627
20 : 4n-6	0.66	0.34	0.48	0.21	0.037	<0.001	<0.001	0.336
Σn-6 PUFA	2.3	1.5	4.4	3.6	0.279	0.019	<0.001	0.847
18 : 3n-3	0.43	0.28	4.56	4.49	0.467	0.093	<0.001	0.131
18 : 4n-3	0.77	0.51	0.51	0.53	0.033	0.025	0.026	0.013
20 : 4n-3	0.30	0.16	0.09	0.05	0.022	<0.001	<0.001	0.459
20 : 5n-3	5.38	2.85	2.60	1.40	0.324	<0.001	<0.001	0.054
21 : 5n-3	0.20	0.06	0.06	0.02	0.016	<0.001	<0.001	0.008
22 : 5n-3	0.81	0.29	0.30	0.13	0.061	<0.001	<0.001	0.011
22 : 6n-3	9.11	3.55	3.84	1.84	0.637	<0.001	<0.001	0.775
Σn-3 PUFA	17.1	7.7	12.0	8.5	0.949	<0.001	0.090	0.025
Σn-3 LC-PUFA	15.9	6.9	7.0	3.5	1.05	<0.001	<0.001	0.483
Ratios								
SFA:PUFA	1.6	4.4	2.3	3.2	0.291	<0.001	0.853	0.020
n3:n6	7.5	5.4	2.7	2.3	0.453	<0.001	<0.001	0.482

FO, fish oil; VO, blend of vegetable oils; CH content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch; n-3 LC-PUFA, n-3 long chain PUFA.

* Significant differences at P < 0.05 (two-way ANOVA).

† FA ≥ 0.02%; <0.02% was not considered in the table as it was below detection.

Dietary carbohydrate also promoted higher whole-body lipid retention, and most of it was deposited in the viscera, as reflected in the positive correlation between VSI and dietary starch. Such a relationship was also previously reported in European sea bass by Peres & Oliva-Teles⁽⁴²⁾. In the muscle, an increase in glycogen content but not in lipid content was also observed in fish fed the CH+ diet.

Fish, particularly carnivorous fish, have limited ability to use dietary carbohydrate, and a persistent hyperglycaemia is observed in several species after administration of glucose or a carbohydrate-rich diet^(23,45). European sea bass is, however, able to restore basal plasma glucose levels within 12 h after feeding, and present data on glucose levels 18 h after feeding are in accordance with such previous evidence^(22,32). In the liver, excess dietary glucose is converted to glycogen or lipids or burned for energy purposes. Glucose can also enter the pentose phosphate pathway, yielding reducing power (NADPH) for biosynthesis of FA and cholesterol⁽⁴⁴⁾. In the present study, hepatic transcriptional regulation of rate-limiting glycolytic and gluconeogenic enzymes by dietary carbohydrate in European sea bass was also observed^(23,47). Accordingly,

gene expression of GK, the first enzyme involved in the glycolytic pathway, was induced by dietary carbohydrate, whereas expression of PEPCK, the first enzyme of gluconeogenesis, was depressed by dietary carbohydrate. Transcriptional regulation of GK by dietary carbohydrate intake was at first observed in European sea bass by Enes *et al.*⁽⁴⁸⁾. Lack of transcriptional regulation of PK by dietary carbohydrate is possibly linked to a post-transcriptional mechanism, as Enes *et al.*⁽⁴⁸⁾ have shown that PK activity was induced by dietary carbohydrate intake. In European sea bass, modulation of PEPCK activity by dietary carbohydrates was not evaluated⁽²³⁾. However, hepatic activity of other key gluconeogenic enzymes, fructose-1,6-bisphosphatase (FBPase) and G6Pase, revealed a lack of regulation by dietary carbohydrate⁽⁴⁸⁾. In this study, we also observed no transcriptional regulation of G6Pase, but PEPCK was down-regulated by dietary carbohydrate. Together, these results indicate that carbohydrate catabolism seems to be adequately regulated at nutritional level in European sea bass. This is in agreement with the conclusion of Enes *et al.*⁽⁴⁹⁾ that the activity of key enzymes of the glycolytic pathway is enhanced by dietary carbohydrate. On the other hand, available

Table 7. Muscle fatty acid profile (expressed as % of total fatty acids; FA) of European seabass fed the experimental diets† (Mean values with their standard errors; *n* 6)

Lipid source (LS)...	Experimental diets							
	FO		VO		SEM	P*		
	CH-	CH+	CH-	CH+		CH	LS	CH × LS
FA								
14 : 0	4.4	4.1	2.6	2.1	0.219	0.006	<0.001	0.164
15 : 0	0.42	0.36	0.25	0.20	0.019	<0.001	<0.001	0.679
16 : 0	19.1	20.7	20.0	21.0	0.211	<0.001	0.067	0.282
17 : 0	0.32	0.26	0.25	0.20	0.009	<0.001	<0.001	0.359
18 : 0	3.7	4.1	4.2	4.4	0.111	0.154	0.101	0.593
20 : 0	0.12	0.10	0.16	0.15	0.006	0.095	<0.001	0.805
ΣSFA	28.0	29.6	27.5	28.1	0.235	0.006	0.009	0.150
14 : 1n-5	0.12	0.10	0.05	0.07	0.008	0.765	0.003	0.218
16 : 1n-7	6.7	6.4	3.7	3.3	0.353	0.166	<0.001	0.717
18 : 1n-9	20.2	21.6	25.5	27.5	0.649	0.002	<0.001	0.451
20 : 1n-9	3.35	3.62	0.88	0.87	0.288	0.968	<0.001	0.694
22 : 1n-9	2.04	2.01	0.17	0.17	0.205	0.779	<0.001	0.783
ΣMUFA	32.4	33.7	30.3	31.9	0.540	0.162	0.069	0.848
18 : 2n-6	3.6	3.5	8.5	9.1	0.562	0.617	<0.001	0.169
18 : 3n-6	0.21	0.18	0.17	0.13	0.008	0.011	0.003	0.982
20 : 2n-6	0.29	0.27	0.32	0.30	0.006	0.044	0.005	0.970
20 : 4n-6	0.99	0.86	0.76	0.64	0.043	0.101	0.005	0.904
Σn-6 PUFA	5.2	4.9	9.8	10.3	0.533	0.769	<0.001	0.080
18 : 3n-3	1.1	1.1	12.0	12.6	1.19	0.780	<0.001	0.601
18 : 4n-3	1.36	1.28	0.59	0.49	0.087	0.044	<0.001	0.440
20 : 4n-3	0.50	0.44	0.22	0.16	0.031	0.001	<0.001	0.210
20 : 5n-3	8.5	7.5	5.1	4.0	0.384	<0.001	<0.001	0.815
21 : 5n-3	0.27	0.25	0.15	0.11	0.017	0.136	<0.001	0.506
22 : 5n-3	1.22	0.99	0.71	0.56	0.055	<0.001	<0.001	0.189
22 : 6n-3	13.4	11.8	8.6	7.4	0.569	0.019	<0.001	0.936
Σn-3 PUFA	26.5	23.3	27.4	25.4	0.414	<0.001	0.015	0.344
Σn-3 LC-PUFA	24.0	21.0	14.8	12.3	1.03	0.001	<0.001	0.708
Ratios								
SFA:PUFA	0.84	1.00	0.72	0.77	0.024	<0.001	<0.001	0.063
<i>n</i> 3:n6	5.1	4.8	2.8	2.5	0.254	0.027	<0.001	0.989

FO, fish oil; VO, blend of vegetable oils; CH content, 0% (CH-) or 20% (CH+) gelatinised maize starch; *n*-3 LC-PUFA, *n*-3 long chain-PUFA.

* Significant differences at *P* < 0.05 (two-way ANOVA).

† FA ≥ 0.02%; <0.02% was not considered in the table.

Table 8. Plasma metabolites levels (mmol/l) in European seabass fed the experimental diets (Mean values with their standard errors; *n* 18)

Lipid source (LS)...	Experimental diets							
	FO		VO		SEM	P*		
	CH-	CH+	CH-	CH+		CH	LS	CH × LS
Metabolites								
GLU	4.7	4.6	4.6	4.2	0.115	0.357	0.270	0.530
TAG	2.5	2.6	2.5	2.6	0.018	0.056	0.327	0.603
CHOL	5.1	6.5	4.7	6.2	0.162	<0.001	0.144	0.807
PL	10.3	11.6	8.6	11.1	0.265	<0.001	0.015	0.201

FO, fish oil; VO, blend of vegetable oils; CH content, 0% (CH-) or 20% (CH+) gelatinised maize starch; GLU, glucose; CHOL, total cholesterol; PL, phospholipids.

* Significant differences at *P* < 0.05 (two-way ANOVA).

data on regulation of the gluconeogenic pathway by dietary carbohydrate are still not consistent. According to Enes *et al.*⁽⁴⁹⁾ an inverse regulation of the gluconeogenic enzyme activities with dietary carbohydrate intake is not observed in European sea bass and gilthead sea bream (*Sparus aurata*), but the

present results on *PEPCK* suggest that gluconeogenesis is in part regulated at the transcriptional level.

Increased whole-body lipids, hepatic and muscular glycogen levels and hepatic lipids (mainly 16 : 0) in fish fed the CH+ diet indicate that both glycogenesis and lipogenesis constitute

Table 9. Enzymatic activity (mU/mg protein) of selected enzymes involved in lipogenesis in European seabass fed the experimental diets (Mean values with their standard errors; n 9)

Lipid source (LS)...	Experimental diets							
	FO		VO		SEM	P^*		
	CH-	CH+	CH-	CH+		CH	LS	CH \times LS
G6PDH	269.3	539.3	294.7	571.5	27.6	<0.001	0.089	0.724
ME	6.8	12.5	6.9	14.0	0.700	<0.001	0.319	0.410
FAS	5.0	4.6	5.3	6.0	0.425	0.841	0.351	0.580

FO, fish oil; VO, blend of vegetable oils; CH content, 0% (CH-) or 20% (CH+) gelatinised maize starch; G6PDH, Glucose-6-phosphate dehydrogenase; ME, malic enzyme; FAS, fatty acid synthase.

* Significant differences at $P < 0.05$ (two-way ANOVA).

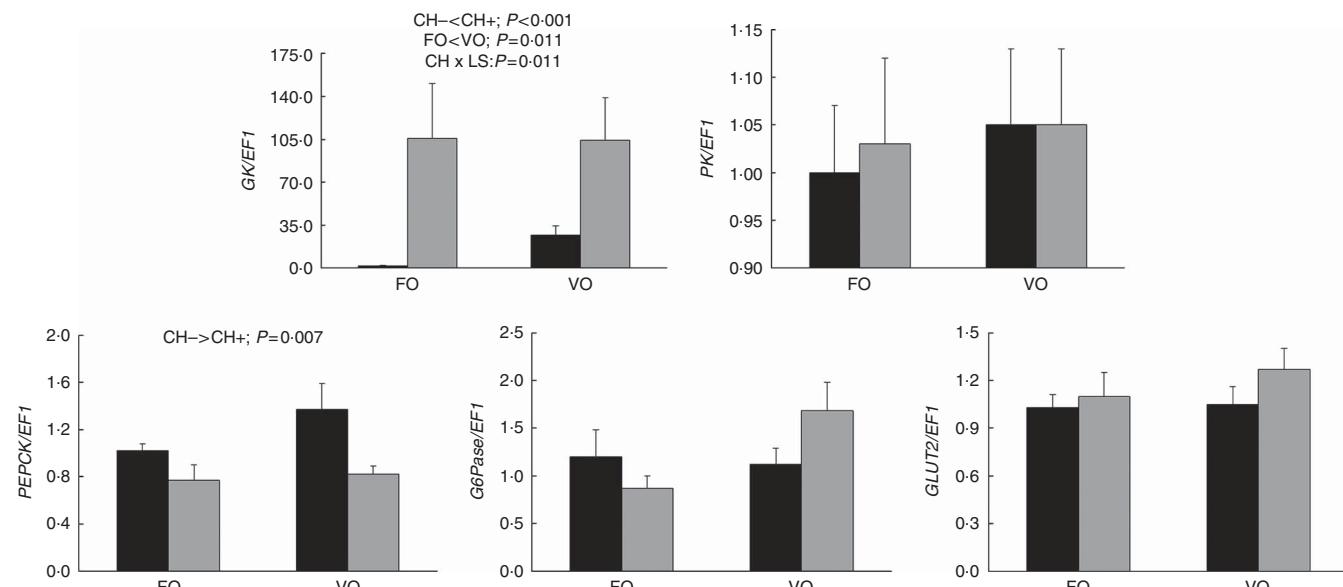


Fig. 1. mRNA levels of proteins involved in glycolysis (GK, glucokinase; PK, pyruvate kinase), gluconeogenesis (PEPCK, phosphoenolpyruvate carboxykinase; G6Pase, glucose 6-phosphatase), and glucose transport (GLUT type 2, GLUT2) in the liver of European sea bass fed the experimental diets. Expression values are normalised by elongation factor-1 α (EF1 α)-expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content, 0% (CH-) or 20% (CH+) gelatinised maize starch. ■, CH-; □, CH+. Values are means (n 6), with their standard errors represented by vertical bars. Significant differences at $P < 0.05$ (two-way ANOVA).

important routes for the excess circulating glucose, as described in this species⁽⁵⁰⁾ and other fish species^(51,52). Present data on the activity of enzymes G6PDH and ME, which are the main providers of NADPH required for lipogenesis, partially corroborate these observations. On the other hand, no changes on FAS activity were observed, despite the increased hepatic accumulation of 16 : 0, the final product of FAS activity^(53,54). As 16 : 0 exerts a negative feedback on FAS activity, this may explain the lack of variation in FAS activity. Similarly, Dias *et al.*⁽²¹⁾ also found no correlation between starch intake and FAS activity in European sea bass.

Interestingly, dietary carbohydrate intake promoted an increase in cholesterol levels in the plasma but not in the liver. An increase in plasma cholesterol concentration was also recorded in rabbits and monkeys⁽⁵⁵⁻⁵⁷⁾ and in rainbow trout⁽²⁵⁾ fed carbohydrate-rich diets. Besides hypercholesterolaemia, dietary carbohydrate also induces alterations of plasma lipoprotein profile, including increased levels of VLDL

and LDL^(56,57). Increased VLDL may be related to an inductor effect of carbohydrate on cholesterol synthesis. Accordingly, in the present study, dietary carbohydrate induced hepatic up-regulation of HMGCR transcript levels, a key protein involved in cholesterol biosynthesis.

Cholesterol biosynthesis is an energy-demanding process and also requires high amounts of NADPH^(2,58), which are mainly obtained through G6PDH activity⁽¹⁰⁾. Thus, in the present trial, the increased activity of G6PDH observed in the CH+ groups might be at least partially related to cholesterol biosynthesis and not to FA synthesis. However, there were no differences on hepatic cholesterol content among dietary groups.

Transcript levels of CYP3A27 were also increased in fish fed the carbohydrate-rich diet. This enzyme belongs to a superfamily of cytochrome P450 (CYP) haem containing mono-oxygenases involved in oxidative metabolism of many xenobiotics^(59,60). Similarities between CYP3A27 and human CYP3A4 were described in rainbow trout⁽⁶¹⁾. CYP3A4

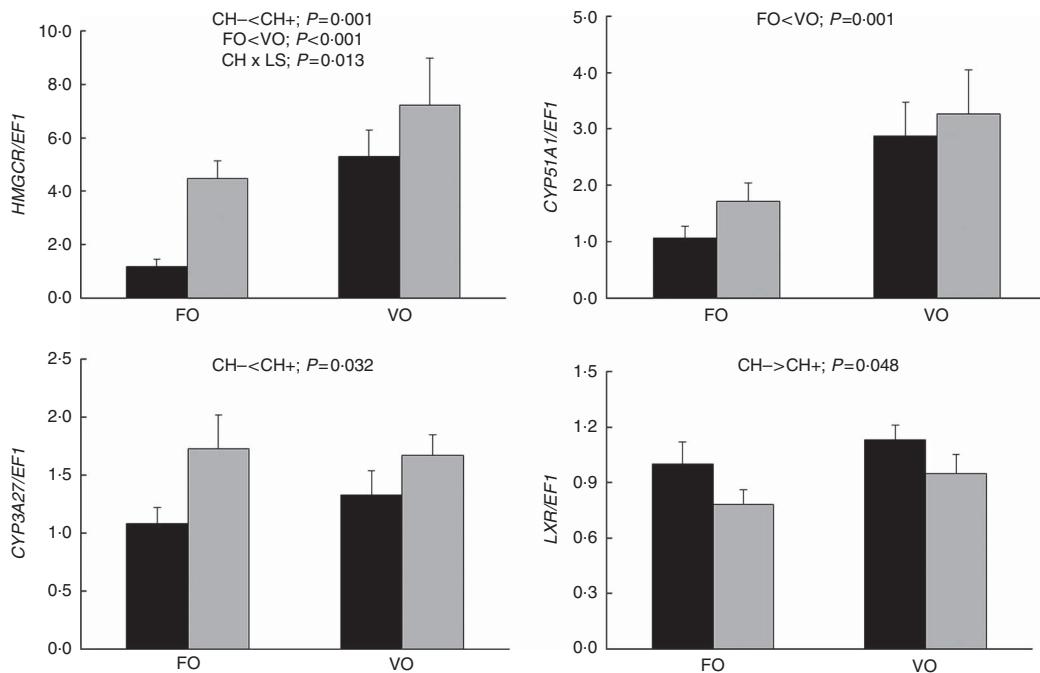


Fig. 2. mRNA levels of proteins involved in cholesterol biosynthesis (*HMGCR*, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; *CYP51A1*, cytochrome P450 51 (lanosterol 14- α -demethylase); *CYP3A27*, cytochrome P450 3A27; *LXR*, liver X receptor) in the liver of European sea bass fed the experimental diets. Expression values are normalised by elongation factor-1 α (*EF1* α)-expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch. ■, CH-; □, CH+. Values are means (n 6), with their standard errors represented by vertical bars. Significant differences at $P < 0.05$ (two-way ANOVA).

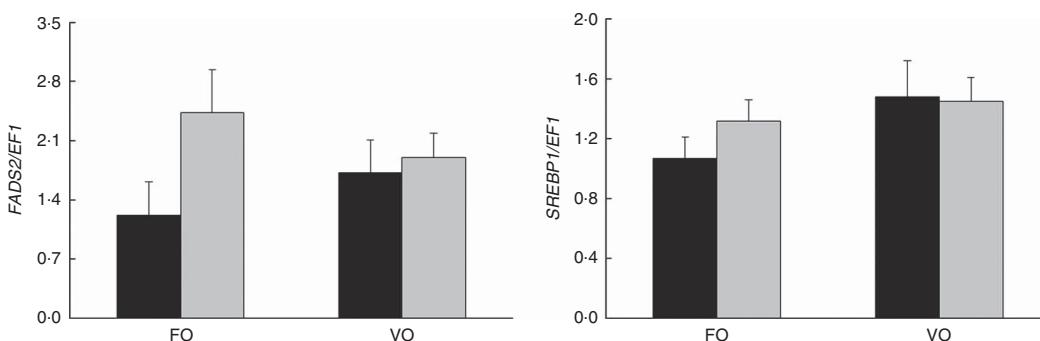


Fig. 3. mRNA levels of proteins involved in LC-PUFA biosynthesis (*FADS2*, Δ 6 fatty acyl desaturase; *SREBP1*, sterol response element binding protein-1) in the liver of European sea bass fed the experimental diets. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch. Expression values are normalised by elongation factor-1 α (*EF1* α)-expressed transcripts. ■, CH-; □, CH+. Values are means (n 6), with their standard errors represented by vertical bars. Significant differences at $P < 0.05$ (two-way ANOVA).

participates in the upstream steps of one of a number of pathways involved in cholesterol catabolism. Specifically, it is responsible for the conversion of cholesterol to 4β -hydroxycholesterol before its elimination in bile salts. However, considering that the 4β -hydroxycholesterol formation rate is very slow, it is believed that *CYP3A4* may play a greater role in the transcriptional regulation of lipid metabolism than in cholesterol elimination⁽⁵⁹⁾. In fact, the endogenous oxidised cholesterol derivate oxysterol 4β -hydroxycholesterol is described to activate the nuclear receptor LXR^(59,62). This nuclear receptor, besides its role in activation of transcription of target genes that protect cells from cholesterol overload⁽⁶²⁾, also activates lipogenic genes and regulate the transcription of genes

involved in glucose metabolism⁽⁶³⁾, either alone or in conjunction with other nutrient-sensing transcription factors such as *SREBP1*⁽⁶⁴⁾. However, contradictory to what was expected, there was a down-regulation of hepatic X receptor (*LXR*) gene expression in fish fed a carbohydrate-rich diet, although the effect was very small ($P = 0.048$). Direct regulation of *SREBP1* by *LXR* was previously described⁽⁶⁴⁾, although in the present study such modulation was not observed, which may be related to the small differences in *LXR* expression among groups.

The inductor effect of dietary carbohydrate on the LC-PUFA biosynthesis pathway through increased transcript levels of *elovl5* and *FADS2* that was reported in salmonids^(17,25) was not observed in the present study. On the contrary, the proportion

of *n*-3 LC-PUFA, particularly EPA and DHA, decreased in the liver of fish fed a carbohydrate-rich diet.

Although PL biosynthesis pathway regulation or tissue PL content was not evaluated, the higher plasma PL levels observed in the CH+ groups suggest that dietary carbohydrate may also induce PL biosynthesis. Further studies are, however, required to confirm this assumption.

Effect of lipid source

Similar to previous results in this species^(10,65–67), no effects on growth parameters and feed utilisation of European sea bass were observed due to dietary FO replacement by VO blend. On the other hand, the well-established effect of FO replacement by VO at tissue FA compositional level, such as increased C18 FA (18 : 3*n*-3; 18 : 2 *n*-6) and decreased levels of LC-PUFA (EPA and DHA), was also observed in this study.

Contrary to humans and other mammals, in which LC-PUFA of FO reduce triglyceridaemia and lipogenesis^(68–71), in fish such effects are not clear. Studies in fish showed that FO either depressed⁽¹⁵⁾, had no effects^(9,10,12,14) or had opposite effects⁽¹³⁾ on lipogenesis.

Contrary to our previous study in European sea bass fed a similar VO blend⁽³²⁾ or plant-based diets^(6,40), in the present study no up-regulation of *FADS2* was observed at hepatic level in fish fed the VO diets. Also contrary to previous observations⁽³²⁾, we were unable to detect the expression of *elavl5* in the liver. The sampling time in this trial (18 h post-feeding) may explain the apparent lack of nutritional regulation of *FADS2* as by that time almost all ingested feed had been already digested and metabolically processed.

Previous studies in rainbow trout and gilthead sea bream reported higher specificities of C18 PUFA (which are characteristic of VO) to be reacylated into TAG, whereas LC-PUFA are reacylated into PL^(72,73). It is therefore possible that similar processes may have happened in fish fed the VO-based diets, thus leading to the decrease in plasma PL levels in the present study.

Regulation of cholesterol biosynthesis is mainly controlled by the rate-limiting enzyme HMGCR by a feedback mechanism⁽⁵⁴⁾. In the present study, we observed an up-regulation of this enzyme at hepatic level in the VO groups. In European sea bass fed plant-based diets, an up-regulation of hepatic transcript levels of *HMGCR* was also previously observed⁽⁶⁾. We also observed increased mRNA levels of *CYP51A1*, an enzyme involved in the last steps of cholesterol synthesis, specifically in the serial reactions that convert lanosterol to cholesterol^(46,74). Similarly, Leaver *et al.*⁽⁴⁾ and Geay *et al.*⁽⁶⁾ also observed an up-regulation of *CYP51A1* transcript levels in Atlantic salmon fed VO-based diets and in European sea bass fed PF-based diets. In Atlantic salmon fed plant-based diets increased hepatic cholesterol biosynthesis and impaired intestinal cholesterol absorption were found^(3,75). Phytosterols and/or PF were advanced as the possible causes for the reduced intestinal cholesterol absorption. In our study, expression of genes encoding for cholesterol uptake was not assessed. With dietary cholesterol levels being similar among groups fed FO and VO, we can speculate that in the present study VO diets also

promoted a reduction of intestinal cholesterol absorption, and this might have induced endogenous production of cholesterol in the liver to counter-balance the absorbed cholesterol deficit.

Carbohydrate and lipid source interaction

Contrary to previous evidence on the inductor role of carbohydrates on the LC-PUFA biosynthesis pathway of salmonids^(17,25), we failed to find a potential interactive effect of dietary LS and carbohydrate on the transcriptional regulation of LC-PUFA biosynthesis.

At the organ compositional level, in the liver an interaction between dietary carbohydrate and LS on total hepatic *n*-3 PUFA, but not for LC-*n*-3-PUFA, was detected. Within the CH- group, total *n*-3 PUFA content was higher in the FOCH- group, whereas within the CH+ groups no differences on total *n*-3 PUFA between VO and FO groups were observed. An interaction was also observed on the transcript levels of *HMGCR*, as differences were only noticed within CH- and FO groups. These results suggest that, although both VO and carbohydrates seem to have an inductor effect on transcript levels of *HMGCR*, they do not act synergistically.

Among analysed actors involved in carbohydrate metabolism, only transcript levels of *GK* in the liver were modulated by dietary LS. Liver interaction of *GK* transcript levels showed a pattern identical to that described for *HMGCR*. Although the recognised on-off regulation of *GK* at the transcriptional level in response to dietary carbohydrate was evident in the FO groups, this effect was not clear within VO groups. In the CH- groups, *GK* mRNA levels were considerably higher in the VO than in the FO group. Recent studies in salmonids suggest that replacement of marine fishery-derived feedstuffs by plant products affects hepatic carbohydrate metabolism^(5,6,76). Although the present results may suggest an induction of glycolysis by VO in the CH- groups, care must be taken in the interpretation of data because *GK* gene expression was very low in the CH- groups and may not be biologically significant.

Conclusions

In this study no key interactions between dietary LS and carbohydrates were detected in European sea bass juveniles. An inductor role of carbohydrates in the LC-PUFA biosynthesis pathway at the transcriptional level previously observed in salmonids was also not observed in European sea bass juveniles.

This study describes for the first time a direct relation between dietary carbohydrate and the cholesterol biosynthetic pathway in fish. The present results seem to be promising, considering that dietary supply of cholesterol and PL will be limited in future aquafeeds with the replacement of both FM and FO by PF and VO. However, further insights into the regulation of cholesterol metabolism are needed, particularly in specific stages of life as reproduction and larvae phases.

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C. C. carried out the main experimental work and wrote the draft of the manuscript under the direction of the project designer and leaders A. O. T., G. C. and S. P. A. P. J. assisted with the biochemical analyses and draft writing. L. L. performed the fatty acid analyses. M. C. assisted with the gene expression analyses. All authors contributed to and approved the manuscript.

The authors declare that there are no conflicts of interest.

References

- Bell JG, Henderson RJ, Tocher DR, *et al.* (2002) Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. *J Nutr* **132**, 222–230.
- National Research Council (2011) *Nutrient Requirements of Fish*. Washington, DC: National Academy Press.
- Liland NS, Espe M, Rosenlund G, *et al.* (2013) High levels of dietary phytosterols affect lipid metabolism and increase liver and plasma TAG in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **110**, 1958–1967.
- Leaver MJ, Villeneuve LAN, Obach A, *et al.* (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* **9**, 299.
- Panserat S, Hortopan GA, Plagnes-Juan E, *et al.* (2009) Differential gene expression after total replacement of dietary fish meal and fish oil by plant products in rainbow trout (*Oncorhynchus mykiss*) liver. *Aquaculture* **294**, 123–131.
- Geay F, Ferrarese S, Zambonino-Infante J, *et al.* (2011) Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. *BMC Genomics* **12**, 522.
- Morais S, Edvardsen RB, Tocher DR, *et al.* (2012) Transcriptomic analyses of intestinal gene expression of juvenile Atlantic cod (*Gadus morhua*) fed diets with Camelina oil as replacement for fish oil. *Comp Biochem Physiol B Biochem Mol Biol* **161**, 283–293.
- Kaushik SJ, Coves D, Dutto G, *et al.* (2004) Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture* **230**, 391–404.
- Richard N, Kaushik S, Larroquet L, *et al.* (2006) Replacing dietary fish oil by vegetable oils has little effects on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). *Br J Nutr* **96**, 299–309.
- Richard N, Mourente G, Kaushik S, *et al.* (2006) Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture* **261**, 1077–1087.
- Benedito-Palos L, Navarro JC, Sitja-Bobadilla A, *et al.* (2008) High levels of vegetable oils in plant protein-rich diets fed to gilthead sea bream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues. *Br J Nutr* **100**, 992–1003.
- Regost C, Arzel J, Robin J, *et al.* (2003) Total replacement of fish oil by soybean or linseed oil with a return to fish oil in turbot (*Psetta maxima*). I. Growth performance, flesh fatty acid profile, and lipid metabolism. *Aquaculture* **217**, 465–482.
- Menoyo D, Izquierdo MS, Robaina L, *et al.* (2004) Adaptation of lipid metabolism, tissue composition, and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soybean oils. *Br J Nutr* **92**, 41–52.
- Torstensen BE, Froyland L & Lie O (2004) Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil – effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquacult Nutr* **10**, 175–192.
- Jordal AEO, Lie O & Torstensen BE (2007) Complete replacement of dietary fish oil with a vegetable oil blend effects liver and plasma lipoprotein levels in Atlantic salmon (*Salmo salar*). *Aquac Nutr* **13**, 114–130.
- Panserat S, Kolditz C, Richard N, *et al.* (2008) Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. *Br J Nutr* **100**, 953–967.
- Seiliez I, Panserat S, Kaushik S, *et al.* (2001) Cloning, tissue distribution and nutritional regulation of a Delta6-desaturase-like enzyme in rainbow trout. *Comp Biochem Physiol B Biochem Mol Biol* **130**, 83–93.
- Zheng X, Tocher DR, Dickson CA, *et al.* (2005) Highly unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and characterization of a delta6 desaturase of Atlantic salmon. *Lipids* **40**, 13–24.
- Tocher DR, Zheng X, Schlechtriem C, *et al.* (2006) Highly unsaturated fatty acid synthesis in marine fish: cloning, functional characterization, and nutritional regulation of fatty acyl 6 desaturase of Atlantic cod (*Gadus morhua* L.). *Lipids* **41**, 1003–1016.
- González-Rovira A, Mourente G, Zheng X, *et al.* (2009) Molecular and functional characterization and expression analysis of a Delta-6 fatty acyl desaturase cDNA of European sea bass (*Dicentrarchus labrax* L.). *Aquaculture* **298**, 90–100.
- Dias J, Alvarez MJ, Diez A, *et al.* (1998) Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (*Dicentrarchus labrax*). *Aquaculture* **161**, 169–186.
- Peres MH, Goncalves P & Oliva-Teles A (1999) Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). *Aquaculture* **179**, 415–423.
- Enes P, Peres H, Sanchez-Gurmaches J, *et al.* (2011) Insulin and IGF-I response to a glucose load in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* **315**, 321–326.
- Kamalam BS, Medale F, Kaushik S, *et al.* (2012) Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J Exp Biol* **215**, 2567–2578.
- Kamalam BS, Médale F, Larroquet L, *et al.* (2013) Metabolism and fatty acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. *PLOS ONE* **8**, e76570.
- Panserat S, Perrin A & Kaushik S. (2002) High dietary lipids induce liver glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J Nutr* **132**, 137–141.
- Menoyo D, Diez A, Lopez-Bote CJ, *et al.* (2006) Dietary fat type affects lipid metabolism in Atlantic salmon (*Salmo salar* L.) and differentially regulates glucose transporter GLUT4 expression in muscle. *Aquaculture* **261**, 294–304.

28. Beutler HO (1984) Starch. In *Methods of Enzymatic Analysis* Vol. 6, pp. 2–10 [HU Bergmeyer, editor]. Basel: Verlag Chemie Weinheim.
29. Roehrig KL & Allred JB (1974) Direct enzymatic procedure for the determination of liver glycogen. *Anal Biochem* **58**, 414–421.
30. Folch J, Lees M & Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissue. *J Biol Chem* **226**, 497–509.
31. Santha NC & Ackman RG (1990) Nervonic acid versus tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *J Chromatogr* **533**, 1–10.
32. Castro C, Corraze G, Panserat S, et al. (2014) Effects of fish oil replacement by a vegetable oil blend on digestibility, post-prandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquac Nutr* (Epublication ahead of print version 29 October 2014).
33. Stadtman TC (1957) Determination of cholesterol and ergosterol by Liebermann-Burchard reaction. *Biochem J* **3**, 362–365.
34. Morales AE, García-Rejón L & De la Higuera M (1990) Influence of handling and/or anaesthesia on stress response in rainbow trout. Effects on liver primary metabolism. *Comp Biochem Physiol A* **95**, 87–93.
35. Singer TD, Mahadevappa VG & Ballantyne JS (1990) Aspects of the energy metabolism of lake sturgeon, *Acipenser fulvescens*, with special emphasis on lipid and ketone body metabolism. *Can J Fish Aquat Sci* **47**, 873–881.
36. Chang HC, Seidman I, Teebor G, et al. (1967) Liver acetyl CoA carboxylase and fatty acid synthetase: relative activities in the normal state and in hereditary obesity. *Biochem Biophys Res Commun* **28**, 682–686.
37. Chakrabarty K & Leveille GA (1969) Acetyl-CoA carboxylase and fatty acid synthetase activities in the liver and adipose tissue of meal-fed rats. *Proc Soc Exp Biol Med* **131**, 1051–1054.
38. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye-binding. *Anal Biochem* **72**, 248–254.
39. Rozen S & Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365–386.
40. Geay F, Santigosa I, Culí E, et al. (2010) Regulation of FADS2 expression and activity in European sea bass (*Dicentrarchus labrax*, L.) fed a vegetable diet. *Comp Biochem Physiol B Biochem Mol Biol* **156**, 237–243.
41. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.
42. Peres H & Oliva-Teles A (2002) Utilization of raw and gelatinized starch by European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* **205**, 287–299.
43. Couto A, Enes P, Peres H, et al. (2008) Effect of water temperature and dietary starch on growth and metabolic utilization of diets in gilthead sea bream (*Sparus aurata*) juveniles. *Comp Biochem Physiol A Mol Integr Physiol* **151**, 45–50.
44. Coutinho F, Peres H, Guerreiro I, et al. (2012) Dietary protein requirement of sharpsnout sea bream (*Diplodus puntazzo*, Cetti 1777) juveniles. *Aquaculture* **356–357**, 391–397.
45. Polakof S, Panserat S, Soengas JL, et al. (2012) Glucose metabolism in fish: a review. *J Comp Physiol B* **182**, 1015–1045.
46. Nelson DL & Cox MM (2004) Lipid biosynthesis. In *Lehniger Principles of Biochemistry*, 4th ed. 1119, pp. 787–832 [DL Nelson and MM Cox, editors]. New York: W. H. Freeman.
47. Gatesoupe FJ, Huelvan C, Le Bayon N, et al. (2014) The effects of dietary carbohydrate sources and forms on metabolic response and intestinal microbiota in sea bass juveniles, *Dicentrarchus labrax*. *Aquaculture* **422–423**, 47–53.
48. Enes P, Panserat S, Kaushik S, et al. (2006) Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. *Comp Biochem Physiol A Mol Integr Physiol* **143**, 89–96.
49. Enes P, Panserat S, Kaushik S, et al. (2011) Dietary carbohydrate utilization by European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.) juveniles. *Rev Fish Sci* **19**, 201–215.
50. Moreira IS, Peres H, Couto A, et al. (2008) Temperature and dietary carbohydrate levels effects on performance and metabolic utilisation of diets in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* **274**, 153–160.
51. Fernández F, Miquel AG, Córdoba M, et al. (2007) Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *J Exp Mar Biol Ecol* **343**, 1–10.
52. Figueiredo-Silva AC, Saravanan S, Schrama JW, et al. (2013) A comparative study of the metabolic response in rainbow trout and Nile tilapia to changes in dietary macronutrient composition. *Br J Nutr* **109**, 816–826.
53. Leaver MJ, Bautista JM, Björnsson BT, et al. (2008) Towards fish lipid nutrigenomics: current state and prospects for fin-fish aquaculture. *Rev Fish Sci* **16**, 73–94.
54. Engelking LR (2010) Fatty acid biosynthesis. In *Textbook of Veterinary Physiological Chemistry*, 2nd ed. pp. 315–320 [LR Engelking, editor]. Amsterdam: Academic Press (Elsevier).
55. Kritchevsky D & Tepper SA (1968) Experimental atherosclerosis in rabbits fed cholesterol free diets: influence of chow components. *J Atheroscler Res* **8**, 357–369.
56. Sparks JD, Sparks CE & Kritchevsky D (1986) Hypercholesterolemia and aortic glycosaminoglycans of rabbits fed semi-purified diets containing sucrose and lactose. *Atherosclerosis* **60**, 183–196.
57. Srinivasan SR, Radhakrishnamurthy B, Webber LS, et al. (1978) Synergistic effects of dietary carbohydrate and cholesterol on serum lipids and lipoproteins in squirrel and spider monkeys. *Am J Clin Nutr* **31**, 603–613.
58. Norambuena F, Lewis M, Hamid NKA, et al. (2013) Fish oil replacement in current aquaculture feed: is cholesterol a hidden treasure for fish nutrition? *PLOS ONE* **8**, e81705.
59. Pikuleva IA (2006) Cytochrome P450s and cholesterol homeostasis. *Pharmacol Ther* **112**, 761–773.
60. Uno T, Ishizuka M & Itakura T (2012) Cytochrome P450 (CYP) in fish. *Environ Toxicol Pharmacol* **34**, 1–13.
61. Lee SJ & Buhler DR (2002) Functional properties of a rainbow trout CYP3A27 expressed by recombinant baculovirus in insect cells. *Drug Metab Dispos* **30**, 1406–1412.
62. Zhao C & Dahlman-Wright K (2010) Liver X receptor in cholesterol metabolism. *J Endocrinol* **204**, 233–240.
63. Mitro N, Mak PA, Vargas L, et al. (2007) The nuclear receptor LXR is a glucose sensor. *Nature* **445**, 219–223.
64. Grønning-Wang LM, Bindesbøll C & Nebb HI (2013) The role of liver X receptor in hepatic *de novo* lipogenesis and cross-talk with insulin and glucose signaling. In *Lipid Metabolism*, pp. 61–90 [R Valenzuela Baez, editor]. Rijeka, Croatia: InTech.
65. Mourente G, Dick JR, Bell JG, et al. (2005) Effect of partial substitution of dietary fish oil by vegetable oils on desaturation and oxidation of [1^{-14}C]18:3n-3 and [1^{-14}C] 20:5n-3 in hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.). *Aquaculture* **248**, 173–186.

66. Mourente G, Good JE & Bell JG (2005) Partial substitution of fish oil with rapeseed, linseed and olive oils in diet for European sea bass (*Dicentrarchus labrax* L.) effects on flesh fatty acid composition, plasma prostaglandins E₂ and F_{2a}, immune function and effectiveness of a fish oil finishing diet. *Aquac Nutr* **11**, 25–40.
67. Figueiredo-Silva A, Rocha E, Dias J, *et al.* (2005) Partial replacement of fish oil by soybean oil on lipid distribution and liver histology in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquac Nutr* **11**, 147–155.
68. Geelen MJ, Schoots WJ, Bijleveld C, *et al.* (1995) Dietary medium-chain fatty acids raise and (n-3) polyunsaturated fatty acids lower hepatic triacylglycerol synthesis in rats. *J Nutr* **125**, 2449–2456.
69. Ikeda I, Cha JY, Yanagita T, *et al.* (1998) Effects of dietary alpha-linolenic, eicosapentaenoic and docosahexaenoic acids on hepatic lipogenesis and beta-oxidation in rats. *Biosci Biotechnol Biochem* **62**, 675–680.
70. Davidson MH (2006) Mechanisms for the hypotriglyceridemic effect of marine omega-3 fatty acids. *Am J Cardiol* **98**, 27–33.
71. Harris WS, Miller M, Tighe AP, *et al.* (2008) Omega-3 fatty acids and coronary heart disease risk: clinical and mechanistic perspectives. *Atherosclerosis* **197**, 12–24.
72. Pérez JA, Rodríguez C & Henderson RJ (1999) The uptake and esterification of radiolabelled fatty acids by enterocytes isolated from rainbow trout (*Oncorhynchus mykiss*). *Fish Physiol Biochem* **20**, 125–134.
73. Caballero MJ, Izquierdo MS, Kjørsvik E, *et al.* (2003) Morphological aspects of intestinal cells from gilthead sea bream (*Sparus aurata*) fed diets containing different lipid sources. *Aquaculture* **225**, 325–340.
74. Liscum L (2008) Cholesterol biosynthesis. In *Biochemistry of Lipids, Lipoproteins and Membranes*, 5th ed. pp. 399–420 [DE Vance and JE Vance, editors]. Oxford: Elsevier.
75. Kortner TM Gu J, Krogdahl A, *et al.* (2012) Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **30**, 1–12.
76. Morais S, Pratoomyot J, Taggart J, *et al.* (2011) Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genomics* **12**, 255.

Chapter 6-

**Regulation of glucose and lipid metabolism
by dietary carbohydrate levels and lipid
sources in gilthead sea bream juveniles**

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Regulation of glucose and lipid metabolism by dietary carbohydrate levels and lipid sources in gilthead sea bream juveniles

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Abstract

The long-term effects on growth performance, body composition, plasma metabolites, liver and intestine glucose and lipid metabolism were assessed in gilthead sea bream juveniles fed diets without carbohydrates (CH-) or carbohydrate-enriched (20% gelatinized starch, CH+) combined with two lipid sources (fish oil, FO; or vegetable oil, VO). No differences in growth performance among treatments were observed. Carbohydrate intake was associated with increased hepatic transcripts of GK but not of PFK. Expression of PEPCK was down-regulated by carbohydrate intake while, unexpectedly, G6Pase was up-regulated. Lipogenic enzyme activities (G6PD, ME, FAS) and FADS2 transcripts were increased in liver of fish fed CH+diets, supporting an enhanced potential for lipogenesis and LC-PUFA biosynthesis. Despite the lower hepatic cholesterol content in CH+groups, no influence on the expression of genes related to cholesterol efflux (ABCG5) and biosynthesis (CYP51A1; DHCR7) was recorded at hepatic level. At intestinal level, however, induction of CYP51A1 transcripts by carbohydrate intake was recorded. Dietary VO led to decreased plasma phospholipid and cholesterol concentrations but not on the transcripts of proteins involved in phospholipid biosynthesis (GPAT) and cholesterol metabolism at intestinal and hepatic levels. Hepatic and muscular fatty acid profiles reflected that of diets, despite the up-regulation of FADS2 transcripts. Overall, this study demonstrated that dietary

carbohydrates mainly affected carbohydrate metabolism, lipogenesis and LC-PUFA biosynthesis, whereas effects of dietary lipid source were mostly related with tissue fatty acid composition, plasma phospholipid and cholesterol concentrations, and LC-PUFA biosynthesis regulation. Interactions between dietary macronutrients induced modifications in tissue lipid and glycogen content.

Key words: carbohydrate content; cholesterol; FA bioconversion; lipid source; nutrient metabolism.

Introduction

Besides being a source of high quality protein and essential micronutrients for humans, fish are unique sources of n-3 long-chain polyunsaturated fatty acids (LC-PUFA), namely eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which were proven to be beneficial for human health¹. Driven by the crescent awareness of the health beneficial effects of n-3 LC-PUFA in a range of human pathologies (including cardiovascular, inflammatory and neurological diseases) the global fish consumption is rising¹ and an increasing proportion of this fish is now being supplied by aquaculture².

Fish meal (FM) and fish oil (FO) have been widely used as main ingredients in aquafeeds for carnivorous fish species. However, environmental sustainability and economically issues, related to the limited availability of fisheries resources and escalating costs, forced the aquafeed industry to search for alternative and eco-friendly ingredients such as plant feedstuffs and vegetable oils (VO). However, the unbalanced amino acid and fatty acid (FA) profiles, relatively high amounts of carbohydrates and the presence of antinutritional factors may limit their use in aquafeeds, especially for carnivorous fish species that are metabolically adapted to diets rich in LC-PUFA and almost devoid of carbohydrates^{1; 3-5}.

It is well known that modifications of dietary macronutrients (e.g. lipid source, carbohydrates, etc.) may have marked effects on tissue FA composition and lipid deposition of fish⁶⁻⁹. For instance, replacement of FO by VO generally leads to a decrease of n-3 LC-PUFA and an increase of 18C PUFA precursors, linoleic acid (18:2 n-6) and alpha linolenic acid (18:3 n-3), in the fillet^{7; 9-11}. Increased tissue lipid deposition with dietary incorporation of VO^{6; 8; 9} or carbohydrates^{7; 12} were also reported. Such effects may occur due to different mechanisms that are recognized to regulate the quantity and quality of the fish lipid depots, such as modification of lipogenesis, β-oxidation, tissue lipid uptake and transport, or FA desaturation and elongation processes^{13; 14}.

Although not always consistent, several studies in fish reported that dietary lipid source and carbohydrates regulate gene expression, key transcription factors and/or activity of enzymes involved in lipogenesis, β -oxidation and lipid uptake. Accordingly, it was observed that replacing dietary FO by VO decreased^{15; 16} or increased¹⁷⁻¹⁹ gene expression or activity of lipogenic enzymes (such as glucose-6-phosphate dehydrogenase -G6PD, malic enzyme-ME, fatty acid synthase- FAS). Similarly, dietary VO was reported to regulate either positively^{20; 21} or negatively^{19; 20; 22; 23} gene expression or activities of lipolytic enzymes (including carnitine palmitoyltransferase I and II- CPT1,2, lipoprotein lipase- LPL) in a tissue-specific manner. Dietary carbohydrate or glucose administration were also reported to enhance lipogenesis²⁴. However, regarding β -oxidation data is contradictory, as either stimulation²⁵ or inhibition²⁶ effects were reported. Regarding LC-PUFA biosynthesis, strong induction of desaturases ($\Delta 5$ and $\Delta 6$ desaturases, FADS1 and FADS2, respectively) and elongases expression by VO administration was reported in freshwater fish and salmonids^{10; 27}, but in marine fish such induction of gene expression is not so clear^{11; 28-30}. Desaturases and elongases were also shown to be up-regulated by dietary carbohydrates in salmonids^{25; 27} but in marine fish such effect has never been demonstrated⁷.

Besides the reported effects on tissue FA composition and lipid deposition of fish, the use of plant feedstuffs and vegetable oils (VO) can also compromise fish physiological functions and, ultimately, fish health^{31; 32}. For example, there is an increased awareness of potential effects due to reduced dietary phospholipids or cholesterol in plant feedstuffs-based diets. Besides their important roles in membrane structure, cholesterol and phospholipids also have important functional roles. Cholesterol is a precursor of physiologically active compounds such as bile acids, vitamin D, adrenal corticoids, and sex hormones; and phospholipids are precursors of eicosanoids, diacylglycerol, inositol phosphates and platelet activating factors^{33; 34}. In a number of fish species, dietary VO was reported to decrease plasma phospholipids^{7; 35; 36}, cholesterol and LDL-cholesterol^{8; 35; 37}, and modulate the activity or expression of genes involved in phospholipid synthesis³⁸ and cholesterol synthesis and absorption^{7; 10; 39}. Recently, it was also demonstrated that dietary carbohydrates regulate plasma cholesterol and phospholipid concentrations and the transcription of proteins involved in cholesterol metabolism⁷.

Thus, to provide adequate background for successful use of plant feedstuffs in aquafeeds all aspects related to fish physiological functions and the nutritional quality of the final product must be better understood. For that purpose, we assessed the long-

term effect of dietary lipid source, carbohydrate content and interactions between both on growth performance, tissue composition, liver and intestine enzymatic activity and expression of genes related with lipid metabolism (lipogenesis, β -oxidation, FA bioconversion, cholesterol and phospholipid metabolism) in gilthead sea bream juveniles. In this study the selection of the relevant genes involved in lipid metabolism was performed taking advantage of the recent advances in the molecular and functional characterization of a number of new gilthead sea bream sequences related to FA, phospholipid and cholesterol metabolism⁴⁰⁻⁴⁷. Additionally, as an increasing number of studies have reported that glucose metabolism was distinctly regulated by different dietary lipid sources^{7; 17; 48-50}, we also investigated the effects of these dietary manipulations on mechanisms involved in glucose utilisation/metabolism (such as glycolysis and gluconeogenesis pathways).

Methods

Experimental diets

Four diets differing in carbohydrate content (0 and 20 % gelatinized starch, diets CH- and CH+, respectively) and lipid source (diets FO or VO) were formulated (Table 1). The increase in carbohydrate content in CH+ diets was achieved by decreasing protein, which was kept well above the requirements of the species⁵¹. The major lipid source of FO diets was cod liver oil. In VO diets, 100% of the cod liver oil was replaced by a VO blend composed of 20% rapeseed, 50% linseed and 30% palm oils. Fish meal was added as a major dietary protein source to isolate the impacts of dietary VO and to avoid the interference of dietary plant protein on lipid metabolism, especially on cholesterol metabolism.

Table 1. Ingredient and chemical composition of the experimental diets

Lipid source Carbohydrates	Experimental Diets			
	FO		VO	
	CH-	CH+	CH-	CH+
<i>Ingredients (% dry weight)</i>				
Fish meal ^a	87.3	65.1	87.3	65.1
Starch ^b	0	20	0	20
Cod liver oil ^c	9.2	11.4	0	0
Vegetable oil blend ^d	0	0	9.2	11.4
Vitamins	1.5	1.5	1.5	1.5
Minerals	1.0	1.0	1.0	1.0
Binder	1.0	1.0	1.0	1.0
<i>Proximate analyses (% dry matter)</i>				
Dry matter (DM)	87.0	86.8	87.2	87.6
Crude protein (CP)	66.3	50.3	66.3	50.4
Crude fat (CF)	18.4	18.4	18.2	18.3
Starch	-	16.8	-	18.0
Energy (kJ/gDM)	22.7	23.3	23.3	22.7
Ash	14.1	11.2	14.3	11.1
Cholesterol	0.59	0.39	0.49	0.39
Protein/Energy (g/MJ)	29.2	21.6	28.5	22.2

Lipid source: Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

^a Steam dried LT fish meal (Superprime). Inproquisa, Madrid, Spain (CP:74.6% DM; CL: 10.1 % DM).

^b C-Gel Instant-12018. Cerestar. Mechelen. Belgium.

^c Labchem. Laborspirit Lda. Lisboa. Portugal.

^d 30% palm oil (Colmi. Malasia). 50% linseed oil (Sociedade Portuense de Drogas. S.A..Portugal) and 20% rapeseed oil (Huilerie Emile Noël S.A.S.. France)

^eVitamins (mg kg⁻¹ diet): retinol acetate. 18000 (IU kg⁻¹ diet); cholecalciferol. 2000 (IU kg⁻¹ diet); alpha tocopherol acetate. 35; sodium menadione bisulphate. 10; thiamin-HCl. 15; riboflavin. 25; calcium pantothenate. 50; nicotinic acid. 200; pyridoxine HCl. 5; folic acid 10; cyanocobalamin. 0.02; biotin. 1.5; ascorbic acid. 50; inositol. 400. Premix. Viana do Castelo. Portugal.

^fMinerals (mg kg⁻¹ diet): cobalt sulphate. 1.91; copper sulphate. 19.6; iron sulphate. 200; sodium fluoride. 2.21; potassium iodide. 0.78; magnesium oxide. 830; manganese oxide. 26; sodiumselenite. 0.66; zinc oxide. 37.5; dibasic calcium phosphate. 5.93 (g kg⁻¹ diet); potassium chloride. 1.15 (g kg⁻¹ diet); sodium chloride. 0.40 (g kg⁻¹ diet). Premix. Viana do Castelo. Portugal.

^gAquacube (Guar gum. polymethyl carbamide. Manioc starch blend. hydrate calcium sulphate). Agil. England.

Animals, experimental conditions and sampling

The experiment was directed by trained scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was performed at the Marine Zoological Station, University of Porto, Portugal, in a thermoregulated recirculation water system equipped with twelve fiberglass cylindrical tanks of 300 liters water capacity, and supplied with continuous flow of filtered seawater. After 2 weeks of adaptation to the experimental conditions, twelve groups of twenty one gilthead sea bream (*Sparus aurata*) juveniles with an initial body weight of 70.8±0.03g were established and the experimental diets were randomly assigned to triplicate groups of these fish.

The growth trial lasted 81 days and during this period fish were hand-fed twice a day, 6 days a week, to apparent visual satiety. At the end of the trial, fish were unfed for 1 day to empty gut content and then bulk-weighed after mild anesthesia with 0.3 ml/l methylethanol. At the beginning and at the end of the growth trial fifteen fish from the stock population and three fish from each tank were respectively sampled, pooled and frozen until whole-body composition analysis. During the trial, salinity averaged 34.7 ± 0.8 g L⁻¹, dissolved oxygen was kept near saturation, and water temperature was regulated to $24.0 \pm 0.5^\circ\text{C}$.

To eliminate handling stress, after the growth trial fish continued to be fed for one more week and then, 18 h after the last meal (the previous day afternoon meal), nine fish from each tank were randomly sampled for blood, liver, intestine and muscle collection. Blood was collected from the caudal vein using heparinized syringes and centrifuged at 2.500 g for 10 min and the recovered plasma was kept at -20°C until analysis. Thereafter, fish were killed with a sharp blow to the head, and whole body, viscera and liver were weighed for determination of hepatosomatic (HSI) and viscerosomatic (VSI) indexes. Liver, intestine and muscle sections were frozen in liquid nitrogen and then stored at -80°C until biochemical, enzymatic and molecular analyses.

Diets, whole fish, liver, muscle and plasma analysis

Chemical analysis of the diets, whole fish, liver and muscle was conducted according to AOAC⁵² and following the below procedures: dry matter after drying at 105°C until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein content ($\text{Nx}6.25$) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation unit (models 1015 and 1026, Tecator Systems; Höganäs); and lipid by petroleum ether extraction (Soxtec HT System; Höganäs). Starch was determined according to Beutler⁵³ and gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 6200, PARR Instruments, Moline, IL, USA).

Hepatic and muscular glycogen contents were determined as described by Roehrig & Allred⁵⁴ and lipids were determined according to the method of Folch *et al.*⁵⁵. FA methyl esters were prepared by acid transmethylation of total lipids using boron trifluoride (BF₃) in methanol (14 %) as described by Shantha & Ackman⁵⁶ and analyzed by GC (Varian 3900; Varian; for details see Castro *et al.*⁴⁸). Total cholesterol in diets, liver and muscle was assayed on total lipid extract by the Liebermann–Burchard method⁵⁷. Plasma metabolites were analyzed using commercial kits from Spinreact: glucose (ref: 1001191),

TAG (ref: 1001312), total cholesterol (ref: 1001090) and phospholipids (PL; ref: 1001140).

Enzymatic activity assays

The activity of key lipogenesis enzymes was determined in the liver (3 fish/tank). For that purpose, liver was homogenized (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0.1mM-EDTA and 0.1 % triton X-100 (v/v), pH 7.8). All procedures were performed on ice. Homogenates were centrifuged at 30.000 g for 30 min at 4°C. After centrifugation, the resultant supernatant was collected and aliquots were stored at -80°C until analysis. All enzyme activities were measured at 37°C, monitoring the changes in absorbance of NADPH at 340 nm in a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China), using 6.22 mM/cm as the millimolar extinction coefficient for NADPH. The optimal substrate and protein concentrations for measurement of each enzyme activity were established by preliminary assays. Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49), malic enzyme (ME; EC 1.1.1.40) and fatty acid synthase (FAS; EC 2.3.1.38) activities were determined as previously described by Castro *et al.*⁷.

Gene expression

Analyses of mRNA levels were performed on liver and intestine samples (2 fish/tank). Tissues for RNA analyses were homogenized in 2mL tubes containing Trizol reagent (Invitrogen, Carlsbad, CA, USA) using rapid vibration (liver: 2 x 10s, with an interval of 10s, at 5000 rpm; intestine: 3 x 10s, with 10s intervals, at 6500 rpm) in a Precellys®24 (Bertin Technologies, Montigny-le- Bretonneux, France). Extraction of total RNA was then performed according to manufacturer recommendations. RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Nanodrop Labtech, Palaiseau, France). Complementary DNA (cDNA) synthesis was performed with 1µg of the resulting total RNA using SuperScript III RNaseH- Reverse Transcriptase kit (Invitrogen) and random primers (Promega, Charbonnières, France). Real-time quantitative PCR (q-PCR) analyses were performed in a total volume of 6 µL (detailed information of the reaction mix in Castro *et al.*⁷) using LightCycler® 480 II apparatus (Roche Diagnostics, Neuilly sur Seine, France) to assess the gene expression levels. Primers were either obtained in the literature or designed from gilthead sea beam expressed sequence tag sequences available on SIGENAE database (<http://www.sigenae.org>) using Primer3 software⁵⁸ (Table 2).

Table 2. Sequences of the primer pairs used for real-time quantitative PCR determination of the transcript level of several Gilthead sea bream genes involved in hepatic and intestinal lipid and glucose metabolism.

Gene	5'-3'Forward primer	5'-3'Reverse primer	EL	EI	Acession number
ABCG5	CCATCAGATGCCAGAGGTC T	GGGCAGGGAGGAAAGAGT AG	2.00	1.96	AM973022*
β-actin	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATG CT	1.99	1.96	Pérez-Sánchez et al. ⁴⁵
CPT1A	GTGCCCTCGTTCGTCCAT GATC	TGATGCTTATCTGCTGCC TGT TTG	1.95	2.00	Pérez-Sánchez et al. ⁴⁵
CYP51A 1	CGACCATTGGTGAAGCTGAA C	AAAGTGCACGACAACCTGA C	2.00	1.95	FP336501*
DHCR7	GGTATCTAGGCTGGGTG AC	TCAGTACGGCGGAATAGGT C	1.90	1.99	CB184694*
Elovl5	TGCCAGGACACTCACAGTG C	GGACGAAGCTGTTAGGG AGG	1.99	2.00	Agaba et al. ⁴⁰
FADS2	GACCATGCAGTTACAAGCC ACC	TCCCCCTGAGTTCTTCAGTG ACC	1.95	-	Seiliez et al. ⁴⁷
FAS	TGAAACTGAAGCCCTGTGT G	TCTCGGCTGATGTTCTTGT G	1.90	2.00	AM952430*
GK	CAAGAGACGAGGGGACTT CG	TCCTCGCCTTCTACCAGCT C	1.93	-	Enes et al. ⁵⁹
GPAT	GATCCAGTACGGAGTTCTC TACG	AAAGGGCTGGGAAACT	2.00	1.85	Mininni et al. ⁴⁴
G6Pase	CGCTGGAGTCATTACAGGC GT	CAGGTCCACGCCAGAAC TC	1.84	-	Enes et al. ⁵⁹
LXRα	GCACCTCGCCTCCAGGACA AG	CAGTCTTCACACAGCCACA TCAGG	1.93	2.00	Sánchez-Gurmaches et al. ⁴⁶
PEPCK	GCAACACAGAGAGGGAGG AG	TATCCTCCAGTGCCTTCAG C	1.78	1.88	CV133734*
PPARα	TCTCTTCAGCCCACCATCC C	ATCCCAGCGTGTCTCC	2.00	1.96	Sánchez-Gurmaches et al. ⁴⁶
PPARβ	AGGCGAGGGAGAGTGAGG ATGAGGAG	CTGTTCTGAAAGCGAGGGT GACGATGTTG	1.97	1.97	Sánchez-Gurmaches et al. ⁴⁶
PPARγ	CGCCGTGGACCTGTCAGA GC	GGAATGGATGGAGGAGGA GGAGATGG	1.91	2.00	Sánchez-Gurmaches et al. ⁴⁶
PFK.liver type	CATGTGTGATTGGCCTCAA C	AGGGAGCCTAAAACCCAG AG	1.93	2.00	AM968607*

EL and EI-primer efficiency in liver and intestine, respectively. *Sigenae accession number. ABCG5. ATP binding cassette G5; β-actin; CPT1A. carnitine palmitoyltransferase 1A; CYP51A1. Cytochrome P450 51 (Lanosterol 14-Alpha-Demethylase); DHCR7-dehydrocholesterol reductase 7; elovl5. elongase 5; FADS2. Δ6 fatty acyl desaturase; FAS. Fatty acid synthase; GK. glucokinase; GPAT. glycerol-3-phosphate acyl transferase; G6Pase. glucose 6-phosphatase; LXRA. Liver X Receptor; PEPCK. phosphoenolpyruvate carboxykinase; PPAR. peroxisome proliferator-activated receptor; PFK. 6-phosphofructokinase. liver type;- no expression.

For gene targets that had not been previously validated, primers were tested on a pool of cDNA and amplified products were systematically sequenced. The PCR protocol followed the conditions described previously by Castro *et al.*⁷. Each PCR run included duplicates of reverse transcription for each sample and negative controls (reverse transcriptase-free samples, RNA-free samples). PCR run for reference gene included quadruplicates for each sample (duplicates of reverse transcription and PCR amplification, respectively) and negative controls. Quantification of the target gene transcripts in the liver and intestine was done using β actin gene expression as reference, as previously used in gilthead sea bream by Pérez-Sánchez *et al.*⁴⁵, and that was stably expressed in the present study (data not shown). Relative quantification of the target gene transcript with the β actin reference gene transcript was performed using the mathematical model described by Pfaffl⁶⁰. The relative expression ratio (R) of a target gene was calculated on the basis of real-time PCR efficiency (E) and the CT deviation (ΔCT) of the unknown sample compared with a control sample and expressed in comparison with the β actin reference gene:

$$R = [(E_{\text{target gene}}) \Delta CT_{\text{target gene (mean control-mean sample)}}]/[(E_{\beta \text{ actin}}) \Delta CT_{\beta \text{ actin (mean control-mean sample)}}].$$

Efficiency of q-PCR was measured by the slope of a standard curve using serial dilutions of cDNA. The fish fed the FOCH- diet was used as the control group.

Statistical analysis

Data were checked for normality and homogeneity of variances and when appropriate was normalized. Statistical evaluation of data was carried out by a 2x2 factorial arrangement of treatments in a completely randomized experimental design (two-way ANOVA) with carbohydrate content and lipid source as fixed factors. The significance level of 0.05 was used for rejection of the null hypothesis. In cases where interaction was significant, one-way ANOVA was performed for each factor. All statistical analyses were conducted using the SPSS 22.0 software package (IBM Corp.) for Windows.

Results

Dietary FA composition

The four diets presented small differences in the proportions of total saturated fatty acids (SFA), while monounsaturated fatty acids (MUFA) were higher in FO diets, and n-3 and n-6 polyunsaturated fatty acids (PUFA) were higher in VO diets (Table 3). Within MUFA,

high levels of oleic acid (18:1 n-9) were recorded in VO diets while the opposite occurred for palmitoleic acid (16:1 n-7), eicosenoic acid (20:1 n-9) and erucic acid (22:1 n-9). Among n-3PUFA, VO diets were particularly rich in linolenic acid (18:3 n-3) and poor in EPA and DHA. The proportion of total n-6 PUFA was strongly higher in VO diets mainly due to linoleic acid (18:2 n-6) levels.

Table 3. Fatty acid composition (% of total fatty acids) of the experimental diets

Lipid source	Experimental Diets			
	FO		VO	
Carbohydrates	CH-	CH+	CH-	CH+
14:0	5.8	5.9	2.5	2.0
15:0	0.7	0.6	0.4	0.3
16:0	18.7	17.2	21.3	21.0
17:0	0.5	0.4	0.4	0.3
18:0	4.0	3.5	4.9	4.5
20:0	0.2	0.1	0.3	0.2
ΣSFA	29.9	27.8	29.9	28.6
16:1n-7	7.1	7.5	2.3	1.8
18:1n-9	18.3	19.1	25.4	27.6
20:1n-9	4.9	5.8	0.9	0.7
22:1 n-9	3.9	4.3	0.5	0.4
ΣMUFA	34.4	36.8	29.2	30.5
18:2 n-6	2.2	2.2	9.0	10.6
18:3 n-6	0.1	0.1	0.0	0.1
20:2 n-6	0.3	0.3	0.1	0.1
20:3 n-6	0.13	0.12	0.08	0.08
20:4 n-6	1.2	1.0	0.9	0.7
Σn-6 PUFA	4.0	3.8	10.4	11.6
18:3 n-3	1.1	1.2	15.7	19.0
18:4 n-3	1.9	2.1	0.5	0.4
20:3 n-3	0.14	0.15	0.06	0.04
20:4 n-3	0.6	0.6	0.2	0.1
20:5 n-3	7.8	8.0	3.5	2.5
21:5 n-3	0.3	0.3	0.1	0.1
22:5 n-3	1.2	1.2	0.7	0.5
22:6 n-3	11.7	10.8	7.2	4.9
Σn-3 PUFA	24.8	24.3	28.0	27.5
<i>Ratios</i>				
Sat/PUFA	1.0	0.9	0.8	0.7
n3 / n6	6.2	6.4	2.7	2.4
unsat. Index	181.4	179.5	169.2	160.2

Lipid source: Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; n-3 LC-PUFA= n-3 long chain polyunsaturated fatty acids; unsat. Index= unsaturation index [sum (fatty acid percentage) x (number of double bonds)].

Growth performance and feed utilization

Fish accepted promptly the experimental diets and no mortality was recorded during the trial. Dietary treatments had no effects on fish growth performance or feed utilization (Table 4). Feed intake (g kg average body weight⁻¹ day⁻¹) was similar among diets. N retention, expressed per unit weight gain, was not affected by diet composition.

However, protein efficiency ratio was higher with the CH+ diets, which had lower protein content. Dietary carbohydrate intake increased lipid retention per unit weight gain only when fish fed the VO diet (Table 4). In fish fed CH- diets lipid retention per unit weight gain was lower in the VO group, and between fish fed VO diets there were also significant differences, lower in fish fed CH-(carbohydrate and lipid source interaction).

Table 4. Growth performance and feed utilization of Gilthead sea bream fed the experimental diets^a.

Lipid source	Experimental diets				p value***		
	FO		VO		CH	LS	CH*LS
Carbohydrates	CH-	CH+	CH-	CH+			
IBW (g)	70.8± 0.03	70.7± 0.03	70.8± 0.03	70.7± 0.02	1.000	0.370	0.094
FBW (g)	222.3± 8.5	221.8 ±8.8	203.9± 18.8	212.1 ±4.6	0.577	0.066	0.524
Daily growth index ^b	2.37 ± 0.1	2.37± 0.1	2.16± 0.22	2.26± 0.05	0.553	0.073	0.503
Feed intake (g/kg ABW§/day)	18.4± 0.6	18.4± 1.5	16.7± 0.8	17.3± 1.3	0.633	0.059	0.716
Feed efficiency ^c	0.7± 0.03	0.69± 0.06	0.71± 0.04	0.72± 0.04	1.000	0.453	0.899
Protein efficiency ratio ^d	1.05± 0.05	1.38± 0.12	1.08± 0.06	1.42± 0.09	<0.001	0.514	0.895
N intake (g/ kg ABW/day)	1.95± 0.08	1.49± 0.14	1.77± 0.18	1.39± 0.13	0.001	0.122	0.637
N retention (g/ kg ABW/day) ^e	0.34± 0.03	0.34± 0.01	0.35± 0.01	0.34± 0.02	0.648	0.878	0.648
Lipid intake (g/ kg ABW/day)	3.37± 0.14	3.39± 0.31	3.04± 0.3	3.15± 0.29	0.681	0.104	0.757
Lipid retention (g/ kg ABW/day) ^f	2.21 ^B ± 0.05	2.31± 0.12	1.84 ^{aA} ± 0.13	2.36 ^b ± 0.07	0.001	0.023	0.006

Lipid source (LS): Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

^aValues are means ± standard deviation (n=3).

ABW: Average body weight: (initial body weight+final body weight)/2.

^bDGI: ((FBW^{1/3}- IBW^{1/3})/time in days) x 100.

^cFE. Wet weight gain/dry feed intake.

^dPER. Wet weight gain/crude protein intake.

^eN retention= ((FBW x carcass N content)-(IBW x carcass N content))/ (ABW x the number of days)

^fLipid retention =((FBW x carcass lipid content)-(IBW x carcass lipid content))/ (ABW x the number of days)

***significant differences at p< 0.05 two-way ANOVA. If interaction was significant, one-way ANOVA was performed for each factor and means in the same line with different capital and small letters indicate significant differences (p<0.05) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different (p>0.05).

Whole-body, liver and muscle composition

At the end of the trial, only whole-body lipid and dry matter contents were affected by dietary treatments. Under a VO based diet regime, whole-body lipid content was higher in CH+ than in CH- groups. In addition, whole-body lipid was lower in VO than in FO groups only when fish were fed no carbohydrate diets (CH- diets) (carbohydrate and lipid

source interaction). Whole body dry matter content was higher in fish fed the CH+ diets (Table 5).

Higher hepatosomatic and viscerosomatic indexes were observed in the CH+ groups, but no effect of dietary lipid source was noticed (Table 5).

Table 5. Whole-body, liver and muscle composition (wet-weight basis), hepatosomatic and viscerosomatic indexes of Gilthead sea bream fed the experimental diets^a

Lipid source Carbohydrates	Experimental diets						p-value***	
	FO		VO		CH	LS		
	CH-	CH+	CH-	CH+				
<i>Whole body composition*</i>								
Protein (%)	16.54± 0.73	16.64± 0.18	17.48± 0.46	16.87± 0.82	0.484	0.134	0.341	
Lipids (%)	14.27 ^B ± 0.25	14.83± 0.64	12.75 ^{aA} ± 0.68	15.35 ^b ± 0.38	0.001	0.123	0.009	
Dry matter (%)	35± 0.23	36.13± 0.62	34.01± 0.72	35.94± 0.28	0.001	0.078	0.208	
Ash	4.01± 0.24	4.31± 0.29	4.5± 0.19	4.39± 0.31	0.095	0.562	0.212	
HSI ^a	1.23± 0.17	1.87± 0.2	1.23± 0.22	1.96± 0.31	<0.001	0.478	0.343	
VSI ^b	6.18± 0.68	6.82± 0.89	6.35± 0.94	6.77± 1.05	0.003	0.755	0.528	
<i>Liver composition</i>								
Lipids (%)	13.88± 3.25	12.01± 2.79	10.39 ^a ± 3.15	13.95 ^b ± 2.98	0.413	0.461	0.021	
Cholesterol (%)	0.34± 0.068	0.26± 0.034	0.35± 0.058	0.257± 0.025	<0.001	0.811	0.809	
Glycogen (mg/ g liver)	73.49 ^{aA} ± 11.75	134.89 ^b ± 7.77	89.80 ^{aB} ± 11.27	125.46 ^b ± 11.21	<0.001	0.338	0.001	
<i>Muscle composition</i>								
Lipids (%)	8.23± 1.06	9.85± 2.1	8.08± 1.17	9.43± 1.05	0.02	0.661	0.877	
Cholesterol (%)	0.097± 0.008	0.088± 0.008	0.09± 0.009	0.097± 0.01	0.811	0.831	0.051	
Glycogen (µg/ gmuscle)	12.28 ^{aA} ± 2.47	29.84 ^b ± 7.89	23.66 ^B ± 3.4	31.35± 8.57	<0.001	<0.001	0.001	

Lipid source (LS): Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

^aValues are means ± standard deviation (n=3 for whole body composition; n=6 for lipids and cholesterol; n=9 for glycogen; n=18 for HSI and VSI).

^b HSI: (liver weight/body weight)×100.

^c VSI: (viscera weight/body weight)×100.

* Initial body composition on the fish: dry matter 28.96%; protein 16.15 %; lipid 7.87%; ash 5.84%.

*** significant differences at $p<0.05$ two-way ANOVA; If interaction was significant, one-way ANOVA was performed for each factor and means in the same line with different capital and small letters indicate significant differences ($p<0.05$) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different ($p>0.05$).

Lipid content in muscle was higher in the CH+ group, while in liver an increase in lipid content with carbohydrate intake was only evident when fish were fed VO based diets (carbohydrate and lipid source interaction). Carbohydrate intake also increased the glycogen content in liver, but in muscle a similar effect was only recorded when fish were fed FO based diets (carbohydrate and lipid source interaction). Dietary lipid source induced no changes on hepatic and muscular lipid content, and glycogen content in both tissues increased in VO group when fish were fed CH-diets (carbohydrate and lipid source interaction). Hepatic cholesterol content was lower in CH+ groups, but no differences were recorded in muscle cholesterol content. Dietary lipid source did not affect hepatic and muscular cholesterol content.

Liver and muscle FA profiles

Muscle and liver FA composition were affected by diet composition and resembled the FA composition of the dietary lipid sources (Table 6 and 7).

Except for muscle SFA content that was similar among CH+ and CH- diets, liver and muscle FA profiles of fish fed the CH+ diets were characterized by higher proportion of SFA (particularly 16:0) and MUFA (particularly 18:1 n-9) and lower proportion of n-3 PUFA and n-6 PUFA. In the liver, replacing FO by VO resulted in higher proportions of SFA (mainly 16:0) and n-6 PUFA (mainly 18:2 n-6), and lower proportion of n-3 PUFA (mainly 22:6 n-3, 20:5 n-3, 22:5 n-3) and MUFA (mainly 16:1 n-7; 20:1 n-9; 22:1 n-9).

Table 6. Liver fatty acid profile (% of total fatty acids) of Gilthead sea bream fed the experimental diets^a

Lipid source	Experimental diets						p-value***
	FO		VO		CH	LS	
	Carbohydrates	CH-	CH+	CH-	CH+	CH*LS	
14:0	4.3 ^{bE} ±0.29	3.5 ^{aB} ±0.46	2.4 ^A ±0.28	2.8 ^A ±0.32	0.185	<0.001	<0.001
15:0	0.51±0.04	0.32±0.03	0.36±0.06	0.25±0.03	<0.001	<0.001	0.073
16:0	19.4±0.9	22.3±0.7	22.03±2.1	25.6±1.5	<0.001	<0.001	0.643
17:0	0.45±0.04	0.31±0.01	0.43±0.04	0.27±0.01	<0.001	0.011	0.305
18:0	4.6 ^{aA} ±0.38	6.4 ^b ±0.42	5.6 ^B ±0.5	5.99±0.3	<0.001	0.074	<0.001
20:0	0.13±0.02	0.14±0.01	0.13±0.04	0.1±0.01	0.215	0.061	0.079
ΣSFA	29.5±0.9	33.0±0.8	31.0±2.3	35.0±1.7	<0.001	0.011	0.737
16:1 n-7	6.5±0.32	6.3 ^B ±0.34	3.5 ^{aA} ±0.18	4.4 ^{bA} ±0.21	0.003	<0.001	<0.001
18:1 n-9	22.1±1.6	27.9±1.2	27.1±1.9	32.2±1.5	<0.001	<0.001	0.475
20:1 n-9	3.64±0.59	3.53±0.31	0.77±0.2	0.48±0.03	0.034	<0.001	0.108
22:1 n-9	2.16±0.35	2.01±0.36	0.3±0.17	0.08±0.03	0.008	<0.001	0.068
ΣMUFA	34.7±1.7	39.9±1.3	31.7±2.2	37.2±1.6	<0.001	0.001	0.795
18:2 n-6	2.41±0.16	1.97±0.22	8.21±0.45	7.9±0.34	0.002	<0.001	0.109
18:3 n-6	0.18 ^B ±0.06	0.19 ^B ±0.01	0.10 ^{aA} ±0.05	0.30 ^{bA} ±0.05	<0.001	0.864	<0.001
20:2 n-6	0.26 ^b ±0.02	0.21 ^{aB} ±0.01	0.25 ^b ±0.05	0.15 ^{aA} ±0.01	<0.001	0.003	0.029
20:3 n-6	0.14±0.02	0.11±0.01	0.08±0.04	0.11±0.02	0.515	0.031	0.071
20:4 n-6	1.4 ^b ±0.22	1.0 ^{aB} ±0.11	1.5 ^b ±0.35	0.64 ^{aA} ±0.06	<0.001	0.003	0.003
Σn-6PUFA	4.61±0.31	3.56±0.21	10.1±0.7	9.1±0.3	<0.001	<0.001	0.141
18:3 n-3	0.91 ^{bA} ±0.09	0.69 ^{aA} ±0.08	11.1 ^B ±0.82	10.5 ^B ±0.84	<0.001	<0.001	0.012
18:4 n-3	1.0 ^{bB} ±0.12	0.84 ^{aB} ±0.1	0.30 ^{aA} ±0.04	0.69 ^{bA} ±0.12	0.002	<0.001	<0.001
20:3 n-3	0.18 ^{bA} ±0.03	0.13 ^{aA} ±0.02	0.65 ^{bB} ±0.16	0.37 ^{aB} ±0.04	<0.001	<0.001	0.020
20:4 n-3	0.85±0.11	0.76±0.06	0.36±0.06	0.34±0.06	0.131	<0.001	0.412
20:5 n-3	5.3 ^{bB} ±0.36	4.2 ^{aB} ±0.3	2.5 ^{bA} ±0.42	1.2 ^{aA} ±0.05	<0.001	<0.001	0.003
21:5 n-3	0.27±0.03	0.21±0.05	0.04±0.05	0.00±0.01	0.023	<0.001	0.439
22:5 n-3	2.6 ^{bB} ±0.31	2.0 ^{aB} ±0.24	1.3 ^{bA} ±0.36	0.47 ^{aA} ±0.05	<0.001	<0.001	0.017
22:6 n-3	14.7 ^{bB} ±1.4	9.6 ^{aB} ±0.7	8.6 ^{bA} ±1.6	3.2 ^{aA} ±0.1	<0.001	<0.001	0.016
Σn-3PUFA	25.8±1.6	18.4±1.0	24.9±1.8	16.7±0.9	<0.001	0.024	0.398
<i>Ratios</i>							
SFA/PUFA	0.93±0.07	1.42±0.08	0.87±0.1	1.33±0.11	<0.001	0.06	0.846
n3/n6	5.6 ^{bB} ±0.18	5.2 ^{aB} ±0.28	2.5 ^{bA} ±0.2	1.8 ^{aA} ±0.05	<0.001	<0.001	0.007
Unsat. Index	191±9	152±5	159±16	123±3	<0.001	<0.001	0.705

Lipid source (LS): Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; n-3 LC-PUFA= n-3 long chain polyunsaturated fatty acids.

Unsat. Index= unsaturation index [sum (fatty acid percentage) x (number of double bonds)].

^aValues are means ± standard deviation (n=6). FA ≥0.02%. < 0.02% was not considered in the table.

*** significant differences at p< 0.05 two-way ANOVA. If interaction was significant, one-way ANOVA was performed for each factor and means in the same line with different capital and small letters indicate significant differences (p<0.05) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different (p>0.05).

The muscle of fish fed the VO diets presented lower proportion of SFA (mainly 14:0) and MUFA (mainly 16:1 n-7; 20:1 n-9; 22:1 n-9) and increased proportion of n-6 (mainly 18:2 n-6) and n-3 (mainly 18:3 n-3) PUFA.

Table 7. Muscle fatty acid profile (expressed as % of total fatty acids) of Gilthead sea bream fed the experimental diets^a

Lipid source	Experimental diets						p-value***	
	FO		VO		CH	LS		
	Carbohydrates	CH-	CH+	CH-	CH+			
14:0	4.76±0.17	4.59±0.19	2.72±0.17	2.39±0.14	0.001	<0.001	0.108	
15:0	0.52±0.02	0.43±0.01	0.34±0.01	0.24±0.01	<0.001	<0.001	0.002	
16:0	19.2±0.5	20.0±0.7	19.1±0.8	19.6±0.3	0.018	0.307	0.754	
17:0	0.39±0.02	0.3±0.01	0.32±0.02	0.23±0.01	<0.001	<0.001	0.179	
18:0	3.3±0.22	3.72±0.17	3.59±0.15	4.01±0.1	<0.001	<0.001	0.875	
20:0	0.15±0.01	0.14±0.00	0.2±0.01	0.18±0.01	0.013	<0.001	0.238	
ΣSFA	28.4±0.6	29.2±0.8	26.3±1.0	26.8±0.6	0.058	<0.001	0.585	
16:1n-7	7.92±0.16	7.8±0.12	4.69±0.44	4.18±0.2	0.009	<0.001	0.058	
18:1n-9	24.4 ^{aA} ±0.9	25.7 ^{bA} ±0.3	28.8 ^{aB} ±0.4	32.2 ^{bB} ±0.4	<0.001	<0.001	<0.001	
20:1n-9	3.83 ^B ±0.29	4.06 ^B ±0.11	1.14 ^{aA} ±0.03	0.95 ^{aA} ±0.08	0.02	<0.001	<0.001	
22:1n-9	2.13 ^{aB} ±0.18	2.40 ^{bB} ±0.12	0.55 ^{bA} ±0.04	0.41 ^{aA} ±0.04	0.778	<0.001	<0.001	
ΣMUFA	38.5 ^{aB} ±0.5	40.2 ^{bB} ±0.3	35.3 ^{aA} ±0.8	37.9 ^{bA} ±0.3	<0.001	<0.001	0.03	
18:2 n-6	4.19±0.32	3.66±0.47	9.13±0.55	9.25±0.35	0.14	<0.001	0.051	
18:3 n-6	0.28±0.04	0.26±0.06	0.2±0.07	0.25±0.04	0.429	0.059	0.081	
20:2 n-6	0.26±0.01	0.23±0.01	0.25±0.01	0.21±0.01	<0.001	0.004	0.241	
20:3 n-6	0.14 ^{bB} ±0.02	0.11 ^{aA} ±0.01	0.11 ^A ±0.01	0.12 ^B ±0.02	0.026	0.180	0.007	
20:4 n-6	0.98 ^{bB} ±0.03	0.79 ^{aB} ±0.04	0.87 ^{bA} ±0.07	0.59 ^{aA} ±0.04	<0.001	<0.001	0.007	
Σn-6PUFA	5.85 ^{bA} ±0.3	5.04 ^{aA} ±0.52	10.6 ^B ±0.6	10.4 ^B ±0.4	0.013	<0.001	0.047	
18:3 n-3	1.10 ^{bA} ±0.03	1.03 ^{aA} ±0.06	11.2 ^{aB} ±0.9	12.6 ^{bB} ±0.6	0.301	<0.001	0.001	
18:4 n-3	1.21±0.09	1.27±0.08	0.45±0.03	0.45±0.04	0.41	<0.001	0.206	
20:3 n-3	0.15±0.02	0.14±0.02	0.42±0.04	0.4±0.03	0.071	<0.001	0.884	
20:4 n-3	0.75±0.03	0.69±0.03	0.38±0.02	0.35±0.03	0.001	<0.001	0.723	
20:5 n-3	5.10 ^B ±0.13	5.01 ^B ±0.07	2.75 ^{bA} ±0.16	1.87 ^{aA} ±0.11	<0.001	<0.001	<0.001	
21:5 n-3	0.25 ^B ±0.01	0.23 ^B ±0.02	0.11 ^{bA} ±0.02	0.03 ^{aA} ±0.04	0.002	<0.001	0.006	
22:5 n-3	2.15 ^{bB} ±0.17	1.86 ^{aB} ±0.12	1.44 ^{bA} ±0.08	0.99 ^{aA} ±0.04	<0.001	<0.001	0.007	
22:6 n-3	11.3 ^{bB} ±0.9	10.2 ^{aB} ±0.1	8.4 ^{bA} ±0.5	5.8 ^{aA} ±0.4	<0.001	<0.001	0.001	
Σn-3PUFA	22.0±1.1	20.4±0.2	25.1±1.3	22.5±0.5	<0.001	<0.001	0.212	
Ratios								
SFA/PUFA	0.97±0.05	1.1±0.05	0.72±0.06	0.8±0.03	<0.001	<0.001	0.423	
n3/n6	3.78 ^B ±0.29	4.08 ^B ±0.46	2.38 ^{bA} ±0.15	2.16 ^{aA} ±0.11	0.943	<0.001	0.023	
Unsat. index	173 ^b ±6	164 ^{aB} ±2	170 ^b ±5	154 ^{aA} ±3	<0.001	0.001	0.029	

Lipid source (LS): Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH):0% (CH-) or 20% (CH+) gelatinized maize starch.

SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; n-3 LC-PUFA= n-3 long chain polyunsaturated fatty acids.

Unsat. Index= unsaturation index [sum (fatty acid percentage) x (number of double bonds)].

^aValues are means ± standard deviation (n=6). FA ≥0.02%. < 0.02% was not considered in the table.

*** significant differences at p< 0.05 two-way ANOVA. If interaction was significant, one-way ANOVA was performed for each factor and means in the same line with different capital and small letters indicate significant differences (p<0.05) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different (p>0.05).

Plasma metabolites and enzymes activity

Eighteen hours after feeding diets CH+ plasma glucose concentration was lower than in fish fed CH- diets (Table 8).

Plasma cholesterol was also lower in fish fed the CH+ diet when combined with VO (carbohydrate and lipid source interaction). On the contrary, plasma TAG was higher in fish fed the CH+ diet, but only in fish fed FO (carbohydrate and lipid source interaction).

Plasma phospholipids and cholesterol were lower in fish fed the VO diets. In fish feeding CH+ diets, plasma TAG was lower in VO than in FO group (carbohydrate and lipid source interaction).

Diets CH+ promoted an increase in fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PD) and malic enzyme (ME) activities. G6PD activity was also responsive to dietary lipid source, being higher in VO diets (Table 8).

Table 8. Plasma metabolite concentrations^a (mmol L⁻¹) and enzymatic activity (mU mg protein⁻¹) of selected enzymes involved in lipogenesis^b in Gilthead sea bream fed the experimental diets.

Lipid source	Experimental diets				p-value***		
	FO		VO				
Carbohydrates	CH-	CH+	CH-	CH+	CH	LS	CH*LS
<i>Plasma metabolites</i>							
GLU	3.73± 0.60	3.16± 0.49	3.47± 0.34	3.19± 0.46	<0.001	0.312	0.203
CHOL	7.37 ^B ± 0.53	7.25 ^B ± 0.95	6.80 ^{bA} ± 0.99	5.82 ^{aA} ± 0.65	0.005	<0.001	0.027
TAG	2.87 ^a ± 0.63	3.73 ^{BB} ± 0.73	3.15±0.86	3.15 ^A ± 0.67	0.015	0.383	0.016
PL	15.37± 1.47	15.45± 1.44	14.46± 2.54	13.42± 1.26	0.246	0.001	0.178
<i>Enzyme activity</i>							
G6PD	140.1± 36.3	203.7± 49.1	198.6± 27.6	240± 68.6	0.004	0.009	0.511
ME	6.97± 3.1	9.58± 3.49	5.51± 1.96	11.63± 4.37	0.001	0.791	0.130
FAS	4.28± 2.76	7.38± 1.75	5.46± 2.91	8.6± 3.61	0.003	0.225	0.981

Lipid source (LS): Fish oil (FO) or blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

^a glucose. GLU; triacylglycerol. TAG; total cholesterol. CHOL; phospholipids. PL. Values are means ± standard deviation (n=18)

^bGlucose-6-phosphate dehydrogenase. G6PD; malic enzyme. ME; Fatty acid synthase. FAS. Values are presented as means ± standard deviation (n=9).

*** Significant differences at p< 0.05 two-way ANOVA. If interaction was significant, one-way ANOVA was performed for each factor and means in the same line with different capital and small letters indicate significant differences (p<0.05) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different (p>0.05).

Gene expression

Hepatic transcript levels of glucokinase (GK), the first glycolytic enzyme, was higher in CH+ than in CH- groups, while 6-phosphofructokinase (PFK) transcript levels were not affected by dietary carbohydrate (Figure 1).

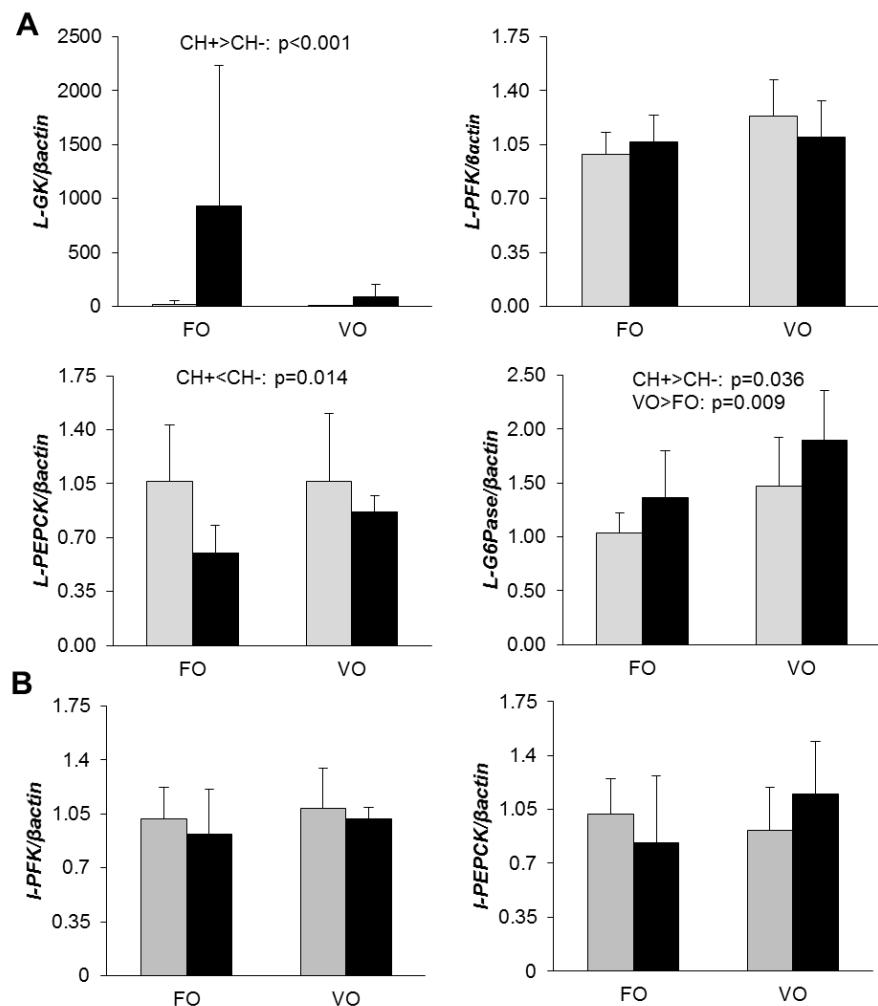


Fig. 1. mRNA levels of genes involved in glycolysis (*glucokinase, GK, 6-phosphofructokinase, PFK*) and gluconeogenesis (*phosphoenolpyruvate carboxykinase, PEPCK; glucose 6-phosphatase, G6Pase*) in the (A) liver and (B) intestine of Gilthead sea bream fed the experimental diets. Expression values are normalized by β -actin- expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0% (□ CH-) or 20% (■ CH+) gelatinized maize starch. Relative fold difference between treatments are presented as means + SD (n = 6); significant differences at p < 0.05 two-way ANOVA.

Hepatic transcript levels of phosphoenolpyruvate carboxykinase (PEPCK), the first key enzyme involved in gluconeogenesis, were lower in CH+ than in CH- groups, while the opposite occurred for hepatic glucose 6-phosphatase (G6Pase) mRNA levels, the enzyme involved last step of gluconeogenesis. Among glycolytic and gluconeogenic enzymes, only hepatic G6Pase transcript levels were up-regulated by dietary VO. At intestinal level, no transcriptional regulation by diet composition of PFK and of PEPCK was observed (Figure 1).

Hepatic and intestinal transcript levels of key enzymes involved in lipogenesis (FAS), β -oxidation (carnitine palmitoyltransferase 1A, CPT1A) and phospholipid synthesis (Glycerol-3-phosphate acyl transferase, GPAT) were not affected by diet composition (Figure 2).

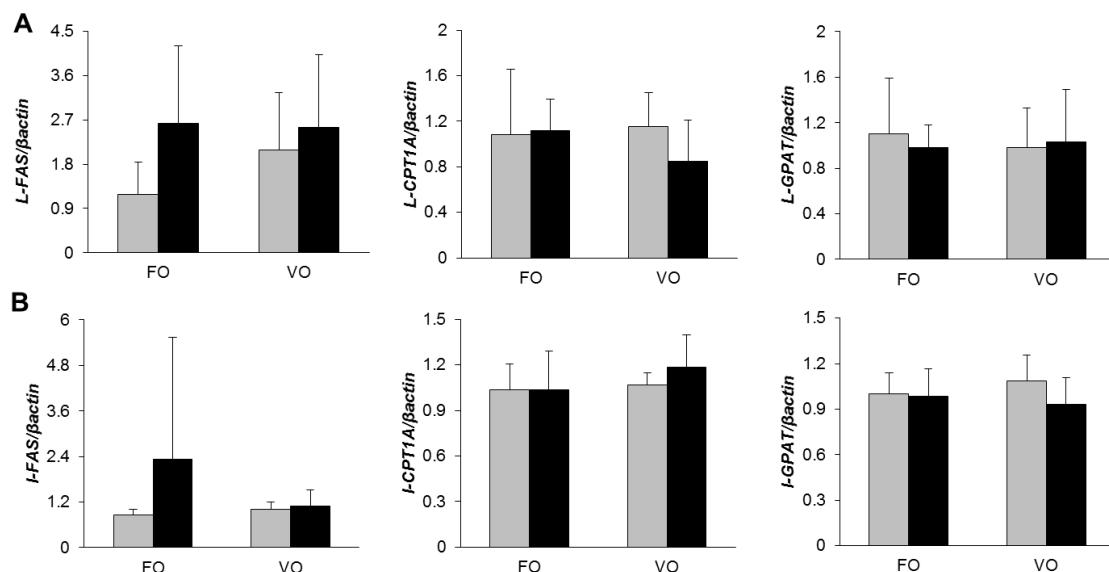


Fig. 2. mRNA levels of genes involved in lipogenesis (*fatty acid synthase*, FAS), β -oxidation (*carnitine palmitoyltransferase 1A*, CPT1A) and phospholipid synthesis (*glycerol-3-phosphate acyl transferase*, GPAT) in the (A) liver and (B) intestine of Gilthead sea bream fed the experimental diets. Expression values are normalized by β -actin- expressed transcripts.

Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0% (□ CH-) or 20% (■ CH+) gelatinized maize starch. Relative fold difference between treatments are presented as means + SD ($n = 6$); significant differences at $p < 0.05$ two-way ANOVA.

With the exception of CYP51A1 transcript levels in the intestine, that were higher in CH+ groups, no variation in hepatic or intestine transcript levels of proteins involved in cholesterol efflux (ATP binding cassette G5, ABCG5), and cholesterol synthesis (7-dehydrocholesterol reductase, DHCR7; Lanosterol 14-Alpha-Demethylase, Cytochrome P450 51- CYP51A1) and catabolism (Liver X Receptor α , LX α) were observed (Figure 3).

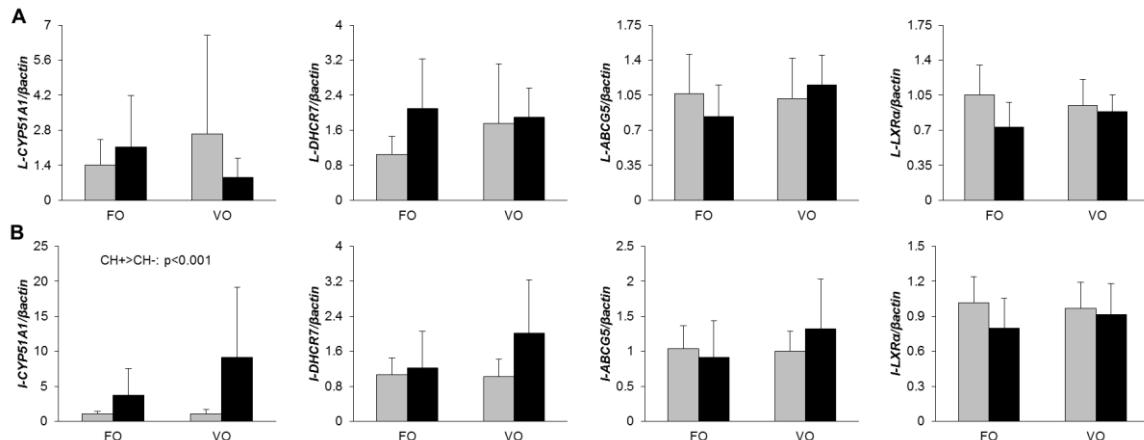


Fig. 3- mRNA levels of genes involved in cholesterol metabolism (*Lanosterol 14-Alpha-Demethylase*, *Cytochrome P450 51- CYP51A1*; *7-dehydrocholesterol reductase*, *DHCR7*; *Liver X Receptor α* , *LXR α*) and transport (*ATP binding cassette G5*, *ABCG5*) in the (A) liver and (B) intestine of Gilthead sea bream fed the experimental diets. Expression values are normalized by β -actin- expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0% (□) CH- or 20% (■) CH+ gelatinized maize starch. Relative fold difference between treatments are presented as means + SD (n = 6); significant differences at p< 0.05 two-way ANOVA.

The expression of genes encoding key proteins involved LC-PUFA-biosynthesis pathway ($\Delta 6$ fatty acyl desaturase, FADS2; elongase 5, elovl5) were nutritionally regulated both in the liver and the intestine (Figure 4). In the liver, FADS2 transcript levels were up-regulated by dietary carbohydrate and VO but in the intestine expression of FADS2 were undetected. Elavl5 expression in the liver was down-regulated in CH+ groups, but in the intestine down-regulation was only evident in fish fed FO based diets (carbohydrate and lipid source interaction). In addition, intestinal elovl5 mRNA levels increased in VO groups when fed a carbohydrate rich diet (carbohydrate and lipid source interaction).

In the liver transcription of proliferator-activated receptors (PPAR) α and PPAR γ was down-regulated in CH+ groups while in the intestine down-regulation of PPAR β transcript levels in CH+ groups was only observed in fish fed the VO diet (carbohydrate and lipid source interaction). (Figure 4). Additionally, in the intestine of fish fed the CH- diets PPAR β transcript levels were up-regulated in fish fed the VO diets (carbohydrate and lipid source interaction).

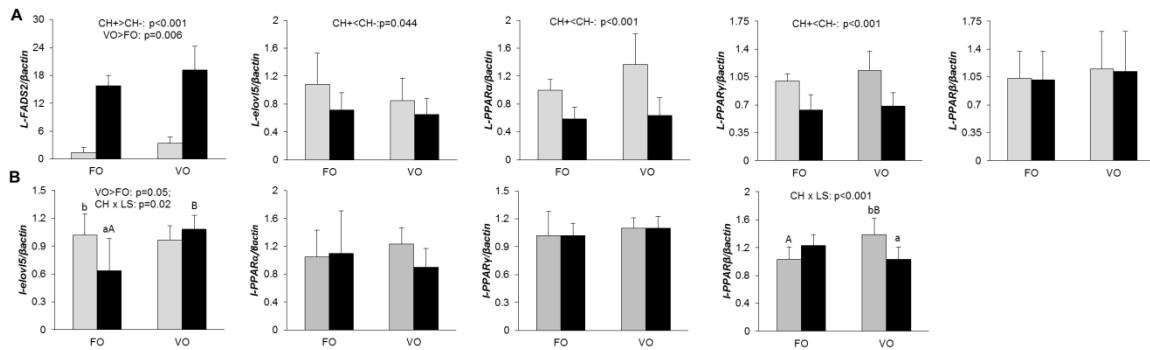


Fig. 4- mRNA levels of genes involved in LC-PUFA-biosynthesis pathway ($\Delta 6$ fatty acyl desaturase, *FADS2*; elongase 5, *elovl5*) and transcription factors involved in several lipid related processes (peroxisome proliferator-activated receptors α, γ , β , *PPAR \alpha, \gamma, \beta*) in the (A) liver and (B) intestine of Gilthead sea bream fed the experimental diets. Expression values are normalized by β -actin- expressed transcripts. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0% (□) or 20% (■) CH+. Relative fold difference between treatments are presented as means + SD (n = 6); significant differences at p< 0.05 two-way ANOVA. If interaction was significant, one-way ANOVA was performed for each factor and means with different capital and small letters indicate significant differences (p<0.05) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different (p>0.05).

Discussion

Effects of dietary carbohydrates

Though gilthead sea bream is a carnivorous species (trophic level 3.3–3.5 according to Fish Base), it tolerates up to 20% dietary starch without detrimental effects in growth performance and feed efficiency. These results support previous evidences^{61, 62} and point out for the possibility of reduce aquafeed costs and alleviate the overexploitation of fisheries marine resources through the use of the carbohydrate component in gilthead sea bream diets.

The low glycaemia values at 18h after feeding are in accordance to results of Peres & Oliva-Teles⁶³ that observed in a glucose tolerance test that seabream was able to restore glucose levels within 12h after receiving an over-dose of glucose.

Hepatic GK is a key player in blood glucose homeostasis by catalyzing the phosphorylation of glucose and providing the first substrate for glycolysis, glycogenesis and the pentose phosphate pathway⁶⁴. An induction of hepatic GK transcripts by dietary carbohydrates was recorded in the present study, as in a previous study in this species⁶⁵. On the contrary, dietary carbohydrates did not affect transcription levels of hepatic PFK, another key glycolytic enzyme.

Hepatic transcript levels of PEPCK, the first key-enzyme of gluconeogenesis, were down-regulated by dietary carbohydrate but the transcript levels of G6Pase, another key-enzyme of gluconeogenesis, was up-regulated by dietary carbohydrates. Although a similar response of hepatic G6Pase by dietary carbohydrate at the transcriptional level

was previously reported in carnivorous rainbow trout²⁴, present data apparently contradicts results of Panserat *et al.*⁶⁶ in this species, whom observed that G6Pase and FBPase were down-regulated by dietary carbohydrates at transcriptional level while PEPCK was not affected. At enzymatic activity level, it was reported that dietary carbohydrates induced minor effects^{62; 67} or even increased⁶¹ hepatic activities of gluconeogenesis enzymes in gilthead sea bream. Recent studies pointed intestine as having important functions in glucose homeostasis^{68; 69}. However, present results do not support that assumption, as expression of glycolytic (PFK) and gluconeogenesis (PEPCK) enzymes in this tissue did not respond to dietary carbohydrate.

In parallel with the hepatic up-regulation of GK transcripts, increased HSI and VSI were recorded in fish fed carbohydrate rich diets which may indicate that the hepatic glucose pool was directed towards glycogen and/or FA synthesis. Indeed, in liver, an increased deposition of glycogen and a higher lipogenic potential, indicated by FAS, G6PD and ME activities, were recorded in CH+ groups. Increased HSI may have unwanted physiological effects, therefore histomorphological analysis of liver should be consider in future studies to discard any histopathological damage induced by diet.

Despite the fact that lipogenesis was nutritionally regulated by dietary carbohydrates, the contribution to the overall lipid deposition in liver or whole body of fish fed FO based diet seemed to be minor, as lipids in liver and whole body increased with carbohydrate intake only under a VO based diet regime.

Recently it was suggested that dietary carbohydrates play a role in cholesterol biosynthesis by inducing (at least at a molecular level) the capacity to produce it⁷. In the present study it was observed that gilthead sea bream fed carbohydrate-rich diets exhibited reduced liver and plasma (only in VOCH+ group) cholesterol content. Further, an increased expression at intestinal level of one gene encoding for an enzyme involved in the last steps of cholesterol biosynthesis, CYP51A1 was also observed. We hypothesize that the depletion of the cholesterol pool was promoted by a lower dietary input of cholesterol, and the up-regulation of CYP51A1 may reflect an increased synthesis of sterols by the intestinal cells in response to the low dietary supply. Metabolic adjustments in de novo cholesterol biosynthesis in response to the dietary load of cholesterol were previously described in Atlantic salmon⁷⁰.

In this work we demonstrated a transcriptional up-regulation by dietary carbohydrate of liver FADS2, a key desaturase involved in LC-PUFA synthesis. With the up-regulation of

FADS2 gene, it was expected to observe increased transcript levels of elovl5, a fatty acyl elongase involved in LC-PUFA biosynthesis. However, and somehow surprisingly, the reverse pattern was observed both in the intestine (only in FOCH+ group) and in the liver of gilthead sea beam fed CH+ diets. In the present state of knowledge, we have no clear explanation for these conflicting results, and therefore further studies are needed to better understand the apparent atypical molecular regulation of some enzymes of LC-PUFA synthesis pathway by dietary carbohydrate in gilthead sea beam. Irrespective of the regulation of FADS2 expression, increased liver or muscle n-3 LC-PUFA content of gilthead sea bream fed CH+ diets were not observed. On the contrary, reduced n-3 LC-PUFA content in CH+ groups was recorded, which could be related to an increase in saturated FA derived by lipogenesis from carbohydrates as previously reported in others species like rainbow trout and European sea bass^{7; 71}.

PPARs are a family of nuclear receptors that have three isoforms in mammals known as PPAR α , PPAR β and PPAR γ that play key roles in regulation of lipid metabolism⁷². It is believed that PPARs control metabolic pathways that support fatty acid β -oxidation (especially PPAR α), tissue lipid deposition and lipogenesis (especially PPAR γ), and the overall lipid homeostasis (especially PPAR β), mainly by means of the action of its ligands (such as FA and their derivatives, and also glucose)^{72; 73}. In this study, despite the down-regulation of PPAR α, β gene expression in the liver and of PPAR β in the intestine (only in VO diets) by dietary carbohydrates, gene expression of CPT1, a marker of mitochondrial FA β -oxidation, was unaffected both in liver and intestine of fish fed CH+ diets. In previous studies, a synchronized regulation between piscine PPAR α, β gene expression and the activity or expression of other enzymes involved in FA β -oxidation, such as acyl-CoA oxidase L-3-hydroxyacyl-CoA dehydrogenase, was described^{74; 75}. However, present results are not completely unexpected, as in a number of studies the expression of the CPT1 gene was not nutritionally regulated or directly linked to the FA β -oxidation capacity^{24; 37; 76}. Furthermore, FA β -oxidation and lipogenesis are two pathways usually regulated in opposite directions^{77; 78}. Therefore, as lipogenic potential increased in liver of fish fed CH+ diets it is possible that FA β -oxidation might have been repressed. Additional data on the activity or expression of the other FA β -oxidation related enzymes would be necessary to confirm this hypothesis.

In the above discussion differences in metabolic responses were assumed to be related to differences in dietary carbohydrate (0% versus 17-18%). However, a potential effect of dietary protein cannot be discarded, particularly in relation to regulation of gluconeogenesis and lipogenesis, as there is increasing evidence that protein and amino acids also modulate these pathways⁷⁹⁻⁸².

Effects of dietary lipid source

In the present study gilthead sea bream performed as well as in previous studies with gilthead sea bream juveniles where dietary FO was partially (70%) replaced by VO in fish meal^{83; 84} or plant protein^{85; 86} based diets. However, plasma cholesterol and phospholipid concentrations were lower in fish fed the VO diets. Replacing FO with VO usually increases dietary phytosterol and reduces cholesterol content. Such dietary modifications been reported to induce a decrease in plasma cholesterol and LDL-cholesterol both in humans^{87; 88} and in fish^{35-37; 89}.

Phytosterols are structurally similar to cholesterol and may induce a relative cholesterol deficiency by mechanisms that interfere with cholesterol absorption such as competition for space in mixed micelles or competition with cholesterol transporters (such as ABC transporters)^{87; 90}. For instance, in Atlantic salmon, dietary inclusion of soybean meal and soya saponins decreased the expression of ABCG5 in the distal intestine⁹¹ and in the liver⁹². In the present study, however, no repression of ABCG5 expression related to diet composition was observed at hepatic or intestinal levels.

Furthermore, no induction in the expression of the selected genes (CYP51A1 and DHCR7) involved in the cholesterol biosynthesis was recorded in the intestine and liver of fish fed the VO diets. This differ from other studies where hepatic or intestinal gene expression of key enzymes involved in cholesterol biosynthesis pathways (such as 3-hydroxy-3-methyl-glutaryl-CoA reductase, isopentenyl-diphosphate Δisomerase, CYP51A1 or DHCR7) increased in response to the decreased plasma cholesterol concentration in fish fed plant based⁹¹⁻⁹³ or VO^{7; 10; 39} diets. Whether, the lack of activation of cholesterol biosynthesis at molecular level as a response to the reduced plasma cholesterol concentration that was observed in the present study is due to variations in the dietary cholesterol supply or to species specific sensitivities to alternative ingredients, such as as VO remains to be clarified.

An intestinal and hepatic induction of expression or increased activity of enzymes involved in phospholipid biosynthesis has been reported in carnivorous fish fed VO³⁸ or

plant feedstuffs-based diets⁹³. However, the biosynthesis rate seems to be insufficient to avoid an accumulation of lipid droplets in the enterocytes^{94; 95}. This condition is usually associated to a phospholipid deficit that promotes an impairment in lipoprotein assembly and export from enterocytes and reduces phospholipid concentration in plasma^{7; 35; 36}.

In the present study, despite the lower plasma phospholipid concentration in VO groups, a nutritional regulation of transcript levels of GPAT was not observed in the intestine or the liver of gilthead sea bream fed the experimental diets. GPAT is involved in the first steps of glycerol-3-phosphate pathway, which according to Caballero *et al.*³⁸ is the main pathway implicated in intestinal phospholipid synthesis in gilthead sea bream. Thus, further investigation on the nutritional regulation of the enzymes involved in downstream steps of the phospholipids biosynthesis pathways is required to understand phospholipid biosynthesis regulation in gilthead sea bream.

Dietary lipid source is known to regulate lipogenesis and FA bioconversion pathways, which may affect tissue lipid deposition and FA composition. While in some studies increased hepatic lipogenic enzymes activity and lipid content have been reported in fish fed diets where dietary FO was replaced with VO^{6; 19}, in other studies such effects were not demonstrated^{7; 35; 36; 96}. In the present study dietary lipid source did not affect hepatic and muscular lipid content and, a decrease in whole-body lipid content was reported within CH-groups when FO was substituted by VO. On the other hand, liver and muscle FA composition was strongly influenced by dietary replacement of FO by VO, as previously reported in gilthead sea bream⁹⁷ and in other species^{7; 8; 29; 98}. According to the predicted effects of dietary oils in muscle FA composition of one-year-old gilthead sea bream with different nutritional backgrounds⁹⁷, in the present study liver and muscle lipids increased content of 18:1 n-9, 18:2 n-6 and 18:3 n-3, while total lipid reduced content of 20:5 n-3 and 22:6 n-3 when FO was replaced by VO. Thus, despite the hepatic up-regulation of FADS2 in fish fed the VO-based diets, the low proportion of LC-PUFA or their intermediary products such as 18:3 n-6 and 18:4 n-3 in VO groups suggest a limited FADS2 activity. Maximal FADS2 efficiency is thought to be modulated by the levels of substrate and product availability³⁰. In this sense, in the present study the limited accumulation of FA intermediates could partly have been caused by an inadequate dietary supply of substrate and/or product availability. Accordingly, in a previous study with this species using a diet completely devoid of LC-PUFA and containing olive oil as the sole lipid source, Seiliez *et al.*⁴⁷ observed higher transcript levels of FADS2 and increased accumulation of FA intermediates (18:2 n-9, 20:2 n-9 and 18:3 n-6) in fish

fed that diet comparatively to fish fed a LC-PUFA-rich diet, which suggested the existence of FADS2 activity.

In this study, the low efficacy in induction of FADS2 expression and of nutritional regulation of elovl5 transcripts by VO observed along with lack of $\Delta 5$ desaturase activity observed in vitro⁹⁹, may explain the low capacity of conversion of C18PUFA into LC-PUFA at appreciable rate in gilthead sea bream.

Dietary carbohydrate content and lipid source interaction

The interactions between dietary carbohydrate content and lipid source that were recorded on the whole-body, liver and plasma lipid content suggest that the overall effect of starch intake on lipid deposition was disturbed by the change in dietary lipid source. Indeed, an increase in lipid retention with dietary starch intake was more evident when fish were fed the VO based diet. This assumption is supported by the higher, whole-body and liver lipid content in VOCH+ group than in VOCH- group and can be related with the coupled increase in the lipogenic potential of dietary carbohydrates (higher liver FAS, ME and G6PD activities) and VO (higher liver G6PD activity). On the other hand, under a FO based diet regime, the unaltered lipid retention, liver and whole body lipid content, but elevated TAG levels following starch intake, suggest a higher lipid mobilization, transport and/or utilization in FOCH+ group than in FOCH- group. No molecular markers for lipid uptake were assessed in this study in liver and intestine and the one evaluated here and related to catabolism (CPT1A) do not help to understand or clarify this hypothesis because transcriptional regulation of these proteins by nutrients was not recorded.

At the same time, we also noticed that the stimulatory effect of dietary starch on muscle glycogen deposition was attenuated and became non-significant under the VO based dietary regime, probably related with the fact that dietary VO per se, but not in combination with carbohydrates, had an inductor effect on glycogenesis as suggested by the higher muscle and liver glycogen levels in VOCH- group than in FOCH- group.

Furthermore, it was observed that intake of starch when coupled with dietary VO seemed to enhance the hypocholesterolemic effects of dietary VO as plasma cholesterol concentration decreased with carbohydrate intake only when fish were fed VO based diet. It is important to note here that within the molecular actors involved in cholesterol related processes (CYP51A1, DHC7, ABCG5, LXRA) assessed in liver and intestine in the present study, no molecular difference induced by a nutritional interaction of nutrients

that could reflect this phenotype was found. Therefore, the physiological or metabolic mechanisms underlying this finding remain to be demonstrated.

Conclusion

To our knowledge, this is the first study in a marine fish species reporting a transcriptional induction of FADS2 by dietary carbohydrate, in addition to VO. Although the n-3 LC-PUFA biosynthesis pathway was insufficient to compensate for the reduced dietary EPA and DHA in VO-based diets, this study provides new perspectives on the use of nutritional strategies for inducing LC-PUFA biosynthesis in marine fish species.

Furthermore, change in dietary lipid source seemed to modify the overall effect of starch intake on mechanisms involved in cholesterol body pools, lipid and glycogen body allocation. Considering that these metabolites greatly influence the fish quality, the present findings highlight the critical need to assess the potential effects between dietary nutrients on metabolic related processes involved in tissue metabolites deposition, especially in the context of alternative aquafeeds rich in VO and carbohydrates.

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CC carried out the main experimental work and wrote the draft of the manuscript under the direction of the project designer and leaders AOT, GC and SP; AD assisted with the biochemical analyses; LL performed the fatty acid analyses; All authors contributed to and approved the manuscript. The authors declare that there are no conflicts of interest.

References

1. Tocher DR (2015) Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture* **449**, 94-107.
2. FAO (2014) *The State of World Fisheries and Aquaculture 2014*. Rome: Food and Agriculture Organization of the United Nations.
3. Enes P, Panserat S, Kaushik S et al. (2009) Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiol Biochem* **35**, 519-539.
4. Gatlin DM, Barrows FT, Brown P et al. (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquacult Res* **38**, 551-579.
5. Stone DAJ (2003) Dietary carbohydrate utilization by fish. *Rev Fish Sci* **11**, 337-369.
6. Betancor M, Sprague M, Usher S et al. (2015) A nutritionally-enhanced oil from transgenic *Camelina sativa* effectively replaces fish oil as a source of eicosapentaenoic acid for fish. *Scientific reports* **5**.
7. Castro C, Corraze G, Pérez-Jiménez A et al. (2015) Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Br J Nutr* **114**, 1143-1156.
8. Jordal AEO, Lie O & Torstensen BE (2007) Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). *Aquacult Nutr* **13**, 114-130.
9. Kjaer MA, Vegusdal A, Gjoen T et al. (2008) Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochim Biophys Acta* **1781**, 112-122.
10. Leaver MJ, Villeneuve LA, Obach A et al. (2008) Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* **9**, 299.
11. Morais S, Edvardsen RB, Tocher DR et al. (2012) Transcriptomic analyses of intestinal gene expression of juvenile Atlantic cod (*Gadus morhua*) fed diets with Camelina oil as replacement for fish oil. *Comp Biochem Physiol B* **161**, 283-293.
12. Brauge C, Medale F & Corraze G (1994) Effect of Dietary Carbohydrate Levels on Growth, Body Composition and Glycaemia in Rainbow Trout, *Oncorhynchus-mykiss*, Reared in Seawater. *Aquaculture* **123**, 109-120.
13. Sheridan MA (1988) Lipid dynamics in fish: aspects of absorption, transportation and mobilization. *Comp Biochem Physiol B* **90**, 679-690.

14. Tocher DR (2003) Metabolism and functions of lipids and fatty acids in teleost fish. *Rev Fish Sci* **11**, 107-184.
15. Menoyo D, Izquierdo MS, Robaina L *et al.* (2004) Adaptation of lipid metabolism, tissue composition and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soyabean oils. *Br J Nutr* **92**, 41-52.
16. Panserat S, Kolditz C, Richard N *et al.* (2008) Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. *Br J Nutr* **100**, 953-967.
17. Morais S, Pratoomyot J, Taggart JB *et al.* (2011) Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genomics* **12**, 255.
18. Morais S, Silva T, Cordeiro O *et al.* (2012) Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (*Salmo salar*). *BMC Genomics* **13**, 448.
19. Peng M, Xu W, Mai K *et al.* (2014) Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. *Aquaculture* **433**, 442-449.
20. Liang XF, Ogata HY & Oku H (2002) Effect of dietary fatty acids on lipoprotein lipase gene expression in the liver and visceral adipose tissue of fed and starved red sea bream *Pagrus major*. *Comp Biochem Physiol A* **132**, 913-919.
21. Stubhaug I, Froyland L & Torstensen BE (2005) beta-oxidation capacity of red and white muscle and liver in Atlantic salmon (*Salmo salar* L.) - Effects of increasing dietary rapeseed oil and olive oil to replace capelin oil. *Lipids* **40**, 39-47.
22. Jordal AEO, Torstensen BE, Tsoi S *et al.* (2005) Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). *J Nutr* **135**, 2355-2361.
23. Turchini GM, Mentasti T, Froyland L *et al.* (2003) Effects of alternative dietary lipid sources on performance, tissue chemical composition, mitochondrial fatty acid oxidation capabilities and sensory characteristics in brown trout (*Salmo trutta* L.). *Aquaculture* **225**, 251-267.
24. Kamalam BS, Médale F, Kaushik S *et al.* (2012) Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J Exp Biol* **215**, 2567-2578.

25. Kamalam BS, Médale F, Larroquet L *et al.* (2013) Metabolism and Fatty Acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. *PLoS One* **8**, e76570.
26. Jin J, Médale F, Kamalam BS *et al.* (2014) Comparison of Glucose and Lipid Metabolic Gene Expressions between Fat and Lean Lines of Rainbow Trout after a Glucose Load. *PLoS ONE* **9**, e105548.
27. Seiliez I, Panserat S, Kaushik S *et al.* (2001) Cloning, tissue distribution and nutritional regulation of a Delta 6-desaturase-like enzyme in rainbow trout. *Comp Biochem Physiol B* **130**, 83-93.
28. González-Rovira A, Mourente G, Zheng XZ *et al.* (2009) Molecular and functional characterization and expression analysis of a Delta 6 fatty acyl desaturase cDNA of European Sea Bass (*Dicentrarchus labrax* L.). *Aquaculture* **298**, 90-100.
29. Tocher DR, Zheng X, Schlechtriem C *et al.* (2006) Highly Unsaturated Fatty Acid Synthesis in Marine Fish: Cloning, Functional Characterization, and Nutritional Regulation of Fatty Acyl Δ6 Desaturase of Atlantic Cod (*Gadus morhua* L.). *Lipids* **41**, 1003-1016.
30. Vagner M & Santigosa E (2011) Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: A review. *Aquaculture* **315**, 131-143.
31. Montero D & Izquierdo M (2010) Welfare and Health of Fish Fed Vegetable Oils as Alternative Lipid Sources to Fish Oil. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, pp. 439-485: CRC Press.
32. Torstensen B & Tocher D (2010) The Effects of Fish Oil Replacement on Lipid Metabolism of Fish. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, pp. 405-437 [GM Turchini, WK Ng and DR Tocher, editors]: CRC Press.
33. NRC (2011) *Nutrient Requirements of Fish and Shrimp*: The National Academies Press.
34. Tocher DR, Bendiksen EA, Campbell PJ *et al.* (2008) The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* **280**, 21-34.
35. Richard N, Kaushik S, Larroquet L *et al.* (2006) Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). *Br J Nutr* **96**, 299-309.
36. Richard N, Mourente G, Kaushik S *et al.* (2006) Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture* **261**, 1077-1087.

37. Morais S, Pratoomyot J, Torstensen BE *et al.* (2011) Diet x genotype interactions in hepatic cholesterol and lipoprotein metabolism in Atlantic salmon (*Salmo salar*) in response to replacement of dietary fish oil with vegetable oil. *Br J Nutr* **106**, 1457-1469.
38. Caballero MJ, Gallardo G, Robaina L *et al.* (2006) Vegetable lipid sources affect in vitro biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*). *Br J Nutr* **95**, 448-454.
39. Liland NS, Espe M, Rosenlund G *et al.* (2013) High levels of dietary phytosterols affect lipid metabolism and increase liver and plasma TAG in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **110**, 1958-1967.
40. Agaba MK, Tocher DR, Zheng X *et al.* (2005) Cloning and functional characterisation of polyunsaturated fatty acid elongases of marine and freshwater teleost fish. *Comp Biochem Physiol B* **142**, 342-352.
41. Benedito-Palos L, Ballester-Lozano G & Perez-Sanchez J (2014) Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene* **547**, 34-42.
42. Benedito-Palos L, Calduch-Giner JA, Ballester-Lozano GF *et al.* (2013) Effect of ration size on fillet fatty acid composition, phospholipid allostasis and mRNA expression patterns of lipid regulatory genes in gilthead sea bream (*Sparus aurata*). *Br J Nutr* **109**, 1175-1187.
43. Calduch-Giner JA, Davey G, Saera-Vila A *et al.* (2010) Use of microarray technology to assess the time course of liver stress response after confinement exposure in gilthead sea bream (*Sparus aurata* L.). *BMC Genomics* **11**, 193.
44. Mininni AN, Milan M, Ferrarese S *et al.* (2014) Liver transcriptome analysis in gilthead sea bream upon exposure to low temperature. *BMC Genomics* **15**, 1-12.
45. Pérez-Sánchez J, Borrel M, Bermejo-Nogales A *et al.* (2013) Dietary oils mediate cortisol kinetics and the hepatic mRNA expression profile of stress-responsive genes in gilthead sea bream (*Sparus aurata*) exposed to crowding stress. Implications on energy homeostasis and stress susceptibility. *Comp Biochem Physiol D* **8**, 123-130.
46. Sánchez-Gurmaches J, Cruz-Garcia L, Ibarz A *et al.* (2013) Insulin, IGF-I, and muscle MAPK pathway responses after sustained exercise and their contribution to growth and lipid metabolism regulation in gilthead sea bream. *Domest Anim Endocrinol* **45**, 145-153.
47. Seiliez I, Panserat S, Corraze G *et al.* (2003) Cloning and nutritional regulation of a Delta 6-desaturase-like enzyme in the marine teleost gilthead seabream (*Sparus aurata*). *Comp Biochem Physiol B* **135**, 449-460.

48. Castro C, Corraze G, Panserat S *et al.* (2015) Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquacult Nutr* **21**, 592-603.
49. Librán-Pérez M, Figueiredo-Silva AC, Panserat S *et al.* (2013) Response of hepatic lipid and glucose metabolism to a mixture or single fatty acids: Possible presence of fatty acid-sensing mechanisms. *Comp Biochem Physiol A* **164**, 241-248.
50. Menoyo D, Diez A, Lopez-Bote CJ *et al.* (2006) Dietary fat type affects lipid metabolism in Atlantic salmon (*Salmo salar* L.) and differentially regulates glucose transporter GLUT4 expression in muscle. *Aquaculture* **261**, 294-304.
51. Oliva-Teles A (2000) Recent advances in European sea bass and gilthead sea bream nutrition. *Aquacult Int* **8**, 477-492.
52. AOAC (2012) *Official Methods of Analysis of Association of Official Analytical Chemists INTERNATIONAL*: AOAC International.
53. Beutler HO (1984) Starch. In *Methods of Enzymatic Analysis*, pp. 2-10 [HU Bergmeyer, editor]. Basel: Verlag Chemie Weinheim.
54. Roehrig KL & Allred JB (1974) Direct enzymatic procedure for the determination of liver glycogen. *Anal Biochem* **58**, 414-421.
55. Folch J, Lees M & Stanley GHS (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497-509.
56. Shantha N & Ackman R (1990) Nervonic acid versus tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n—3 polyunsaturated fatty acid methyl esters. *J Chromatogr B Biomed Sci Appl* **533**, 1-10.
57. C. ST (1957) Determination of cholesterol and ergosterol by Liebermann-Burchard reaction. *Methods Enzymol* **3**, 362-365.
58. Rozen S & Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365-386.
59. Enes P, Panserat S, Kaushik S *et al.* (2008) Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (*Sparus aurata*) juveniles reared at two temperatures. *Comp Biochem Physiol A* **149**, 80-86.
60. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.
61. Couto A, Enes P, Peres H *et al.* (2008) Effect of water temperature and dietary starch on growth and metabolic utilization of diets in gilthead sea bream (*Sparus aurata*) juveniles. *Comp Biochem Physiol A* **151**, 45-50.

62. Enes P, Panserat S, Kaushik S *et al.* (2008) Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. *Aquaculture* **274**, 101-108.
63. Peres H, Gonçalves P & Oliva-Teles A (1999) Glucose tolerance in gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*). *Aquaculture* **179**, 415-423.
64. Engelking L (2010) Overview of Carbohydrate Metabolism. In *Textbook of Veterinary Physiological Chemistry*, updated 2nd ed ed., pp. 120–124 [L Engelking, editor]. Amsterdam Netherlands: Academic Press (Elsevier).
65. Panserat S, Medale F, Blin C *et al.* (2000) Hepatic glucokinase is induced by dietary carbohydrates in rainbow trout, gilthead seabream, and common carp. *Am J Physiol Regul Integr Comp Physiol* **278**, R1164-R1170.
66. Panserat S, Plagnes-Juan E & Kaushik S (2002) Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). *Biochim Biophys Acta* **1579**, 35-42.
67. Fernández F, Miquel AG, Córdoba M *et al.* (2007) Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead sea bream (*Sparus aurata*, L.) fingerlings. *J Exp Mar Biol Ecol* **343**, 1-10.
68. Kamalam BS, Panserat S, Aguirre P *et al.* (2013) Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp Biochem Physiol A* **164**, 417-427.
69. Polakof S, Álvarez R & Soengas JL (2010) Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity. *Am J Physiol Regul Integr Comp Physiol* **299**, R19-R32.
70. Kortner TM, Björkhem I, Krasnov A *et al.* (2014) Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **111**, 2089-2103.
71. Álvarez MJ, Lopez-Bote CJ, Diez A *et al.* (1998) Dietary fish oil and digestible protein modify susceptibility to lipid peroxidation in the muscle of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*). *Br J Nutr* **80**, 281-289.
72. Viana Abrantes M, Esteves de Oliveira FC & Bressan J (2011) Peroxisome proliferator-activated receptor: effects on nutritional homeostasis, obesity and diabetes mellitus. *Nutr Hosp* **26**, 271-279.

73. Leaver MJ, Bautista JM, Bjornsson BT *et al.* (2008) Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture. *Rev Fish Sci* **16**, 73-94.
74. Diez A, Menoyo D, Perez-Benavente S *et al.* (2007) Conjugated linoleic acid affects lipid composition, metabolism, and gene expression in Gilthead sea bream (*Sparus aurata* L.). *J Nutr* **137**, 1363-1369.
75. Du ZY, Demizieux L, Degrace P *et al.* (2004) Alteration of 20:5n-3 and 22:6n-3 fat contents and liver peroxisomal activities in fenofibrate-treated rainbow trout. *Lipids* **39**, 849-855.
76. Kennedy SR, Leavera MJ, Campbell PJ *et al.* (2006) Influence of dietary oil content and conjugated linoleic acid (CLA) on lipid metabolism enzyme activities and gene expression in tissues of Atlantic salmon (*Salmo salar* L.). *Lipids* **41**, 423-436.
77. Bonacic K, Estevez A, Bellot O *et al.* (2016) Dietary Fatty Acid Metabolism is Affected More by Lipid Level than Source in Senegalese Sole Juveniles: Interactions for Optimal Dietary Formulation. *Lipids* **51**, 105-122.
78. Zheng JL, Luo Z, Zhuo MQ *et al.* (2014) Dietary L-carnitine supplementation increases lipid deposition in the liver and muscle of yellow catfish (*Pelteobagrus fulvidraco*) through changes in lipid metabolism. *Br J Nutr* **112**, 698-708.
79. Dai W, Panserat S, Plagnes-Juan E *et al.* (2015) Amino Acids Attenuate Insulin Action on Gluconeogenesis and Promote Fatty Acid Biosynthesis via mTORC1 Signaling Pathway in trout Hepatocytes. *Cell Physiol Biochem* **36**, 1084-1100.
80. Ekmann KS, Dalsgaard J, Holm J *et al.* (2013) Effects of dietary energy density and digestible protein:energy ratio on de novo lipid synthesis from dietary protein in gilthead sea bream (*Sparus aurata*) quantified with stable isotopes. *Br J Nutr* **110**, 1771-1781.
81. Kirchner S, Kaushik S & Panserat S (2003) Low protein intake is associated with reduced hepatic gluconeogenic enzyme expression in rainbow trout (*Oncorhynchus mykiss*). *J Nutr* **133**, 2561-2564.
82. Lansard M, Panserat S, Plagnes-Juan E *et al.* (2010) Integration of insulin and amino acid signals that regulate hepatic metabolism-related gene expression in rainbow trout: role of TOR. *Amino Acids* **39**, 801-810.
83. Izquierdo MS, Montero D, Robaina L *et al.* (2005) Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long terin period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture* **250**, 431-444.
84. Montero D, Mathlouthi F, Tort L *et al.* (2010) Replacement of dietary fish oil by vegetable oils affects humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream *Sparus aurata*. *Fish Shellfish Immunol* **29**, 1073-1081.

85. Benedito-Palos L, Saera-Vila A, Calduch-Giner JA *et al.* (2007) Combined replacement of fish meal and oil in practical diets for fast growing juveniles of gilthead sea bream (*Sparus aurata* L.): Networking of systemic and local components of GH/IGF axis. *Aquaculture* **267**, 199-212.
86. Fountoulaki E, Vasilaki A, Hurtado R *et al.* (2009) Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: Recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. *Aquaculture* **289**, 317-326.
87. Brufau G, Canela MA & Rafecas M (2008) Phytosterols: physiologic and metabolic aspects related to cholesterol-lowering properties. *Nutr Res* **28**, 217-225.
88. MacKay DS & Jones PJH (2011) Phytosterols in human nutrition: Type, formulation, delivery, and physiological function. *Eur J Lipid Sci Tech* **113**, 1427-1432.
89. Gilman CI, Leusch FD, Breckenridge WC *et al.* (2003) Effects of a phytosterol mixture on male fish plasma lipoprotein fractions and testis P450scc activity. *Gen Comp Endocrinol* **130**, 172-184.
90. Ostlund RE, Jr. (2002) Phytosterols in human nutrition. *Annu Rev Nutr* **22**, 533-549.
91. Kortner TM, Gu J, Krogdahl A *et al.* (2013) Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **109**, 593-604.
92. Gu M, Kortner TM, Penn M *et al.* (2014) Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **111**, 432-444.
93. Geay F, Ferrarese S, Zambonino-Infante JL *et al.* (2011) Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. *BMC Genomics* **12**, 522.
94. Caballero MJ, Izquierdo MS, Kjorsvik E *et al.* (2003) Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. *Aquaculture* **225**, 325-340.
95. Olsen RE, Dragnes BT, Myklebust R *et al.* (2003) Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *Oncorhynchus mykiss* Walbaum. *Fish Physiol Biochem* **29**, 181-192.
96. Torstensen BE, Froyland L & Lie O (2004) Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil - effects on Atlantic salmon (*Salmo salar* L.) tissue

- and lipoprotein lipid composition and lipogenic enzyme activities. *Aquacult Nutr* **10**, 175-192.
97. Benedito-Palos L, Bermejo-Nogales A, Karampatos AI *et al.* (2011) Modelling the predictable effects of dietary lipid sources on the fillet fatty acid composition of one-year-old gilthead sea bream (*Sparus aurata* L.). *Food Chem* **124**, 538-544.
98. Benitez-Dorta V, Caballero MJ, Izquierdo M *et al.* (2013) Total substitution of fish oil by vegetable oils in Senegalese sole (*Solea senegalensis*) diets: effects on fish performance, biochemical composition, and expression of some glucocorticoid receptor-related genes. *Fish Physiol Biochem* **39**, 335-349.
99. Tocher DR & Ghioni C (1999) Fatty acid metabolism in marine fish: Low activity of fatty acyl Delta 5 desaturation in gilthead sea bream (*Sparus aurata*) cells. *Lipids* **34**, 433-440.

Chapter 7-

Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles

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Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles

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Abstract

This study aimed to evaluate the effects of dietary lipid source and carbohydrate content on the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. For that purpose, four diets were formulated with fish oil (FO) and vegetable oils (VO) as the lipid source and with 20 or 0 % gelatinised starch as the carbohydrate source, in a 2 × 2 factorial design. Liver and intestine antioxidant enzyme activities (catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD)), hepatic and intestinal lipid peroxidation (LPO), as well as hepatic oxidative stress index (OSI), were measured in fish fed the experimental diets for 73 d (n 9 fish/diet). Carbohydrate-rich diets promoted a decrease in hepatic LPO and OSI, whereas the lipid source induced no changes. Inversely, dietary lipid source, but not dietary carbohydrate concentration, affected LPO in the intestine. Lower intestinal LPO was observed in VO groups. Enzymes responsive to dietary treatments were GR, G6PD and CAT in the liver and GR and GPX in the intestine. Dietary carbohydrate induced GR and G6PD activities and depressed CAT activity in the liver. GPX and GR activities were increased in the intestine of fish fed VO diets. Overall, effects of diet composition on oxidative status were tissue-related: the liver and intestine were strongly responsive to dietary carbohydrates and lipid sources, respectively. Furthermore, different metabolic routes were more active to deal with the oxidative stress in the two organs studied.

Key words: Antioxidant enzymes; Feedstuffs; Lipid oxidative damage; Carbohydrates; Vegetable oils

Substitution of fishmeal (FM) and fish oil (FO) in aquafeeds by more readily available and sustainable alternative ingredients such as plant feedstuffs (PF) has become a priority issue in aquaculture⁽¹⁾. However, dietary replacement of fishery by-products by PF may have negative health-related effects, especially in carnivorous species. For instance, vegetable oils (VO) were reported to induce alterations of the immune response in different fish species^(2–7), change the fatty acid profile of immune cells or modify eicosanoid production^(2,5,6,8). Inflammation and pathological morphological modifications in the intestine and steatosis in the liver were also reported in several species fed VO-rich diets^(9–13). Inadequate dietary nutrient balance, high fibre or carbohydrate content and the presence of anti-nutritional factors in PF are possible causes for such effects^(14,15).

Oxidative stress occurs when balance between the generation of reactive oxygen species (ROS) exceeds that of ROS removal, thus inducing oxidative damage. ROS are produced at

a controlled rate during normal cell metabolism⁽¹⁶⁾. However, under stressful conditions such as nutritional alterations, ROS may dramatically increase and induce oxidative stress. Accordingly, variations in diet macronutrient sources^(7,17–25), dietary protein:non-protein ratios^(26–29) or non-protein energy sources⁽³⁰⁾ have been linked to modifications of fish susceptibility to lipid peroxidation (LPO).

The knowledge about the effects of macronutrients on oxidative stress is still limited and the available data are contradictory. Regarding the effect of dietary lipid source, VO were reported to either decrease^(7,18,20,23,24), increase⁽²²⁾ or promote no effects⁽³¹⁾ in fish hepatic and/or muscular LPO susceptibility. High dietary protein and/or lipid concentrations were observed to increase muscular LPO susceptibility^(26,28), whereas dietary carbohydrates decreased LPO in muscles and the liver^(27,28,30).

To prevent oxidative damage, organisms have evolved effective antioxidant systems, consisting of both non-enzymatic (GSH, vitamins and carotenoids, among others) and enzymatic

Abbreviations: CAT, catalase; FO, fish oil; G6PD, glucose-6-phosphate dehydrogenase; GPX, glutathione peroxidase; GR, glutathione reductase; LPO, lipid peroxidation; OSI, oxidative stress index; SOD, superoxide dismutase; VO, vegetable oil.

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mechanisms. Enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GR) play key roles in the antioxidant defence mechanism^(32,33). Available literature suggests that dietary lipid source has no effect on the liver and muscle activities of SOD, CAT and/or GPX^(22,31,34) or decreased SOD activity in VO-rich diets⁽³⁵⁾ in a number of fish species.

Concerning macronutrient modifications, it was reported that in common dentex (*Dentex dentex*) high dietary lipid concentrations induced an increase in hepatic SOD activity at high dietary protein concentrations, and of GPX, independent of dietary protein concentrations⁽²⁸⁾. The authors also observed an interaction between dietary protein and carbohydrate or lipid concentrations in muscular GR and hepatic SOD and CAT activities. In yellow catfish (*Pelteobagrus fulvidraco*), a decrease in CAT and GPX activities as dietary carbohydrate:lipid ratio increased was also observed⁽³⁰⁾. In contrast, partial substitution of dietary proteins with carbohydrates induced no major alterations in antioxidant enzyme activities in Senegalese sole (*Solea senegalensis*) liver⁽²⁹⁾.

Overall, current knowledge about the effects of alternative feed ingredients, which may be rich in carbohydrates or VO, on the oxidative status of animals is still very scarce. Thus, in this study, we evaluated the effect of the dietary lipid source and carbohydrate manipulation on oxidative defence mechanisms in the liver and intestine of European sea bass (*Dicentrarchus labrax*) juveniles.

Methods

Experimental diets

Four diets differing in carbohydrate content (0 and 20% gelatinised starch, CH- and CH+, respectively) and lipid source (fish oil (FO) or a VO blend, FO and VO, respectively) were formulated (Table 1). The dietary carbohydrate content was increased at the expense of dietary protein. The VO blend was composed of 20% rapeseed, 50% linseed and 30% palm oils and replaced approximately 70% of dietary lipids, which were provided by cod liver oil and FM. All the ingredients were finely ground, well-mixed and dry-extruded in a laboratory pellet mill (California Pellet Mill), through a 3-mm die. The pellets were air-dried for 24 h and stored in a refrigerator (4°C) until used.

Animals, experimental conditions and sampling

This experiment was directed by accredited scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was performed at the Marine Zoological Station, University of Porto, Portugal, in a thermo-regulated recirculation water system, equipped with twelve fibre-glass cylindrical tanks of 300 litre water capacity, and supplied with continuous flow of filtered seawater.

After 2 weeks of adaptation to the experimental conditions, twelve groups of twenty European sea bass juveniles (initial body weight 74.0 (sd 1.5) g) were established and randomly

Table 1. Ingredients and chemical composition of the experimental diets

Lipid source...	Experimental diets			
	FO	VO	CH-	CH+
Carbohydrates...	CH-	CH+	CH-	CH+
Ingredients (% dry weight)				
Fishmeal*	86.5	64.5	86.5	64.5
Gelatinised maize starch†	0	20	0	20
Cod liver oil‡	10	12	0	0
Vegetable oil blend§	0	0	10	12
Vitamins	1.5	1.5	1.5	1.5
Minerals¶	1.0	1.0	1.0	1.0
Binder**	1.0	1.0	1.0	1.0
Proximate analyses (% DM)				
DM	89.5	90.4	91.3	91.0
Crude protein (CP)	62.4	46.6	62.4	47.1
Crude lipid (CL)	18.4	18.4	18.2	18.3
Gross energy (kJ/g)	26.2	25.8	26.7	25.6
Starch	0.8	18.7	0.8	17.5
Ash	17.6	14.0	17.6	13.8
Cholesterol	0.5	0.4	0.4	0.3

FO, fish oil; VO, blend of vegetable oils; carbohydrate content, 0% (CH-) or 20% (CH+) gelatinised maize starch.

* Steam-dried low-temperature fishmeal; Pesquera Diamante (CP: 71.1% DM; CL: 8.8% DM).

† C-Gel Instant-12018; Cerestar.

‡ Labchem, Laborspirit Lda.

§ 30% Palm oil (Colmi), 50% linseed oil (Sociedade Portuense de Drogas) and 20% rapeseed oil (Huilerie Emile Noël SAS).

|| Vitamins (mg/kg diet): retinyl acetate, 18 000 IU (6.19 mg)/kg diet; cholecalciferol, 2000 IU (0.04 mg)/kg diet; α-tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400 (Premix).

¶ Minerals (mg/kg diet): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodiumselenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g/kg diet); potassium chloride, 1.15 (g/kg diet); sodium chloride, 0.40 (g/kg diet) (Premix).

** Aquacube (Guar gum, polymethyl carbamide, Manioc starch blend, hydrate calcium sulphate) (Agil).

distributed into the tanks. The experimental diets were randomly assigned to triplicate groups of fish (*n* 3 tanks/diet). During the trial, salinity averaged 35 (sd 1.0) g/l, dissolved oxygen was kept near saturation and water temperature was regulated to 25.4 (sd 0.5) °C. The photoperiod was the natural one for June–August. The trial lasted 73 d, and during that period fish were hand-fed twice a day, 6 d/week, to apparent visual satiety. Food intake and mortality were recorded daily, and fish in each tank were bulk-weighted at the end of the trial, after 1 d of feed deprivation to empty gut contents. To eliminate handling stress, the fish were continued to be fed for one more week, and subsequently 18 h after the last meal (previous day afternoon meal) three fish per tank (*n* 9 fish/diet) were killed with a sharp blow to the head; the liver and intestines were removed, immediately frozen in liquid N₂ and stored at -80°C until enzymatic analyses.

Diet analysis

Chemical analyses of the experimental diets were carried out according to Association of Official Analytical Chemists⁽³⁶⁾ as follows: in brief, DM after drying at 105°C until constant weight;

ash by incineration in a muffle furnace at 450°C for 16 h; protein content ($N \times 6.25$) by the Kjeldahl method after acid digestion using a Kjeltec digestion and distillation units (models 1015 and 1026, respectively; Tecator Systems); starch content according to Beutler⁽³⁷⁾; gross energy by direct combustion in an adiabatic bomb calorimeter (Parr model 6200; Parr Instruments); and lipid content by petroleum diethyl ether extraction (Soxtec HT System; Höganäs). Fatty acid methyl esters were prepared by acid-catalysed transmethylation of total lipids using boron trifluoride in methanol (14%) according to Santha & Ackman⁽³⁸⁾ and were analysed by GC (Varian 3900; Varian) as described by Castro *et al.*⁽³⁹⁾.

Enzymatic activity assays

Liver and whole intestine samples were homogenised (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0.1 mM-EDTA and 0.1% triton X-100 (v/v), pH 7.8). All the procedures were performed on ice. Homogenates were centrifuged at 30 000 **g** for 30 min at 4°C. After centrifugation, the resultant supernatant was collected, and the aliquots were stored at -80°C until analysis.

All enzyme assays were carried out at 37°C. Changes in absorbance were monitored using a microplate reader (ELx808; BioTek Instruments) to determine enzyme activities. The optimal substrate and protein concentrations for maximal activity measurement of each enzyme were established by preliminary assays. The molar extinction coefficients used for H₂O₂ and NADPH were 0.039 and 6.22 $\text{mm}^{-1} \times \text{cm}^{-1}$, respectively.

The specific assay conditions for each enzyme were as follows.

SOD (*EC* 1.15.1.1) activity was measured at 550 nm by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals⁽⁴⁰⁾. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.8), 0.1 mM-EDTA, 0.1 mM-xanthine, 0.012 mM-cytochrome C and 0.025 IU/ml xanthine oxidase. Activity was reported as units per mg of protein. One unit of activity was defined as the amount of enzyme necessary to produce 50% inhibition of ferricytochrome C reduction rate.

CAT (*EC* 1.11.1.6) activity was determined according to Aebi⁽⁴¹⁾ by measuring the decrease in H₂O₂ concentration at 240 nm. The reaction mixture contained 50 mM-potassium phosphate buffer (pH 7) and 10 mM-H₂O₂ freshly added.

GPX (*EC* 1.11.1.9) activity was assayed as described by Flohé & Günzler⁽⁴²⁾. The GSSG generated by GPX was reduced by GR, and NADPH consumption rate was monitored at 340 nm. The reaction mixture consisted of 50 mM-potassium phosphate buffer (pH 7.1), 1 mM-EDTA, 3.9 mM-GSH, 3.9 mM-sodium azide, 1 IU/ml GR, 0.2 mM-NADPH and 0.05 mM-H₂O₂.

GR (*EC* 1.6.4.2) activity was determined at 340 nm by measuring the oxidation of NADPH as described by Morales *et al.*⁽⁴³⁾. The reaction mixture consisted of 0.1 M-sodium phosphate buffer (pH 7.5), 1 mM-EDTA, 0.63 mM-NADPH and 0.16 mM-GSSG.

Glucose-6-phosphate dehydrogenase (G6PD; *EC* 1.1.1.49) activity was assayed by measuring the reduction of NADP⁺ at 340 nm as previously described by Morales *et al.*⁽⁴⁴⁾, using a reaction mixture containing 50 mM-imidazole-HCl buffer (pH 7.4), 5 mM-MgCl₂, 2 mM-NADP and 1 mM-glucose-6-phosphate.

Except for SOD, whose units of expression were described above, all other enzyme activities were expressed as units (CAT) or milliunits (G6PD, GPX and GR) per mg of soluble protein. One unit of enzyme activity was defined as the amount of enzyme required to transform 1 μmol of substrate/min under the above assay conditions. Protein concentration was determined according to Bradford⁽⁴⁵⁾ using a protein assay kit (Sigma) and bovine serum albumin as the standard.

Total glutathione, GSH and oxidative stress index

A portion of liver was homogenised in nine volumes of ice-cold solution containing 1.3% 5-sulphosalicylic acid (w/v) and 10 mM-HCl. The procedure was carefully performed always on ice in order to avoid the GSH. Homogenates were centrifuged at 14 000 **g** for 10 min at 4°C, and the resulting supernatants were immediately analysed.

Total glutathione (tGSH) and GSSG were measured following the method described by Griffith⁽⁴⁶⁾ and Vandepitte *et al.*⁽⁴⁷⁾ with modifications. Both tGSH and GSSG analyses were carried out at 37°C, and the changes in absorbance as a consequence of the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were monitored at 405 nm using a microplate reader (ELx808; BioTek Instruments). The optimal substrate and protein concentrations for the measurement of maximal activity for each enzyme were established by preliminary assays. The molar extinction coefficient used for DTNB was 13.6 $\text{mm}^{-1} \times \text{cm}^{-1}$.

tGSH was determined using a reaction mixture containing 133 mM-phosphate buffer with 5.8 mM-EDTA at pH 7.4, 0.71 mM-DTNB, 0.24 mM-NADPH and 1.2 IU/ml GR.

GSSG was measured using an aliquot from the solution obtained after 60 min of incubation of 100 μl of the sample with 2 μl vinylpyridine and 6 μl 1.5 M-triethanolamine. The reaction mixture contained 122 mM-phosphate buffer with 5.4 mM-EDTA at pH 7.4, 0.71 mM-DTNB, 0.24 mM-NADPH and 1.2 IU/ml GR.

The results were calculated using the standard curves of GSH and GSSG for tGSH and GSSG measurements, respectively. GSH level was calculated by subtracting GSSG from tGSH values. The results are expressed as nmol per g of tissue.

Oxidative stress index (OSI) was calculated as follows:

$$\text{OSI} (\%) = 100 \times (2 \times \text{GSSG/tGSH}).$$

Lipid peroxidation

Concentration of malondialdehyde (MDA) was determined as a marker for LPO following the methodology described by Buege & Aust⁽⁴⁸⁾. An aliquot of supernatant from the homogenate (100 μl) was mixed with 500 μl of a previously prepared solution containing 15% (w/v) TCA, 0.375% (w/v) thiobarbituric acid (TBA), 80% (v/v) HCl 0.25 N and 0.01% (w/v) butylated hydroxytoluene. The mixture was heated to 100°C for 15 min. After being cooled to room temperature and centrifuged at 1500 **g** for 10 min, the absorbance was measured at 535 nm in the supernatant. MDA concentration was expressed as nmol MDA per g of tissue, calculated from a calibration curve.

Statistical analysis

Data are presented as mean values and standard deviations. Data were checked for normality and homogeneity of variances

and when appropriate were normalised (log, inverse and $\arcsin(\sqrt{(x / 100)})$ transformations). Statistical evaluation of the data was carried out using a 2×2 factorial arrangement of treatments in a completely randomised experimental design (two-way ANOVA), with carbohydrate concentration and lipid source as fixed factors. The significance level of 0.05 was used for rejection of the null hypothesis. All the statistical analyses were carried out using SPSS 21.0 software package (IBM Corp.) for Windows.

Results

The diets presented small differences in the proportions of total SFA, which were slightly higher in the VO diets, and of MUFA, which were higher in the FO diets (Table 2). Within MUFA, higher concentrations of oleic acid (18 : 1n-9) were found in the VO diets, whereas the opposite occurred for palmitoleic acid (16 : 1n-7), eicosenoic acid (20 : 1n-9) and erucic acid (22 : 1n-9). Linoleic acid (LA, 18 : 2n-6), n-6 PUFA, was considerably higher in the VO diets. Regarding n-3 PUFA, VO diets were particularly rich in linolenic acid (LNA, 18 : 3n-3) and poor in EPA(20 : 5n-3) and DHA (22 : 6n-3).

Table 2. Fatty acid composition (% of total fatty acids) of the experimental diets

Lipid source...	Experimental diets			
	FO		VO	
Carbohydrates...	CH-	CH+	CH-	CH+
SFA				
14 : 0	5.2	5.0	2.7	2.0
15 : 0	0.4	0.4	0.2	0.2
16 : 0	14.5	13.6	17.8	17.2
17 : 0	0.3	0.3	0.3	0.2
18 : 0	2.9	2.7	3.7	3.5
20 : 0	0.2	0.1	0.3	0.3
Σ SFA	23.7	22.2	25.3	23.8
MUFA				
16 : 1n-7	7.5	7.5	3.3	2.4
18 : 1n-9	16.7	17.7	24.6	27.1
20 : 1n-9	5.9	7.0	0.5	0.5
22 : 1n-9	4.6	5.5	0.1	0.1
Σ MUFA	34.9	37.8	28.5	30.0
n-6 PUFA				
18 : 2n-6	1.5	1.7	9.2	10.8
18 : 3n-6	0.2	0.2	0.1	0.1
20 : 2n-6	0.3	0.3	0.1	0.1
20 : 4n-6	0.8	0.7	0.5	0.4
Σ n-6 PUFA	5.9	5.7	11.7	12.5
n-3 LC-PUFA				
18 : 3n-3	0.9	1.0	17.4	21.3
18 : 4n-3	2.3	2.4	0.7	0.5
20 : 4n-3	0.7	0.7	0.3	0.2
20 : 5n-3	10.9	10.5	5.6	4.2
21 : 5n-3	0.5	0.5	0.2	0.2
22 : 5n-3	1.6	1.5	0.8	0.6
22 : 6n-3	13.9	13.1	7.3	5.4
Σ n-3 LC-PUFA	30.8	29.7	32.4	32.4
SFA:PUFA	0.6	0.6	0.6	0.5
n 3: n 6	10.5	10.1	3.2	2.9

FO, fish oil, VO, blend of vegetable oils; carbohydrate content, 0% (CH-) or 20% (CH+) gelatinised maize starch; n-3 LC-PUFA, n-3 long-chain PUFA.

Results of the feeding trial were not the aim of this study and are presented elsewhere⁽⁴⁹⁾.

In brief, growth performance and feed efficiency were not affected by dietary treatments. Final body mass averaged 221.7 (sd 5.0) g, feed efficiency (wet weight gain(g)/dry feed intake (g)) averaged 0.8 (sd 0.03) and feed intake (g/kg average body mass per d) averaged 16.6 (sd 0.7). Liver fatty acid composition is summarised in Table 3. Accordingly, liver samples of fish fed VO-based diets were characterised by higher concentrations of LA (18 : 2n-6), γ -LNA (GLA; 18 : 3n-6) and LNA (18 : 3n-3) and by lower concentration of arachidonic acid (ARA; 20 : 4n-6), EPA (20 : 5n-3) and DHA (22 : 6n-3) as well as n-3 PUFA:n-6 PUFA ratio. Except for ARA concentrations, lower in groups fed the CH+ diets, no other differences were found for hepatic n-6 PUFA concentrations. Regarding n-3 PUFA, groups fed the CH+ diets presented similar LNA concentrations but lower EPA and DHA concentrations compared with those fed CH- diets. Unsaturation index was lower in fish fed the CH+ diets. On the other hand, differences between lipid sources on unsaturation index were only evident in the CH- group. During the trial, no mortality occurred.

Overall, both in the liver and in the intestines, there were no interactions between dietary carbohydrate and lipid source in the activities of oxidative stress enzymes or in LPO values. In the liver, dietary lipid source did not affect the activities of oxidative stress enzymes or LPO values, whereas dietary carbohydrates promoted a decrease in LPO values and in CAT activity and an increase in GR and G6PD activities (Table 4). Moreover, in the liver, SOD and GPX activities were not affected by dietary carbohydrates. In the intestine, the dietary carbohydrate level did not affect the activities of oxidative stress enzymes or LPO values, whereas dietary VO promoted a decrease in LPO and an increase in GPX and GR activities (Table 5). No effects on activities of CAT, SOD or G6PD in relation to diet composition were observed in the intestine.

In the liver, higher GSH content and lower GSSG levels and OSI were observed in the carbohydrate groups (Fig. 1), but no effects of dietary lipid source on glutathione (total, reduced and oxidised) or OSI were observed.

Discussion

In this study, low susceptibility to LPO was observed in the liver of fish fed carbohydrate-rich diets, which is in agreement with previous observations on muscles of this and other fish species^(27,28). The beneficial effect of dietary carbohydrates on lipid oxidative damage may be related to the nature of the glucose molecule, which can scavenge ROS by itself⁽⁵⁰⁾, or through increased activity of the pentose phosphate pathway^(27,28,50,51). The pentose phosphate pathway, via G6PD, generates reducing power in the form of NADPH, which is required for the activity of two H₂O₂-scavenging pathways involving CAT and GPX^(43,52). In fact, along with lower hepatic LPO levels, higher G6PD activity was observed in European sea bass fed carbohydrate-rich diets. Furthermore, dietary carbohydrates also promoted an increase in hepatic GR activity, an enzyme that also requires NADPH to regenerate reduced glutathione

Table 3. Liver fatty acid (FA) profile (% of total FA) of European sea bass fed the experimental diets
(Mean values and standard deviations; *n* 6 fish)

Lipid source (LS)...	Experimental diets										
	FO				VO				<i>P</i> *		
	CH-		CH+		CH-		CH+				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
FA											
<i>n</i> -6 PUFA											
18 : 2 <i>n</i> -6	1.3	0.5	0.9	0.3	3.5	0.9	3.1	0.8	0.153	<0.001	0.922
18 : 3 <i>n</i> -6	0.1	0.1	0.1	0.1	0.2	0.03	0.2	0.1	0.700	0.001	0.376
20 : 4 <i>n</i> -6	0.7	0.1	0.3	0.1	0.5	0.1	0.2	0.02	<0.001	<0.001	0.336
<i>n</i> -3 PUFA											
18 : 3 <i>n</i> -3	0.4	0.1	0.3	0.1	4.6	1.2	4.5	1.3	0.093	<0.001	0.131
20 : 5 <i>n</i> -3	5.4	0.9	2.9	0.6	2.6	0.6	1.4	0.2	<0.001	<0.001	0.054
22 : 6 <i>n</i> -3	9.1	2.6	3.6	0.9	3.8	1.3	1.8	0.4	<0.001	<0.001	0.775
Ratios											
<i>n</i> -3: <i>n</i> -6	7.5	1.2	5.4	0.6	2.7	0.2	2.3	0.1	<0.001	<0.001	0.482
Unsaturation index	146	20	92	11	109	15	91	9	<0.001	0.004	0.008

FO, fish oil; VO, blend of vegetable oils; CH content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch.

* Significant differences at *P* < 0.05 (two-way ANOVA).

Table 4. Specific activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein) and lipid peroxidation (LPO) (nmol malondialdehyde per g tissue) in the liver of European sea bass fed the experimental diets
(Mean values and standard deviations; *n* 9 fish)

Lipid source (LS)...	Experimental diets										
	FO				VO				<i>P</i> *		
	CH-		CH		CH-		CH+				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
G6PD											
G6PD	269	44	539	154	295	34	572	56	<0.001	0.089	0.724
GPX	57	15	52	15	56	21	49	20	0.342	0.703	0.894
GR	7.6	2.2	9.5	2.9	7.6	3.0	9.1	1.4	0.044	0.832	0.778
CAT	618	90	518	68	620	78	552	56	0.002	0.474	0.520
SOD	232	29	220	58	220	40	178	45	0.071	0.080	0.325
LPO	11	1.0	9.0	1.6	12	2	8.7	0.7	<0.001	0.591	0.232

FO, fish oil; VO, blend of vegetable oils; CH content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch.

* Significant differences at *P* < 0.05 (two-way ANOVA).

Table 5. Specific activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), superoxide dismutase (SOD), catalase (CAT) (U/mg protein) and lipid peroxidation (LPO) (nmol malondialdehyde per g tissue) in the intestine of European sea bass fed the experimental diets
(Mean values and standard deviations; *n* 9 fish)

Lipid source (LS)...	Experimental diets										
	FO				VO				<i>P</i> *		
	CH-		CH+		CH-		CH+				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	CH	LS	CH × LS
G6PD											
G6PD	16	13	27	13	19	20	22	18	0.258	0.857	0.477
GPX	122	75	99	40	185	87	190	66	0.748	0.009	0.613
GR	18	6	15	3	22	6	20	6	0.250	0.029	0.782
CAT	89	26	84	20	94	22	88	15	0.475	0.570	0.932
SOD	196	43	194	30	201	50	145	15	0.052	0.087	0.051
LPO	75	24	51	17	47	15	46	13	0.076	0.025	0.107

FO, fish oil; VO, blend of vegetable oils; CH content, 0 % (CH-) or 20 % (CH+) gelatinised maize starch.

* Significant differences at *P* < 0.05 (two-way ANOVA).

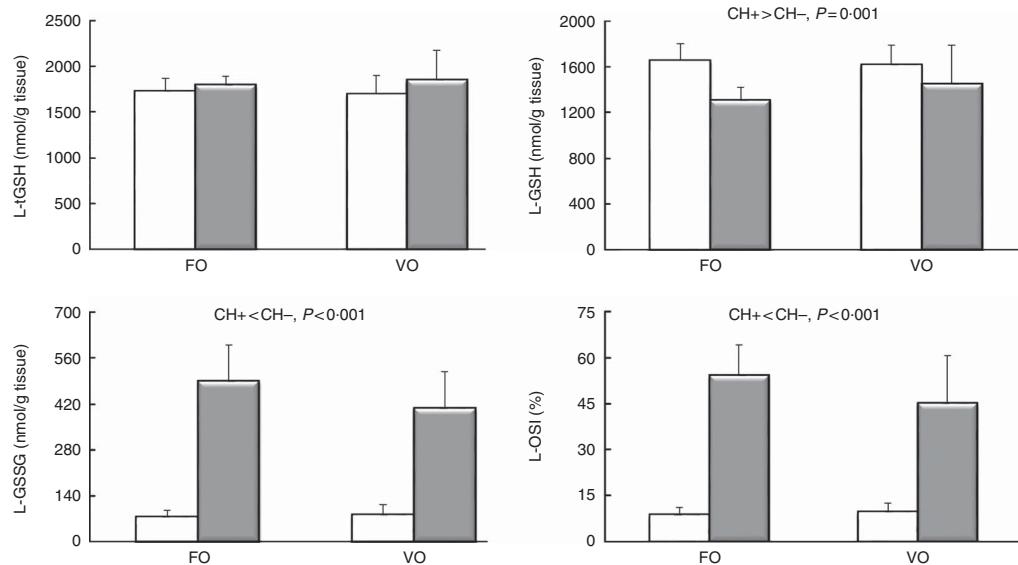


Fig. 1. Total glutathione (tGSH), GSH, GSSG and oxidative stress index (OSI) in the liver (L) of European sea bass fed experimental diets. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0 % (■, CH-) or 20 % (□, CH+) gelatinised maize starch. Values are means ($n=9$ fish), with standard deviations represented by vertical bars. Significant differences at $P<0.05$ (two-way ANOVA).

(GSH) to its oxidised form (GSSG). Consistent with this observation, higher concentration of GSH and lower concentration of GSSG were observed in the liver of fish fed carbohydrate-rich diets. GSH is an essential substrate for GPX, but as it is also an effective ROS scavenger it plays an important role by preventing some of the deleterious effects of oxidative stress⁽⁵³⁾. Accordingly, liver OSI, a biomarker of the cellular redox state, was also lower in groups fed carbohydrate-rich diets. Studies using OSI to assess the effects of oxidative stress in fish are scarce⁽⁵⁴⁻⁵⁸⁾ and mainly report a huge variation in OSI values (from 0·6 to 30 %) at the hepatic level⁽⁵⁵⁻⁵⁸⁾. As several variables (such as fish species, abiotic factors, fish size, time elapsed from the last meal or nutritional history) may regulate OSI values, comparisons among studies or establishment of reliable reference values for this parameter are difficult. Furthermore, a number of parameters or techniques have traditionally been used as measurements of oxidative stress. Among the most studied are antioxidant defence enzymes, TBA reactive substances (TBARS), concentrations of tGSH and GSSG and resulting OSI. In this study, the response of most parameters analysed (including OSI) was well correlated. However, other studies^(55,58) have demonstrated that OSI was not a suitable biomarker of oxidative stress under stressful conditions. As stated by Peres *et al.*⁽⁵⁹⁾, reliable parameters are those that respond to stressful conditions but have low variation under normal conditions. This highlights the importance of taking into consideration the response of more than one parameter involved in the oxidative status^(55,58).

On the other hand, the carbohydrate-rich diets promoted a lower hepatic CAT activity. Previously, Lygren & Hemre⁽⁵⁰⁾ also reported that hepatic CAT and SOD activities decreased in Atlantic salmon (*Salmo salar*) fed high-carbohydrate diets. These observations suggest that the lower LPO observed in fish fed the carbohydrate diets was mainly related with the higher concentrations of reducing equivalents such as NADPH and

GSH and that activation of primary antioxidant enzymes, which are more energy-demanding, was not required.

Fatty acid composition of reserve lipids in tissues (liver, muscle, intestine, etc.) reflects dietary fatty acid profile⁽⁶⁰⁾. In the present study, the reduced *n*-3:n-6 ratios (2·3 to 2·7 in VO groups v. 7·5 to 5·4 in FO groups) in the hepatic fatty acid profile of European sea bass fed VO-blend diets are consistent with previously reported values (1·9 to 2·5 in VO groups v. 4·3 in FO group) for this species also fed similar VO-blend based diets⁽⁵⁾. FO is rich in long-chain PUFA (LC-PUFA), whereas VO is rich in PUFA, and it is well established that due to their high degree of unsaturation LC-PUFA are more susceptible to peroxidation than PUFA or MUFA, thus promoting the formation of more free radicals⁽¹⁶⁾. Accordingly, in this study, higher hepatic LC-PUFA content was observed in European sea bass fed the FO-based diets than the VO-rich diets⁽⁴⁹⁾, although liver oxidative status was not affected by dietary lipid source. In fact, although unsaturation index was lower in fish fed the carbohydrate-rich diets, differences related to lipid sources were only evident in the CH- group (lower in VO diet). Similarly, partial replacement of FO by VO did not induce changes in hepatic lipid oxidative damages and antioxidant enzyme activities of Atlantic cod (*Gadus morhua*) or rockfish (*Sebastodes schlegeli*)^(31,34) or in LPO concentration in Japanese sea bass (*Lateolabrax japonicas*)⁽²³⁾.

In contrast to what was observed in the liver, in the present study, the dietary lipid source affected the intestinal oxidative status in European sea bass juveniles. Overall, oxidative lipid stress measured as LPO concentration was higher in fish fed the FO-based diets than the VO-rich diets. Studies addressing the effect of dietary lipid source on fish oxidative status were mainly focused on liver and muscle tissues^(7,18,20,22-24,31,35). To the best of the authors' knowledge, the impact of dietary lipid source on intestinal oxidative status of fish was only evaluated in Japanese sea bass, and it was limited to the assessment of LPO

concentration⁽²³⁾. In that study, dietary inclusion of palm oil led to decreased intestinal LPO concentration. Available studies dealing with the modulator role of nutrition on intestinal antioxidant enzymatic mechanisms were mainly dedicated to the role of amino acids^(61,62), anti-nutritional factors⁽⁶³⁾ and NSP⁽⁶⁴⁾.

GPX catalyses the reduction of H₂O₂ and lipid peroxides (ROOH) into the corresponding stable alcohols (ROH) at the expense of GSH⁽³²⁾. GR catalyses the reduction of GSSG to GSH and its activity is tightly bound to that of GPX, in an attempt to keep the adequate GSSG:GSH ratio necessary for GPX function. In the present study, GPX and GR activities were responsive to dietary treatments and had increased activities in groups fed the VO diets. Although flavonoids and phenolic compounds of VO were not measured in the present study, their potential role in the intestinal GPX and GR activation cannot be discarded. There is evidence that some plant constituents (flavonoids, phenolic compounds) also present in VO have antioxidant functions in mammals^(65–67). This antioxidant effect may result from a direct free-radical scavenging action by these compounds or from their role in activation of important antioxidant enzymes^(65–67). Accordingly, Nagata *et al.*⁽⁶⁵⁾ observed *in vitro* that in rat hepatocytes two flavonoids (quercetin and catechin) exert their antioxidant activity against H₂O₂ through activation of GPX. In human hepatocytes, it was also demonstrated that cocoa flavonoids exert an antioxidant protective effect against tert-butyl hydroperoxide via induction of GPX and GR activities. In fish, studies are not yet available. However, Sitjà-Bobadilla *et al.*⁽⁶⁸⁾ suggested that replacement of FM by a mixture of plant ingredients in diets for gilthead sea bream (*Sparus aurata*) exerts an antioxidative effect through activation of glutathione metabolism, as plant-containing diets increased muscular GSH and hepatic glutathione redox status, as well as GR activity, in both hepatic and muscular tissues. According to the authors, this increased antioxidant potential related to plant ingredients was probably linked to the presence of phenolic compounds and/or flavonoids. Further, Saera-Vila *et al.*⁽⁶⁹⁾ reported that FO replacement by VO in plant protein-based diets increased hepatic GSH:GSSG ratio, despite the reduction of absolute glutathione concentrations. According to the authors, this suggests that fish fed these diets may be less susceptible to LPO and oxidative damage. Overall, both the fatty acid profile of VO-based diets and the presence of plants constituents with antioxidant potential may explain the lower intestinal susceptibility to oxidative stress/damages in VO groups.

Transcriptional and proteomic studies have also evidenced that intestinal oxidative status may be affected by changes in dietary lipid source^(70,71). Accordingly, Morais *et al.*⁽⁷¹⁾ reported that in Atlantic salmon the dietary FO substitution by VO led to an up-regulation of transcripts and proteins involved in detoxification and protection from oxidative stress, CAT and selenoprotein, as well as haemopexin-like protein and peroxiredoxin-1 proteins. On the contrary, a previous study on the same species showed that dietary FO replacement by VO decreased the transcription levels of antioxidant-related genes such as glutathione S-transferase and GR⁽⁷⁰⁾.

Similarly to our previous studies on sturgeon (*Acipenser naccarii*) and rainbow trout (*Oncorhynchus mykiss*)⁽⁷²⁾, in this study, European sea bass intestines also showed LPO damage

values and GPX activity 2- to 5-fold higher compared with the liver. GPX and GR activities were also 2-fold higher in the intestine than in the liver of European sea bass. On the contrary, G6PD and CAT activities were, respectively, more than 10- and 5-fold lower in the intestine than in the liver. These differences between the two organs are a reflex of the distinct antioxidant mechanism strategies that are more relevant in each organ. The intestine being a tissue with high cell turnover is highly susceptible to oxidative status, and seems to require high activity of antioxidant enzymes.

In conclusion, results of the present study indicate that liver and intestine oxidative status balance were regulated through different metabolic pathways, and that dietary macronutrients affected metabolic pathways differently in each tissue. In addition, the observed LPO values suggest that susceptibility to oxidative stress was more important in the intestine than in the liver. Dietary carbohydrates contributed to reduced oxidative stress only in the liver, whereas the intestine was exclusively responsive to the dietary lipid source, with VO having a positive effect on the reduction of oxidative stress. Nowadays a wide range of PF are already used in commercial aquafeeds, including cereals and peas, which are rich sources of starch, and may therefore have a protective effect on liver oxidative stress. Given the importance of the intestine on whole-body homoeostasis and health, increased attention should be paid to this tissue in future studies evaluating the impact of dietary alternative ingredients on oxidative stress.

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C. C. carried out the main experimental work and wrote the draft of the manuscript under the direction of the project designer and leader A. O.-T., G. C., S. P. A. P.-J., F. C., P.D.-R., C. R. S. and H. P. assisted with the biochemical analyses and draft writing. All the authors contributed to and approved the manuscript.

The authors declare that there are no conflicts of interest.

References

- National Research Council (2011) Replacement of marine resources: effects of alternative ingredients and strategies on nutritional quality. In *Nutrient Requirements of Fish*, pp. 304–322. Washington, DC: National Academies Press.
- Montero D, Kalinowski T, Obach A, *et al.* (2003) Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on fish health. *Aquaculture* **225**, 353–370.

3. Montero D, Grasso V, Izquierdo MS, *et al.* (2008) Total substitution of fish oil by vegetable oils in gilthead seabream (*Sparus aurata*) diets: effects on hepatic Mx expression and some immune parameters. *Fish Shellfish Immunol* **24**, 147–155.
4. Mourente G, Good JE & Bell JG (2005) Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass (*Dicentrarchus labrax* L.): effects on flesh fatty acid composition, plasma prostaglandins E2 and F2 α , immune function and effectiveness of a fish oil finishing diet. *Aquac Nutr* **11**, 25–40.
5. Mourente G, Good JE, Thompson KD, *et al.* (2007) Effects of partial substitution of dietary fish oil with blends of vegetable oils, on blood leucocyte fatty acid compositions, immune function and histology in European sea bass (*Dicentrarchus labrax* L.). *Br J Nutr* **98**, 770–779.
6. Bell JG, Strachan F, Good JE, *et al.* (2006) Effect of dietary echium oil on growth, fatty acid composition and metabolism, gill prostaglandin production and macrophage activity in Atlantic cod (*Gadus morhua* L.). *Aquac Res* **37**, 606–617.
7. Lin YH & Shiao SY (2007) Effects of dietary blend of fish oil with corn oil on growth and non-specific immune responses of grouper, *Epinephelus malabaricus*. *Aquac Nutr* **13**, 137–144.
8. Ganga R, Bell JG, Montero D, *et al.* (2005) Effect of feeding gilthead sea bream (*Sparus aurata*) with vegetable lipid sources on two potential immunomodulator products: prostanoids and leptins. *Comp Biochem Physiol B* **142**, 410–418.
9. Olsen RE, Myklebust R, Kaino T, *et al.* (1999) Lipid digestibility and ultrastructural changes in the enterocytes of Arctic charr (*Salvelinus alpinus* L.) fed linseed oil and soybean lecithin. *Fish Physiol Biochem* **21**, 35–44.
10. Caballero MJ, Obach A, Rosenlund G, *et al.* (2002) Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **214**, 253–271.
11. Benedito-Palos L, Navarro JC, Sitjá-Bobadilla A, *et al.* (2008) High levels of vegetable oils in plant protein-rich diets fed to gilthead seabream (*Sparus aurata* L.): growth performance, muscle fatty acid profiles and histological alterations of target tissues. *Br J Nutr* **100**, 992–1003.
12. Kjaer MA, Vegusdal A, Gjøen T, *et al.* (2008) Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. *Biochim Biophys Acta* **1781**, 112–122.
13. Kortner TM, Gu J, Krogdahl A, *et al.* (2012) Transcriptional regulation of cholesterol and bile acid metabolism after dietary soyabean meal treatment in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **30**, 1–12.
14. Gatlin DM III, Barrows FT, Brown P, *et al.* (2007) Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquac Res* **38**, 551–579.
15. Montero D & Izquierdo M (2010) Welfare and health of fish fed vegetable oils as alternative lipid sources to fish oil. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, pp. 439–485 [GM Turchini, WK Ng and DR Tocher, editors]. Boca Raton, FL: CRC Press, Taylor & Francis.
16. Halliwell B & Chirico S (1993) Lipid peroxidation: its mechanism, measurement, and significance. *Am J Clin Nutr* **57**, 715S–725S.
17. López-Bote CJ, Diez A, Corraze G, *et al.* (2001) Dietary protein source affects the susceptibility to lipid peroxidation of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*) muscle. *Anim Sci* **73**, 443–449.
18. Menoyo D, Izquierdo MS, Robaina L, *et al.* (2004) Adaptation of lipid metabolism, tissue composition and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soyabean oils. *Br J Nutr* **92**, 41–52.
19. Rueda-Jasso R, Conceição LEC, Dias J, *et al.* (2004) Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. *Aquaculture* **231**, 417–433.
20. Peng SM, Chen LQ, Qin JG, *et al.* (2008) Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, *Acanthopagrus schlegeli*. *Aquaculture* **276**, 154–161.
21. Kiron V, Thawonwan J, Panigrahi A, *et al.* (2011) Antioxidant and immune defences of rainbow trout (*Oncorhynchus mykiss*) offered plant oils differing in fatty acid profiles from early stages. *Aquac Nutr* **17**, 130–140.
22. Ostbye TK, Kjaer MA, Rora AMB, *et al.* (2011) High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. *Aquac Nutr* **17**, 177–190.
23. Gao J, Koshio S, Ishikawa M, *et al.* (2012) Effects of dietary palm oil supplements with oxidized and non-oxidized fish oil on growth performances and fatty acid compositions of juvenile Japanese sea bass, *Lateolabrax japonicas*. *Aquaculture* **324–325**, 97–103.
24. Ng WK, Chong CY, Wang Y, *et al.* (2013) Effects of dietary fish and vegetable oils on the growth, tissue fatty acid composition, oxidative stability and vitamin E content of red hybrid tilapia and efficacy of using fish oil finishing diets. *Aquaculture* **372**, 97–110.
25. Bañuelos-Vargas I, López LM, Pérez-Jiménez A, *et al.* (2014) Effect of fishmeal replacement by soy protein concentrate with taurine supplementation on hepatic intermediary metabolism and antioxidant status of totoaba juveniles (*Totoaba macdonaldi*). *Comp Biochem Physiol B* **170**, 18–25.
26. Alvarez MJ, López-Bote CJ, Díez A, *et al.* (1998) Dietary fish oil and digestible protein modify susceptibility to lipid peroxidation in the muscle of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*). *Br J Nutr* **80**, 281–289.
27. Alvarez ML, López-Bote CJ, Díez A, *et al.* (1999) The partial substitution of digestible protein with gelatinized starch as an energy source reduces susceptibility to lipid oxidation in rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*) muscle. *J Anim Sci* **77**, 3322–3329.
28. Pérez-Jiménez A, Hidalgo MC, Morales AE, *et al.* (2009) Antioxidant enzymatic defenses and oxidative damage in *Dentex dentex* fed on different dietary macronutrient levels. *Comp Biochem Physiol C* **150**, 537–545.
29. Castro C, Pérez-Jiménez A, Guerreiro I, *et al.* (2012) Effects of temperature and dietary protein level on hepatic oxidative status of Senegalese sole juveniles (*Solea senegalensis*). *Comp Biochem Physiol A* **163**, 372–378.
30. Wang LN, Liu WB, Lu KL, *et al.* (2014) Effects of dietary carbohydrate/lipid ratios on non-specific immune responses, oxidative status and liver histology of juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture* **426–427**, 41–48.
31. Kjaer MA, Aursnes IA, Berge GM, *et al.* (2014) The influence of different dietary oil qualities on growth rate, feed utilization and oxidative stress in Atlantic cod. *Aquac Nutr* **20**, 192–204.
32. Mourente G, Bell JG & Tocher DR (2007) Does dietary tocopherol level affect fatty acid metabolism in fish? *Fish Physiol Biochem* **33**, 269–280.
33. Lushchak VI (2011) Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol* **101**, 13–30.

34. Aminikhoei Z, Choi J, Lee SM, *et al.* (2013) Effects of different dietary lipid sources on growth performance, fatty acid composition, and antioxidant enzyme activity of juvenile rockfish, *Sebastes schlegeli*. *J World Aquacult* **44**, 716–725.
35. Kjaer MA, Todorcevic M, Torsløsen BE, *et al.* (2008) Dietary n-3 HUFA affects mitochondrial fatty acid beta-oxidation capacity and susceptibility to oxidative stress in Atlantic salmon. *Lipids* **43**, 813–827.
36. Association of Official Analytical Chemists (2000) *Official Methods of Analysis*. Gaithersburg, MD: Association of Official Analytical Chemists.
37. Beutler HO (1984) Starch. In *Methods of Enzymatic Analysis* 6, pp. 2–10 [HU Bergmeyer, editor]. Basel: Verlag, Chemie Weinheim.
38. Santha NC & Ackman RG (1990) Nervonic acid versus tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *J Chromatogr Biomed Appl* **533**, 1–10.
39. Castro C, Corraze G, Panserat S, *et al.* (2014) Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquac Nutr* (epublication ahead of print version 29 October 2014).
40. McCord JM & Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein. *J Biol Chem* **244**, 6049–6055.
41. Aebi H (1984) Catalase *in vitro*. *Methods Enzymol* **105**, 121–126.
42. Flohé L & Günzler WA (1984) Assay of glutathione peroxidase. *Methods Enzymol* **105**, 115–121.
43. Morales AE, Pérez-Jiménez A, Hidalgo MC, *et al.* (2004) Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. *Comp Biochem Physiol C* **139**, 153–161.
44. Morales AE, García Rejón L & de la Higuera M (1990) Influence of handling and/or anaesthesia on stress response in rainbow trout. Effects on liver primary metabolism. *Comp Biochem Physiol A* **95**, 87–93.
45. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein dye-binding. *Anal Biochem* **72**, 248–254.
46. Griffith OW (1980) Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal Biochem* **106**, 207–212.
47. Vandepitte C, Guizon I, Genestie-Denis I, *et al.* (1994) A microtitre plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. *Cell Biol Toxicol* **10**, 415–421.
48. Buege JA & Aust SD (1978) Microsomal lipid peroxidation. *Methods Enzymol* **52**, 302–310.
49. Castro C, Corraze G, Pérez-Jiménez A, *et al.* (2015) How dietary carbohydrates and lipid source affect lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles? *Br J Nutr* (epublication ahead of print version 26 August 2015).
50. Lygren B & Hemre GI (2001) Influence of dietary carbohydrate on antioxidant enzyme activities in liver of Atlantic salmon (*Salmo salar* L.). *Aquac Int* **9**, 421–427.
51. Sagone AL, Greenwald J, Kraut EH, *et al.* (1983) Glucose: a role as a free radical scavenger in biological systems. *J Lab Clin Med* **101**, 97–103.
52. Nelson DL & Cox MM (2004) Glycolysis, gluconeogenesis, and the pentose phosphate pathway. In *Lehninger Principles of Biochemistry*, 4th ed., pp. 521–559 [DL Nelson and MM Cox, editors]. New York: W.H. Freeman.
53. Wu G, Fang YZ, Yang S, *et al.* (2004) Glutathione metabolism and its implications for health. *J Nutr* **134**, 489–492.
54. Olsvik PA, Kristensen T, Waagbo R, *et al.* (2005) mRNA expression of antioxidant enzymes (SOD, CAT and GSHPx) and lipid peroxidative stress in liver of Atlantic salmon (*Salmo salar*) exposed to hyperoxic water during smoltification. *Comp Biochem Physiol C* **141**, 314–323.
55. Olsvik PA, Kristensen T, Waagbo R, *et al.* (2006) Effects of hypo- and hyperoxia on transcription levels of five stress genes and the glutathione system in liver of Atlantic cod *Gadus morhua*. *J Exp Biol* **209**, 2893–2901.
56. Heier LS, Lien IB, Strømseng AE, *et al.* (2009) Speciation of lead, copper, zinc and antimony in water draining a shooting range – time dependant metal accumulation and biomarker responses in brown trout (*Salmo trutta* L.). *Sci Tot Environ* **407**, 4047–4055.
57. Karlsson A, Heier LS, Rosseland BO, *et al.* (2011) Changes in arterial PO₂, physiological blood parameters and intracellular antioxidants in free-swimming Atlantic cod (*Gadus morhua*) exposed to varying levels of hyperoxia. *Fish Physiol Biochem* **37**, 249–258.
58. Pérez-Jiménez A, Peres H, Cruz Rubio V, *et al.* (2012) The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (*Sparus aurata*) fed on diets supplemented with methionine and white tea. *Comp Biochem Physiol C* **155**, 506–516.
59. Peres H, Santos S & Oliva-Teles A (2012) Selected plasma biochemistry parameters in gilthead seabream (*Sparus aurata*) juveniles. *J Appl Ichthyol* **29**, 1–7.
60. Rosenlund G, Corraze G, Izquierdo M, *et al.* (2010) The effects of fish oil replacement on nutritional and organoleptic qualities of farmed fish. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds* CRC Press, pp. 487–522 [GM Turchini, WK Ng and DR Tocher, editors]. Boca Raton, FL: CRC Press, Taylor & Francis.
61. Wen H, Feng L, Jiang W, *et al.* (2014) Dietary tryptophan modulates intestinal immune response, barrier function, anti-oxidant status and gene expression of TOR and Nrf2 in young grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol* **40**, 275–287.
62. Zhao Y, Hu Y, Zhou XQ, *et al.* (2015) Effects of dietary glutamate supplementation on growth performance, digestive enzyme activities and antioxidant capacity in intestine of grass carp (*Ctenopharyngodon idella*). *Aquac Nutr* (epublication ahead of print version 10 January 2015).
63. Zhang JX, Guo LY, Feng L, *et al.* (2013) Soybean β-conglycinin induces inflammation and oxidation and causes dysfunction of intestinal digestion and absorption in fish. *PLOS ONE* **8**, e58115.
64. Enes P, Pérez-Jiménez A, Peres H, *et al.* (2012) Oxidative status and gut morphology of white sea bream, *Diplodus sargus* fed soluble non-starch polysaccharide supplemented diets. *Aquaculture* **358–359**, 79–84.
65. Nagata H, Takekoshi S, Takagi T, *et al.* (1999) Antioxidative action of flavonoids, quercetin and catechin, mediated by the activation of glutathione peroxidase. *Tokai J Exp Clin Med* **24**, 1–11.
66. Martín MÁ, Serrano ABG, Ramos S, *et al.* (2010) Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *J Nutr Biochem* **21**, 196–205.
67. Procházková D, Boušová I & Wilhelmová N (2011) Review: antioxidant and prooxidant properties of flavonoids. *Fitoterapia* **82**, 513–523.
68. Sitjà-Bobadilla A, Peña-Llopis S, Gómez-Requeni P, *et al.* (2005) Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* **249**, 387–400.

69. Saera-Vila A, Benedito-Palos L, Sitjà-Bobadilla A, *et al.* (2009) Assessment of the health and antioxidant trade-off in gilthead sea bream (*Sparus aurata* L.) fed alternative diets with low levels of contaminants. *Aquaculture* **296**, 87–95.
70. Olsvik PA, Torstensen BE & Berntssen MHG (2007) Effects of complete replacement of fish oil with plant oil on gastrointestinal cell death, proliferation and transcription of eight genes encoding proteins responding to cellular stress in Atlantic salmon *Salmo salar* L. *J. Fish Biol.* **71**, 550–568.
71. Morais S, Silva T, Carneiro O, *et al.* (2012) Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (*Salmo salar*). *BMC Genomics* **13**, 448.
72. Trenzado C, Hidalgo MC, García-Gallego M, *et al.* (2006) Antioxidant enzymes and lipid peroxidation in sturgeon *Acipenser naccarii* and trout *Oncorhynchus mykiss*. A comparative study. *Aquaculture* **254**, 758–767.

Chapter 8-

**Liver and intestine oxidative status of
gilthead sea bream fed in vegetable oil and
carbohydrate rich diets**

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Liver and intestine oxidative status of gilthead sea bream fed in vegetable oil and carbohydrate rich diets

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Abstract

This study investigated the effects of dietary lipid source and carbohydrate content on liver and intestine oxidative status of gilthead sea bream juveniles, by assessing antioxidant defences enzymes (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX; glutathione reductase, GR; and glucose-6-phosphate dehydrogenase, G6PD); total, reduced, and oxidized glutathione (GSH); and lipid peroxidation (LPO). Fish were fed for 13 weeks with four diets (2x2 factorial design) differing in lipid source (FO or a blend of VO) and carbohydrate level (0 or 20% of digestible starch). Dietary VO reduced liver and intestine LPO, and enhanced liver GSH redox status (total and reduced GSH), GPX, GR ,and G6PD activities, while in the intestine a decrease of G6PD activity and an increase of catalase activity and of reduced GSH (only in the carbohydrate diet) were recorded. Dietary carbohydrate promoted an increase of hepatic SOD and G6PD activities, and a decrease of CAT activity, total, oxidized, and reduced GSH, LPO, and oxidative stress index (OSI) (only in the FO diet). In the intestine, dietary carbohydrate did not induce alterations in LPO or enzymatic antioxidant defences but negatively affected GSH redox status (OSI, reduced and oxidized GSH). Overall, few interactions between dietary lipid source and carbohydrates were recorded. Dietary VO appeared to have a protective role against LPO in both tissues. Dissimilarities in liver and intestine susceptibility to LPO by dietary carbohydrate may reflect differences in glucose and GSH metabolism in the two tissues.

Keywords: Antioxidant defences; carbohydrates; intestine; liver; vegetable oils

Introduction

Fisheries-related feedstuffs such as fishmeal (FM) and fish oil (FO) have traditionally been used as main sources of protein and lipids in aquafeeds for marine carnivorous fish¹. However, given that FM and FO are expensive and limited resources, the current trend in aquaculture is toward their replacement with more cost-effective and sustainable feed ingredients, such as terrestrial plant feedstuffs (PF) and vegetable oils (VO).

Nevertheless, incorporation levels of PF in aquafeeds for carnivorous marine fish have several nutritional drawbacks, such as having high amounts of carbohydrates, non-starch polysaccharides, antinutrients, and lacking n-3 LC-PUFA (long-chain polyunsaturated fatty acids). Although equipped with all enzymatic apparatus required for digestion and metabolic utilization of carbohydrates, carnivorous fish use carbohydrates inefficiently, and dietary carbohydrate inclusion levels above 20% generally lead to decreased growth performance and feed utilization^{2; 3}. On the other hand, marine fish have limited ability for elongating and desaturating the C18 PUFA characteristics of VO into n-3 LC-PUFA. Thus, diets for marine fish must include n-3 LC-PUFA, namely eicosapentaenoic (EPA; 20:5 n-3) and docosahexaenoic acid (DHA; 22:6 n-3)^{4; 5}. Besides being required for normal membrane structure and function, these fatty acids (FA) play vital roles on the inflammatory response as they are precursors of eicosanoids^{6; 7}.

Research undertaken until now has demonstrated that feeds for marine carnivorous fish with PF and VO replacing up to 50% and 70% of dietary FM and FO, respectively, have minimal impacts on fish growth performance and feed utilization. However, the shift in diet formulation from marine resources towards PF and/or VO promotes marked modifications in whole-body lipid deposits and in fillet FA composition^{8; 9}. This, because dietary lipid composition and fillet FA composition are generally linearly correlated¹⁰. Thus, a decrease in dietary FO leads to an unavoidable decrease of tissue n-3 LC-PUFA levels with a consequent loss of the beneficial effects of fish for human health. In recent years, research has been devoted to the impact of these new dietary formulas on the metabolic processes involved in lipid deposition and FA composition of fish tissues¹¹. Changes of fish lipid composition may also affect membrane phospholipids and therefore affect structure, integrity, and function of cell and intracellular membranes. Among other effects, these changes may induce imbalances on fish oxidative status¹².

It is well known that all aerobic organisms are susceptible to attack by oxidizing agents - free radicals or reactive oxygen species (ROS) - that are produced in the body primarily as result of aerobic metabolism but that are also promoted by external factors, including nutritional ones^{7; 13; 14}. Fish are rich in n-3 LC-PUFA and are therefore particularly prone to ROS attack, which can lead to lipids peroxidation (LPO) and eventually to oxidative stress, if ROS attack is not prevented and cellular damage is not repaired^{7; 12; 13}. Detrimental effects of LPO include decreased membrane structure or fluidity, increased membrane permeability to normally impermeable substances, and inactivation of membrane-bound enzymes, with potential pathological effects on cells, tissues, quality and palatability of the final product to consumers^{7; 12}. To prevent oxidative injury, organisms developed an antioxidant protection system that involves low molecular weight antioxidants (vitamins and other molecules, such as glutathione, GSH) and enzymatic antioxidants (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPX; glutathione reductase, GR)¹³.

An adequate knowledge of dietary PF effects on fish's susceptibility to oxidative damage and on defence mechanisms to counteract it is of upmost importance to ensure that PF-rich aquafeeds do not compromise fish health and nutritional value to humans. However, potential interactions between VO and carbohydrates on processes that affect fish oxidative status are poorly known and available data is still apparently contradictory. For instance, there is growing evidence that high levels of dietary LC-PUFA increased LPO in the muscle of rainbow trout, European sea bass, and javelin goby^{15; 16}. On the other hand, dietary substitution of FO by single VO decreased susceptibility to LPO in the liver of turbot, grouper, and black seabream¹⁷⁻¹⁹ and in the muscle of turbot, gilthead sea bream, Japanese sea bass, and tilapia¹⁹⁻²². However, no differences in LPO due to dietary substitution of FO by single VO were reported in the liver Japanese sea bass, Atlantic cod, and European sea bass^{20; 23; 24}, or in the muscle of Atlantic salmon²⁵.

Although dietary FA were reported to affect tissue LPO susceptibility in fish, their role in the regulation of enzymatic and non-enzymatic antioxidant defence mechanisms is not clearly understood, as studies simultaneously assessing enzymatic and non-enzymatic antioxidant responses and LPO damages are still scarce²³⁻²⁵.

The potential effects of dietary carbohydrate on fish oxidative status has also received limited attention. However, it seems that dietary carbohydrate protect tissues against oxidative damage^{23; 26-29}. This is possibly due to the nature of glucose molecule, which

can scavenge ROS by itself, or through increased activity of the pentose phosphate pathway, that leads to increased production of NADPH via glucose-6-phosphate dehydrogenase (G6PD) activity. NADPH is required for the generation of reduced glutathione (GSH) by GR, and GSH is required for GPX to reduce H₂O₂. Recently, it was shown that dietary carbohydrate increased GSH concentration and GR and G6PD activities, and promoted a decrease of LPO in the liver of European sea bass²³.

Most studies on lipids effects in oxidative status focused on liver and muscle, the target tissues involved in lipid deposition. However, the intestine is also highly susceptible to oxidative stress^{20; 23; 30; 31} as it has a high cell turnover, and therefore requires more attention regarding this subject.

Thus, the aim of the present study was to evaluate the potential effects of dietary lipid source, carbohydrate content and interactions between both on liver and intestine oxidative status in gilthead sea bream juveniles.

Methods

Experimental diets

Four diets were formulated differing in carbohydrate content (0 and 20 % gelatinized starch, diets CH- and CH+, respectively) and lipid source (diets FO and VO) (Table 1). Carbohydrate inclusion in the diets was achieved by replacing protein, which was kept above requirement for the species in all diets³². The VO was a blend of rapeseed (20 %), linseed (50 %), and palm (30 %) oils, and replaced circa 70 % of lipids of the FO diets, which were provided by cod liver oil and oil from FM. All ingredients were finely ground, well mixed, and dry pelleted in a laboratory pellet mill (California Pellet Mill), through a 3-mm die. The pellets were air-dried for 24 h and stored in a refrigerator at 4°C until use.

Table 1. Ingredient and chemical composition of the experimental diets

Lipid source Carbohydrates	Experimental Diets			
	FO		VO	
	CH-	CH+	CH-	CH+
<i>Ingredients (% dry weight)</i>				
Fish meal ^a	87.3	65.1	87.3	65.1
Starch ^b	0	20	0	20
Cod liver oil ^c	9.2	11.4	0	0
Vegetable oil	0	0	9.2	11.4
Vitamins	1.5	1.5	1.5	1.5
Minerals	1.0	1.0	1.0	1.0
Binder	1.0	1.0	1.0	1.0
<i>Proximate analyses (% dry matter)</i>				
Dry matter (DM)	87.0	86.8	87.2	87.6
Crude protein	66.3	50.3	66.3	50.4
Crude fat (CF)	18.4	18.4	18.2	18.3
Starch	-	16.8	-	18.0
Energy	22.7	23.3	23.3	22.7
Ash	14.1	11.2	14.3	11.1

Fish oil (FO). blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch.

^a Steam dried LT fish meal (Superprime). Inproquisa, Madrid, Spain(CP:74.6% DM; CL: 10.1 % DM).

^b C-Gel Instant-12018. Cerestar. Mechelen. Belgium.

^c Labchem. Laborspirit Lda. Lisboa. Portugal.

^d 30% palm oil (Colmi. Malasia). 50% linseed oil (Sociedade Portuense de Drogas. S.A..Portugal) and 20% rapeseed oil (Huilerie Emile Noël S.A.S.. France)

^eVitamins (mg kg⁻¹ diet): retinol acetate. 18000 (IU kg⁻¹ diet); cholecalciferol. 2000 (IU kg⁻¹ diet); alpha tocopherol acetate. 35; sodium menadione bisulphate. 10; thiamin-HCl. 15; riboflavin. 25; calcium pantothenate. 50; nicotinic acid. 200; pyridoxine HCl. 5; folic acid 10; cyanocobalamin. 0.02; biotin. 1.5; ascorbic acid. 50; inositol. 400. Premix. Viana do Castelo. Portugal.

^fMinerals (mg kg⁻¹ diet): cobalt sulphate. 1.91; copper sulphate. 19.6; iron sulphate. 200; sodium fluoride. 2.21; potassium iodide. 0.78; magnesium oxide. 830; manganese oxide. 26; sodiumselenite. 0.66; zinc oxide. 37.5; dibasic calcium phosphate. 5.93 (g kg⁻¹ diet); potassium chloride. 1.15 (g kg⁻¹ diet); sodium chloride. 0.40 (g kg⁻¹ diet). Premix. Viana do Castelo. Portugal.

^gAquacube (Guar gum. polymethyl carbamide. Manioc starch blend. hydrate calcium sulphate). Agil. England.

Animals, experimental conditions, and sampling

The experiment was directed by trained scientists (following FELASA category C recommendations) and conducted according to the European Union Directive (2010/63/EU) on the protection of animals for scientific purposes. The study was performed at the Marine Zoological Station, University of Porto, Portugal, in a thermoregulated recirculation water system equipped with twelve fiberglass cylindrical tanks with a water capacity of 300 liters and supplied with continuous flow of filtered seawater. After 2 weeks of adaptation to the experimental conditions, 12 groups of twenty-one gilthead sea bream (*Sparus aurata*) juveniles with an initial body weight of 71.0 ± 1.5 g were established, and the experimental diets randomly assigned to triplicate groups of these fish. The growth trial lasted 81 days and during this period fish were hand-fed twice a day, 6 days a week, to apparent visual satiety. At the end of the trial fish were unfed for one day to empty gut content and then bulk-weighed after mild

anesthesia with 0.3 ml/l methylethanol. During the trial, salinity averaged 34.7 ± 0.8 g L⁻¹, dissolved oxygen was kept near saturation, and water temperature was regulated to 24.0 ± 0.5 °C. To reduce handling stress, fish continued to be fed for one more week and then, 18 h after the last meal (previous day afternoon meal), three fish per tank ($n=9$ fish/diet) were sampled, killed with a sharp blow to the head, liver and intestine removed and immediately frozen in liquid N₂, and then stored at -80°C until enzymatic analyses.

Diets and liver analysis

Chemical analysis were done according to AOAC (2012)³³ as follows: dry matter by drying at 105°C until constant weight; ash by incineration in a muffle furnace at 450°C for 16 h; protein (Nx6.25) by the Kjeldahl method after acid digestion using Kjeltec digestion and distillation units (models 1015 and 1026, Tecator Systems; Höganäs); gross energy by direct combustion in an adiabatic bomb calorimeter (PARR model 6200, PARR Instruments, Moline, IL, USA); starch, according to Beutler (1984)³⁴; lipid by petroleum ether extraction (Soxtec HT System; Höganäs). FA methyl esters of diets and liver were prepared by acid transmethylation of total lipids using boron trifluoride (BF3) in methanol (14 %) as described by Shantha and Ackman (1990)³⁵ and analyzed by GC (Varian 3900; Varian) according to Castro et al. (2015)³⁶.

Enzymatic activity assays and lipid peroxidation determination

Liver and whole intestine samples were homogenized (dilution 1:4) in ice-cold buffer (100 mM-Tris-HCl, 0.1mM-EDTA, 0.1%triton X-100 (v/v), pH 7.8). Homogenates were centrifuged at 30 000g for 30 min at 4°C. After centrifugation, the resultant supernatant was collected and aliquots stored at -80 °C until analysis. All enzyme assays were carried out at 37°C and changes in absorbance monitored using a Multiskan GO microplate reader (Model 5111 9200; Thermo Scientific, Nanjing, China). The optimal substrate and protein concentration for maximal activity of each enzyme were established by preliminary assays. The molar extinction coefficients used for H₂O₂ and NADPH were 0.039 and 6.22 mM⁻¹×cm⁻¹, respectively.

Activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GR, EC 1.6.4.2), glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) were measured as described by Castro et al. (2015)²³. Protein concentration was determined according to Bradford (1976)³⁷ using a Sigma protein kit (cod. B6916) and bovine serum albumin as standard. Enzyme activity was expressed as units (CAT, SOD) or milliunits (G6PD, GPX, and GR) per mg of soluble protein. Except for SOD, one unit of enzyme activity is defined as the

amount of enzyme required to transform 1 μ mol of substrate per min under the assay conditions. One unit of SOD activity is defined as the amount of enzyme necessary to produce a 50% inhibition of ferricytochrome c reduction rate. Lipid peroxidation levels were determined based on malondialdehyde (MDA) levels, which were measured as described by Castro et al. (2015)²³. Results are expressed as nmol malondialdehyde (MDA) per gram of wet tissue, calculated from a calibration curve.

Glutathione and oxidative stress index

Portions of liver and intestine were homogenized in nine volumes of ice-cold solution containing 1.3 % 5-sulphosalicylic acid (w/v) and 10 mM-HCl. The procedures were performed on ice in order to avoid GSH oxidation. Homogenates were centrifuged at 14 000g for 10 min at 4°C, and the resulting supernatants were immediately analyzed. Total glutathione (tGSH) and oxidized glutathione (GSSG) were determined as described by Castro et al. (2015)²³. Both tGSH and GSSG analyses were carried out at 37°C, and changes in absorbance due to reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were monitored at 405 nm in a Multiskan GO microplate reader. The molar extinction coefficient used for DTNB was 13.6mM⁻¹xcm⁻¹. Results were calculated using standard curves of reduced glutathione (GSH) and GSSG for tGSH and GSSG measurements, respectively. GSH level was calculated by subtracting GSSG from tGSH values. Data is expressed as nmol per g of tissue. The oxidative stress index (OSI) was calculated as follows:

$$\text{OSI (\%)} = 100 \times (2 \times \text{GSSG/tGSH}).$$

Statistical analysis

Data were checked for normality and homogeneity of variances and when appropriate was normalized. Statistical evaluation of data was carried out by a 2x2 factorial arrangement of treatments in a completely randomized design (two-way ANOVA) with carbohydrate level and lipid source as fixed factors. The significance level of 0.05 was used for rejection of the null hypothesis. When interaction was significant, one-way ANOVA was performed for each factor. All statistical analyses were conducted using the SPSS 22.0 software package (IBM Corp.) for Windows.

Results

Diets presented some differences in FA composition due to the lipid sources (Table 2). Proportion of total saturated fatty acids (SFA) was similar among diets, but monounsaturated fatty acids (MUFA) were higher in FO diets, and n-3 and n-6 polyunsaturated fatty acids (n-3, n-6 PUFA) were slight higher in VO diets. Within MUFA, higher levels of oleic acid (18:1 n-9) were recorded in VO diets, while the opposite occurred for palmitoleic acid (16:1 n-7), eicosenoic acid (20:1 n-9) and erucic acid (22:1 n-9). VO diets had high levels of linolenic acid (18:3 n-3) and lower levels of EPA and DHA than the FO diets. Total n-6 PUFA were higher in VO diets, mainly due to linoleic acid (18:2 n-6) levels.

Table 2. Fatty acid composition (% of total fatty acids) of the experimental diets

Lipid source	Experimental Diets			
	FO		VO	
Carbohydrates	CH-	CH+	CH-	CH+
14:00	5.8	5.9	2.5	2.0
15:00	0.7	0.6	0.4	0.3
16:00	18.7	17.2	21.3	21.0
17:00	0.5	0.4	0.4	0.3
18:00	4.0	3.5	4.9	4.5
20:00	0.2	0.1	0.3	0.2
ΣSFA	29.9	27.8	29.9	28.6
16:1n-7	7.1	7.5	2.3	1.8
18:1n-9	18.3	19.1	25.4	27.6
20:1n-9	4.9	5.8	0.9	0.7
22:1 n-9	3.9	4.3	0.5	0.4
ΣMUFA	34.4	36.8	29.2	30.5
18:2 n-6	2.2	2.2	9.0	10.6
18:3 n-6	0.1	0.1	0.0	0.1
20:2 n-6	0.3	0.3	0.1	0.1
20:3 n-6	0.13	0.12	0.08	0.08
20:4 n-6	1.2	1.0	0.9	0.7
Σn-6 PUFA	4.0	3.8	10.4	11.6
18:3 n-3	1.1	1.2	15.7	19.0
18:4 n-3	1.9	2.1	0.5	0.4
20:3 n-3	0.14	0.15	0.06	0.04
20:4 n-3	0.6	0.6	0.2	0.1
20:5 n-3	7.8	8.0	3.5	2.5
21:5 n-3	0.3	0.3	0.1	0.1
22:5 n-3	1.2	1.2	0.7	0.5
22:6 n-3	11.7	10.8	7.2	4.9
Σn-3 PUFA	24.8	24.3	28.0	27.5
<i>Ratios</i>				
Sat/PUFA	1.0	0.9	0.8	0.7
n3 / n6	6.2	6.4	2.7	2.4
Unsat. Index	181.4	179.5	169.2	160.2

Fish oil (FO), blend of vegetable oils (VO); carbohydrate content:0% (CH-) or 20% (CH+) gelatinized maize starch.
SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; n-3 LC-PUFA= n-3 long chain polyunsaturated fatty acids; Unsat. Index= Unsaturation index.

During the trial no mortality occurred. Results of the feeding trial were not the aim of this study and are presented elsewhere³⁸. In short, no differences in growth performance and feed efficiency were observed due to diet composition. Final body mass averaged 215.0 g and feed efficiency averaged 0.7.

Liver fatty acid composition is summarised in Table 3. Replacing FO by VO resulted in higher proportion of n-6 PUFA (mainly 18:2 n-6), and lower proportion of n-3 PUFA (mainly 22:6 n-3, 20:5 n-3, and 22:5 n-3). In fish fed CH+ diets, liver FA had lower proportion of n-3 PUFA and of n-6 PUFA (mainly 18:2 n-6) than fish fed CH- diets. Unsaturation index and n3/n6 ratio were lower in fish fed VO and CH+ diets than the other diets.

Table 3. Liver fatty acid profile (% of total fatty acids) of Gilthead sea bream fed the experimental diets^a

Lipid source	Experimental diets						
	FO		VO		p-value***		
Carbohydrates	CH-	CH+	CH-	CH+	CH	LS	CH*LS
18:2 n-6	2.41±0.16	1.97±0.22	8.21±0.45	7.9±0.34	0.002	<0.001	0.109
18:3 n-6	0.18±0.06	0.19±0.01	0.1±0.05	0.3±0.05	<0.001	0.864	<0.001
20:4 n-6	1.4±0.22	1±0.11	1.5±0.35	0.64±0.06	<0.001	0.003	0.003
Σn-6PUFA	4.61±0.31	3.56±0.21	10.08±0.7	9.1±0.3	<0.001	<0.001	0.141
18:3 n-3	0.91±0.09	0.69±0.08	11.1±0.82	10.5±0.84	<0.001	<0.001	0.012
20:5 n-3	5.3±0.36	4.2±0.3	2.5±0.42	1.2±0.05	<0.001	<0.001	0.003
22:5 n-3	2.6±0.31	2±0.24	1.3±0.36	0.47±0.05	<0.001	<0.001	0.017
22:6 n-3	14.7±1.44	9.6±0.68	8.6±1.55	3.2±0.11	<0.001	<0.001	0.016
Σn-3PUFA	25.82±1.63	18.44±1.02	24.89±1.8	16.74±0.87	<0.001	0.024	0.398
<i>Ratios</i>							
n3/n6	5.6±0.18	5.2±0.28	2.5±0.2	1.8±0.05	<0.001	<0.001	0.007
Unsat.index	191±9	151.5±5	159.3±16	122.9±3	<0.001	<0.001	0.705

Lipid source (LS): Fish oil (FO); blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

SFA=saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; n-3 LC-PUFA= n-3 long chain polyunsaturated fatty acids.

Unsat. index= Unsaturation index.

^aValues are presented as mean± standard deviation (SD), n=6. FA ≥0.02%. < 0.02% was not considered in the table.

*** significant differences at p< 0.05 two-way ANOVA.

Higher activities of GPX, GR, and G6PD, but lower LPO were measured in the liver of fish fed VO diets than FO diets (Table 4). SOD and CAT activities were not affected by dietary lipid source. Dietary carbohydrate increased SOD and G6PD activities and decreased CAT activity and LPO levels. No interaction between dietary lipid source and carbohydrate content was observed in the liver oxidative stress enzymes activities and LPO levels.

Table 4. Specific activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein) and lipid peroxidation (LPO) (nmol malondialdehyde per g tissue) in the liver of gilthead sea bream fed the experimental diets.

Lipid source Carbohydrates	Experimental diets				p-value***		
	FO		VO				
	CH-	CH+	CH-	CH+	CH	LS	CHxLS
SOD	237.1± 69.8	348.1± 60.4	297.1± 37.8	373.3± 61.2	<0.001	0.05	0.416
CAT	140.7± 35.9	128.3± 19.9	181.2± 49.6	129.6± 51.4	0.031	0.15	0.177
GPX	156.8± 65.1	179.0± 44.7	229.3± 53.6	188.4± 48.5	0.612	0.03	0.093
GR	7.5±1.7 140.1±	6.6±1.1 203.7±	9.3±1.8 198.6±	7.8±2.1 240.0±	0.053	0.02	0.565
G6PD	36.3	49.1	27.6	68.6	0.004	0.01	0.511
LPO	9±1.6	6.6±0.8	7.8±0.7	5.9±0.6	<0.001	0.01	0.817

Lipid source (LS): Fish oil (FO), blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

Values are presented as means ± standard deviation (SD), n=9.

*** significant differences at p< 0.05 two-way ANOVA.

Higher tGSH and GSH levels were observed in the liver of fish fed VO diets, but GSSG and OSI were unaffected by dietary lipid source (Fig. 1). Dietary carbohydrate led to a decrease of tGSH, GSH, and GSSG. There was an interaction between dietary lipid source and carbohydrate content in OSI, which was lower in fish fed dietary carbohydrate only in the FO diets (p<0.001).

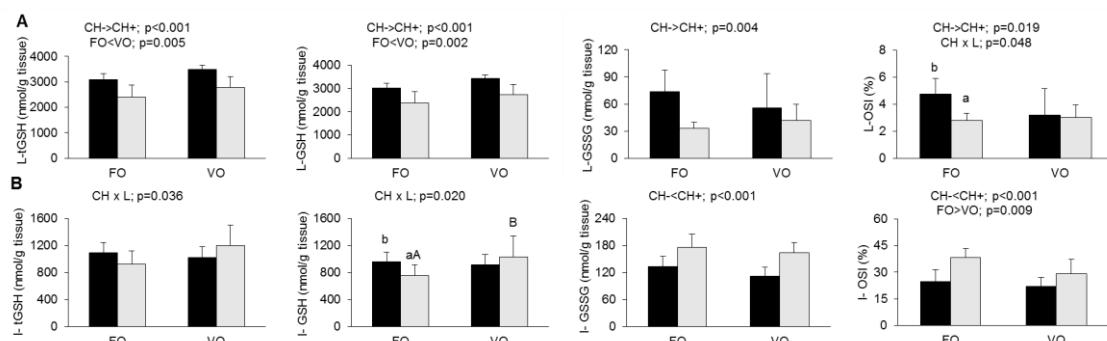


Fig. 1. Total glutathione (tGSH), reduced glutathione (GSH), oxidized glutathione (GSSG) and oxidative stress index (OSI) in (A) liver and (B) intestine of gilthead sea bream fed experimental diets. Fish oil (FO), blend of vegetable oils (VO); carbohydrate content: 0 % (CH-) or 20 % (CH+) gelatinized maize starch. Values are means ± SD (n =9 fish). Significant differences (p<0.05), two-way ANOVA. If interaction was significant, one-way ANOVA was performed for each factor and means with different capital and small letters indicate significant differences (p<0.05) between the two tested lipid sources and two carbohydrate levels, respectively; means with no letters are not significantly different (p>0.05).

In the intestine, CAT activity was higher, while G6PD activity and LPO levels were lower in fish fed VO diets (Table 5). Except for G6PD activity, which was higher in fish fed CH+ diets, no other antioxidant enzymes activities nor lipid peroxidation level were affected by dietary carbohydrate. No interaction between dietary lipid source and carbohydrate content was observed in the intestine oxidative stress enzymes activities and LPO levels.

Table 5. Specific activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione peroxidase (GPX), glutathione reductase (GR) (mU/mg protein), catalase (CAT), superoxide dismutase (SOD) (U/mg protein) and lipid peroxidation (LPO) (nmol malondialdehyde per g tissue) in the intestine of gilthead sea bream fed the experimental diets.

Lipid source	Experimental diets				p-value***			
	FO		VO					
	Carbohydrates	CH-	CH+	CH-	CH+	CH	LS	CHxLS
SOD	201.5± 36.5	204.3± 54.5		199.2± 53.9	182.5± 50.1	0.680	0.475	0.561
CAT	34.7± 21.0	22.3± 21.2		44.0± 15.4	55.7± 16.5	0.956	0.003	0.076
GPX	102.0± 45.8	129.5± 38.9		122.7± 19.1	117.2± 26.8	0.383	0.736	0.193
GR	9.2± 0.9	11±1.3		9.9±1.4	11±1.3	0.083	0.545	0.053
G6PD	7.3±1.7	5.7±2.3		7±1.7	3.1±1.1	<0.001	0.023	0.064
LPO	28.5±3.6	26.8±4.5		23.8±3.8	23.6±4.5	0.542	0.012	0.616

Lipid source (LS): Fish oil (FO), blend of vegetable oils (VO); carbohydrate content (CH): 0% (CH-) or 20% (CH+) gelatinized maize starch.

Values are presented as means ± standard deviation (SD), n=9.

*** significant differences at p< 0.05 two-way ANOVA.

An interaction between the dietary lipid source and carbohydrate content was observed for the intestine tGSH and GSH (Fig. 1). In fish fed CH+ diets, GSH content was higher in VO than in FO group. In fish fed FO diets, but not VO diets, GSH was lower when the diet included carbohydrate. OSI was lower in VO than FO diets, and increased with dietary carbohydrate. GSSG content also increased in fish fed dietary carbohydrate.

Discussion

Effects of dietary lipid source

It is well established that due to the high plasticity in tissue FA makeup, dietary FA play a preponderant role in FA composition of fish organs and lipid stores^{5; 8}. Reflecting this, liver FA composition of fish fed VO diets was characterized by low unsaturation index, with high C18 PUFA (mainly 18:2 n-6 and 18:3 n-3) and low LC-PUFA (mainly 22:6 n-3, 20:5 n-3) levels. As FA susceptibility to oxidation is directly dependent on degree of unsaturation^{14; 39}, the lower LPO in the liver of fish fed VO diets is assumed to be related to the lower unsaturation index of dietary lipids. This is consistent with other studies that

reported decreased susceptibility to LPO in the liver of turbot fed peanut oil replacing cod liver oil¹⁹, in grouper fed diets with corn oil replacing 50% of FO¹⁷, in black seabream fed soybean oil diets¹⁸.

Recently, in a study with European sea bass fed diets similar to those used in the present study, we observed no marked changes in hepatic LPO level, while decreased levels were recorded in the intestine of fish fed VO diets²³. In the present study with gilthead sea bream juveniles lower LPO levels were also recorded in the intestine of fish fed VO diets. Though intestine FA composition was not assessed, it is well documented that tissues with fast turnover rate like intestine readily reflect dietary changes^{8; 40-42}. In this sense, it is probable that the lower LPO in the intestine of fish fed VO diets was related with the lower unsaturation of VO diets. Identical results were also reported for Japanese sea bass, where dietary inclusion of palm oil led to a decrease in LPO in the intestine²⁰.

In the liver, tGSH and GSH concentrations and GPX, GR, and G6PD activities were higher in fish fed VO than FO diets. GSH plays critical roles in cell protection, by directly scavenging ROS or by acting in GPX-mediated catabolism of H₂O₂ and lipid hydroperoxides. In the reaction catalysed by GPX, GSH is oxidized to GSSG, which is regenerated to GSH by NADPH-dependent GR. The provision of NADPH required for GSH reduction is provided by G6PD activity. Thus, present data suggests that glutathione and glutathione-related antioxidant enzymes were the main responsible for the improved antioxidant status of fish fed VO diets.

PF and VO are rich in compounds with strong antioxidant properties, namely various classes of phenolic or polyphenolic compounds such as flavonoids⁴³⁻⁴⁵. Flavonoids, due to their molecular structure, particularly the number and arrangement of the phenolic hydroxyl groups attached to ring structures, function as powerful antioxidant agents by scavenging ROS and, in some instances, chelating transition metal ions, which are also causal factors for ROS formation^{44; 45}. Furthermore, *in vitro* studies with mammal models pointed out that the protective effects of flavonoids against oxidative stress may not only be due to their antioxidant properties per se; flavonoids may also indirectly contribute to increase endogenous antioxidant defences or attenuate ROS producing systems⁴³⁻⁴⁹. Supporting the increased endogenous antioxidant defence potential, several studies with human cells and in mammals also demonstrated that individual flavonoids or polyphenolic extracts exercise antioxidant protection by enhancing glutathione-dependent enzymes activities⁵⁰⁻⁵⁴, the expression of the rate limiting enzyme involved in

glutathione synthesis, γ -glutamylcysteine synthetase, and the intracellular glutathione concentration⁴³.

In fish, pure phenolic compounds and plant polyphenolic extracts were reported to prevent lipid oxidative damage in tissues of a number of fish species but the effects of these compounds on fish enzymatic and non-enzymatic antioxidant defences have been scarcely explored⁵⁵⁻⁶⁴. However, inclusion of PF in the diets at the expense of fisheries related feedstuffs has been reported to enhance the anti-oxidative status of a number of fish species by activation of glutathione metabolism, and this effect has been linked to the action of flavonoids or polyphenolic compounds present in dietary PF. Indeed, Stijà-Bobadilla et al. (2005)⁶⁵ reported that substitution of FM by a mixture of PF exerted an anti-oxidative effect in gilthead sea bream by enhancing muscular GSH content and hepatic GSH/GSSG ratio and GR activity. Also in gilthead sea beam, Saera-Vila et al. (2009)⁶⁶ observed that FO replacement by VO in PF-based diets increased hepatic GSH:GSSG ratio despite the reduction of total glutathione concentration. In European sea bass, the lower LPO concentration recorded in the intestine of fish fed VO diets was also accompanied by increased GPX and GR activities²³. Although flavonoids and phenolic compounds were not measured in the present study, it is reasonable to consider that the lower LPO in the liver of fish fed VO diets could be due to a combined action of the lower dietary unsaturation index and the increased antioxidant potential provided by polyphenolic compounds present in VO.

In contrast to what was observed in the liver and in a previous study in European sea bass²³, glutathione antioxidant enzymes activity in the intestine was not induced by dietary VO. Thus, the lower LPO and OSI recorded in fish fed VO diets may be mainly related to the lower dietary unsaturation index and the antioxidant properties of flavonoids to scavenge ROS.

Effects of dietary carbohydrate level

In this study dietary carbohydrate were included in the diets replacing protein; thus, an effect of dietary protein on antioxidant response cannot be completely discarded, particularly in relation to GSH metabolism, as sulphur amino acids are required for GSH synthesis^{67; 68}. Thus, the lower total and reduced GSH levels recorded in the liver of fish fed CH+ diets may at least in part be explained by the lower dietary protein level and the consequent lower availability of sulphur amino acids for GSH synthesis. On the other hand, the lower GSSG concentration and the increased G6PD activity in the liver of fish fed CH+ diets may be due to increased availability of glucose for the activity of G6PD,

which will generate reducing equivalents (NADPH) that are crucial to maintain normal GSH to GSSG ratio and cell redox status^{67; 69}. A normal cell redox status may additionally be due to the direct role of glucose in ROS scavenging^{27; 29; 69}.

Together with GSSG and G6PD data, the lowest LPO levels and OSI (only in FO diet) were recorded in the liver of fish fed CH+ diets. The lack of dietary carbohydrate effects on OSI in the VO diet was probably related to a masking effect of dietary VO. As in the present study, dietary carbohydrate were also reported to decrease LPO content in the liver and muscle of European sea bass and rainbow trout^{23; 26} through increased ROS scavenging action by glucose, increased G6PD activity^{23; 26} and GSH redox status²³.

The lower LPO content in the liver of fish fed CH+ diets may also be related to a lower degree of unsaturation of liver lipids. In fact, it was reported in rainbow trout and European sea bass that dietary carbohydrate induce lipogenesis and tissue deposition of saturated FA^{15; 70}. As previously observed in Atlantic salmon²⁷ and European sea bass²³, carbohydrate-rich diets promoted a decrease in liver CAT activity. On the contrary, hepatic SOD activity increased in fish fed CH+ diets. The response of SOD to dietary carbohydrate is still contradictory. Dietary carbohydrate were reported to either decrease²⁷, increase⁷¹, or have no effects^{23; 72} on hepatic SOD activity. In the present study, the higher SOD activity may be explained by increased generation of superoxide anion due to increment in the production of reducing equivalents after feeding CH+ diets^{67; 69; 73; 74}. As no increase of CAT and GPX activities were observed in the liver of fish fed CH+ diets, it is possible that removal of the free radicals generated was achieved through direct scavenging actions of reduced GSH and glucose^{27-29; 68; 69}. However, this hypothesis should be further investigated.

Contrary to what was observed in the liver, CH+ diets seem to have no beneficial effects in intestine susceptibility to LPO. Similar results were previously described in European sea bass²³. Intestine is a tissue with high turnover rates and a major consumer of GSH. However, its capacity for GSH synthesis or accumulation is limited, as indicated by the lower total GSH concentration comparatively to that of liver. In the intestine, limited availability of NADPH to maintain an adequate GSSG:GSH ratio may also explain the higher GSSG and OSI in CH+ groups.

The only interaction between dietary lipid source and carbohydrate content observed in this study was related to the GSH redox status. The overall effect of dietary VO on reduced GSH in the intestine was only evident in fish fed CH+ diets, suggesting a synergistic effect between dietary carbohydrate and VO. On the other hand, the beneficial effect of dietary carbohydrate on liver OSI was not evident in fish fed VO diets. Similarly, the negative effect of dietary carbohydrate on intestinal GSSG concentration seemed to be impaired by VO, which may be linked with the capacity of VO diets to maintain an appropriate intracellular GSH/GSSG ratio.

In conclusion, replacing dietary FO by VO reduced LPO in the liver and intestine of gilthead sea bream juveniles. Dietary carbohydrate reduced liver but not intestine susceptibility to LPO. There were no significant interactions between dietary lipid source and carbohydrate content on fish oxidative status.

Dietary carbohydrate seem to exert its protective role against oxidative stress in the liver by a direct role of glucose in scavenging reactive oxygen species and through increased activity of G6PD to generate reducing equivalents for the regeneration of reduced GSH. The antioxidant effect of dietary VO may be related to its lower fatty acid unsaturation composition and by enhanced activity of GSH related enzymes (GPX and GR).

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References

1. Oliva Teles A, Enes P & Peres H (2015) Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. In *Feed and Feeding Practices in Aquaculture*, pp. 203-233 [A Davis, editor]. Cambridge, UK: Woodhead Publishing.
2. Enes P, Panserat S, Kaushik S et al. (2009) Nutritional regulation of hepatic glucose metabolism in fish. *Fish Physiol Biochem* **35**, 519-539.
3. Enes P, Panserat S, Kaushik S et al. (2011) Dietary Carbohydrate Utilization by European Sea Bass (*Dicentrarchus labrax* L.) and Gilthead Sea Bream (*Sparus aurata* L.) Juveniles. *Rev Fish Sci* **19**, 201-215.
4. Tocher DR (2010) Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquacult Res* **41**, 717-732.
5. Turchini GM, Torstensen BE & Ng W-K (2009) Fish oil replacement in finfish nutrition. *Rev Aquacult* **1**, 10-57.
6. Bell J & Koppe W (2010) Lipids in Aquafeeds. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, pp. 21-59 [GM Turchini, WK Ng and DR Tocher, editors]: CRC Press.
7. Sargent JR, Tocher DR & Bell JG (2002) The lipids. In *Fish nutrition, 3rd Edition*, pp. 181-257 [JE Halver and RW Hardy, editors]: Academic Press.
8. Rosenlund G, Corraze Gv, Izquierdo M et al. (2010) The Effects of Fish Oil Replacement on Nutritional and Organoleptic Qualities of Farmed Fish. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, pp. 487-522 [GM Turchini, WK Ng and DR Tocher, editors]: CRC Press.
9. Torstensen BE, Espe M, Stubhaug I et al. (2011) Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.). *Br J Nutr* **106**, 633-647.
10. Benedito-Palos L, Bermejo-Nogales A, Karampatos AI et al. (2011) Modelling the predictable effects of dietary lipid sources on the fillet fatty acid composition of one-year-old gilthead sea bream (*Sparus aurata* L.). *Food Chem* **124**, 538-544.
11. Torstensen B & Tocher D (2010) The Effects of Fish Oil Replacement on Lipid Metabolism of Fish. In *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds*, pp. 405-437 [GM Turchini, WK Ng and DR Tocher, editors]: CRC Press.
12. Crockett EL (2008) The cold but not hard fats in ectotherms: consequences of lipid restructuring on susceptibility of biological membranes to peroxidation, a review. *J Comp Physiol B* **178**, 795-809.

13. Halliwell B & Gutteridge JM (2015) *Free radicals in biology and medicine*: Oxford University Press, USA.
14. Mourente G, Bell JG & Tocher DR (2007) Does dietary tocopherol level affect fatty acid metabolism in fish? *Fish Physiol Biochem* **33**, 269-280.
15. Alvarez MJ, Lopez-Bote CJ, Diez A et al. (1998) Dietary fish oil and digestible protein modify susceptibility to lipid peroxidation in the muscle of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*). *Br J Nutr* **80**, 281-289.
16. Luo Z, Tan XY, Li XD et al. (2012) Effect of dietary arachidonic acid levels on growth performance, hepatic fatty acid profile, intermediary metabolism and antioxidant responses for juvenile *Synechogobius hasta*. *Aquacult Nutr* **18**, 340-348.
17. Lin YH & Shiau SY (2007) Effects of dietary blend of fish oil with corn oil on growth and non-specific immune responses of grouper, *Epinephelus malabaricus*. *Aquacult Nutr* **13**, 137-144.
18. Peng SM, Chen LQ, Qin JG et al. (2008) Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, *Acanthopagrus schlegeli*. *Aquaculture* **276**, 154-161.
19. Stephan G, Guillaume J & Lamour F (1995) Lipid Peroxidation in Turbot (*Scophthalmus maximus*) Tissue - Effect of Dietary Vitamin E and Dietary N-6 or N-3 Polyunsaturated Fatty Acids. *Aquaculture* **130**, 251-268.
20. Gao J, Koshio S, Ishikawa M et al. (2012) Effects of dietary palm oil supplements with oxidized and non-oxidized fish oil on growth performances and fatty acid compositions of juvenile Japanese sea bass, *Lateolabrax japonicus*. *Aquaculture* **324**, 97-103.
21. Menoyo D, Izquierdo MS, Robaina L et al. (2004) Adaptation of lipid metabolism, tissue composition and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soyabean oils. *Br J Nutr* **92**, 41-52.
22. Ng WK, Chong CY, Wang Y et al. (2013) Effects of dietary fish and vegetable oils on the growth, tissue fatty acid composition, oxidative stability and vitamin E content of red hybrid tilapia and efficacy of using fish oil finishing diets. *Aquaculture* **372**, 97-110.
23. Castro C, Peréz-Jiménez A, Coutinho F et al. (2015) Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. *Br J Nutr* **114**, 1584-1593.
24. Kjaer MA, Aursnes IA, Berge GM et al. (2014) The influence of different dietary oil qualities on growth rate, feed utilization and oxidative stress in Atlantic cod. *Aquacult Nutr* **20**, 192-204.

25. Ostbye TK, Kjaer MA, Rora AMB *et al.* (2011) High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. *Aquacult Nutr* **17**, 177-190.
26. Alvarez MJ, Lopez-Bote CJ, Diez A *et al.* (1999) The partial substitution of digestible protein with gelatinized starch as an energy source reduces susceptibility to lipid oxidation in rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*) muscle. *J Anim Sci* **77**, 3322-3329.
27. Lygren B & Hemre GI (2001) Influence of dietary carbohydrate on antioxidant enzyme activities in liver of Atlantic salmon (*Salmo salar* L.). *Aquacult Int* **9**, 421-427.
28. Pérez-Jiménez A, Hidalgo MC, Morales AE *et al.* (2009) Antioxidant enzymatic defenses and oxidative damage in Dentex dentex fed on different dietary macronutrient levels. *Comp Biochem Physiol C* **150**, 537-545.
29. Sagone Jr A, Greenwald J, Kraut EH *et al.* (1983) Glucose: a role as a free radical scavenger in biological systems. *J Lab Clin Med* **101**, 97-104.
30. Morais S, Silva T, Cordeiro O *et al.* (2012) Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (*Salmo salar*). *BMC Genomics* **13**, 448.
31. Olsvik PA, Torstensen BE & Berntssen MHG (2007) Effects of complete replacement of fish oil with plant oil on gastrointestinal cell death, proliferation and transcription of eight genes' encoding proteins responding to cellular stress in Atlantic salmon *Salmo salar* L. *J Fish Biol* **71**, 550-568.
32. Oliva-Teles A (2000) Recent advances in European sea bass and gilthead sea bream nutrition. *Aquacult Int* **8**, 477-492.
33. AOAC (2012) *Official Methods of Analysis of Association of Official Analytical Chemists INTERNATIONAL*: AOAC International.
34. Beutler HO (1984) Starch. In *Methods of Enzymatic Analysis*, pp. 2-10 [HU Bergmeyer, editor]. Basel: Verlag Chemie Weinheim.
35. Shantha N & Ackman R (1990) Nervonic acid versus tricosanoic acid as internal standards in quantitative gas chromatographic analyses of fish oil longer-chain n-3 polyunsaturated fatty acid methyl esters. *J Chromatogr B Biomed Sci Appl* **533**, 1-10.
36. Castro C, Corraze G, Panserat S *et al.* (2015) Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquacult Nutr* **21**, 592-603.

37. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254.
38. Castro C, Corraze G, Diógenes A et al. (2016) Regulation of glucose and lipid metabolism by dietary carbohydrate and lipid source in gilthead sea bream juveniles. *Submitted for publication*.
39. Giordano E & Visioli F (2014) Long-chain omega 3 fatty acids: Molecular bases of potential antioxidant actions. *Prostaglandins Leukot Essent Fatty Acids* **90**, 1-4.
40. Caballero MJ, Izquierdo MS, Kjorsvik E et al. (2003) Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. *Aquaculture* **225**, 325-340.
41. Olsen RE, Dragnes BT, Myklebust R et al. (2003) Effect of soybean oil and soybean lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow trout, *Oncorhynchus mykiss* Walbaum. *Fish Physiol Biochem* **29**, 181-192.
42. Ruyter B, Moya-Falcón C, Rosenlund G et al. (2006) Fat content and morphology of liver and intestine of Atlantic salmon (*Salmo salar*): effects of temperature and dietary soybean oil. *Aquaculture* **252**, 441-452.
43. Moskaug JØ, Carlsen H, Myhrstad MC et al. (2005) Polyphenols and glutathione synthesis regulation. *Am J Clin Nutr* **81**, 277S-283S.
44. Procházková D, Boušová I & Wilhelmová N (2011) Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* **82**, 513-523.
45. Rice-Evans C (2001) Flavonoid antioxidants. *Curr Med Chem* **8**, 797-807.
46. Chang W-S, Lee Y-J, Lu F et al. (1993) Inhibitory effects of flavonoids on xanthine oxidase. *Anticancer Res* **13**, 2165-2170.
47. Masella R, Di Benedetto R, Varì R et al. (2005) Novel mechanisms of natural antioxidant compounds in biological systems: involvement of glutathione and glutathione-related enzymes. *J Nutr Biochem* **16**, 577-586.
48. Mehta SK & Gowder SJT (2015) Members of Antioxidant Machinery and Their Functions. In *Basic principles and clinical significance of oxidative stress*, pp. 59-85 [SJT Gowder, editor]. Rijeka, Croatia: InTech.
49. Vauzour D, Vafeiadou K & Spencer JP (2012) Polyphenols. In *Phytonutrients*, pp. 110-145 [A Salter, H Wiseman and G Tucker, editors]. Chichester, UK: Wiley-Blackwell, Blackwell Publishing Ltd.

50. Alía M, Ramos S, Mateos R *et al.* (2006) Quercetin protects human hepatoma HepG2 against oxidative stress induced by tert-butyl hydroperoxide. *Toxicol Appl Pharmacol* **212**, 110-118.
51. Martín MÁ, Ramos S, Cordero-Herrero I *et al.* (2013) Cocoa Phenolic Extract Protects Pancreatic Beta Cells against Oxidative Stress. *Nutrients* **5**, 2955-2968.
52. Martín MA, Ramos S, Mateos R *et al.* (2015) Chemical characterization and chemo-protective activity of cranberry phenolic powders in a model cell culture. Response of the antioxidant defenses and regulation of signaling pathways. *Food Res Int* **71**, 68-82.
53. Martín MÁ, Serrano ABG, Ramos S *et al.* (2010) Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells. *J Nutr Biochem* **21**, 196-205.
54. Rodríguez-Ramiro I, Martín MÁ, Ramos S *et al.* (2011) Comparative effects of dietary flavanols on antioxidant defences and their response to oxidant-induced stress on Caco2 cells. *Eur J Nutr* **50**, 313-322.
55. Iglesias J, Pazos M, Torres JL *et al.* (2012) Antioxidant mechanism of grape procyanidins in muscle tissues: Redox interactions with endogenous ascorbic acid and α-tocopherol. *Food Chem* **134**, 1767-1774.
56. Ishihara N, Araki T, Tamaru Y *et al.* (2002) Influence of green tea polyphenols on feed performance, growth performance, and fish body component in yellowtail (*Seriola quinqueradiata*). *Jpn J Food Chem* **9**, 7-14.
57. Liu T, Qi H, Ma L *et al.* (2015) Resveratrol Attenuates Oxidative Stress and Extends Life Span in the Annual Fish *Nothobranchius guentheri*. *Rejuvenation Res* **18**, 225-233.
58. Maqsood S, Benjakul S, Abushelaibi A *et al.* (2014) Phenolic Compounds and Plant Phenolic Extracts as Natural Antioxidants in Prevention of Lipid Oxidation in Seafood: A Detailed Review. *Comp Rev Food Sci F* **13**, 1125-1140.
59. Pazos M, Gallardo JM, Torres JL *et al.* (2005) Activity of grape polyphenols as inhibitors of the oxidation of fish lipids and frozen fish muscle. *Food Chem* **92**, 547-557.
60. Pazos M, González MJ, Gallardo JM *et al.* (2005) Preservation of the endogenous antioxidant system of fish muscle by grape polyphenols during frozen storage. *Eur Food Res Technol* **220**, 514-519.
61. Pérez-Jiménez A, Peres H, Rubio VC *et al.* (2012) The effect of dietary methionine and white tea on oxidative status of gilthead sea bream (*Sparus aurata*). *Br J Nutr* **108**, 1202-1209.

62. Thawonsuwan J, Kiron V, Satoh S *et al.* (2010) Epigallocatechin-3-gallate (EGCG) affects the antioxidant and immune defense of the rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol Biochem* **36**, 687-697.
63. Villasante A, Patro B, Chew B *et al.* (2015) Dietary Intake of Purple Corn Extract Reduces Fat Body Content and Improves Antioxidant Capacity and n-3 Polyunsaturated Fatty Acid Profile in Plasma of Rainbow Trout, *Oncorhynchus mykiss*. *J World Aquacult Soc* **46**, 381-394.
64. Villasante A, Powell MS, Moutou K *et al.* (2016) Effects of anthocyanidins on myogenic differentiation and antioxidant defense in primary myogenic cells isolated from rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **454**, 81-89.
65. Sitja-Bobadilla A, Pena-Llopis S, Gomez-Requeni P *et al.* (2005) Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). *Aquaculture* **249**, 387-400.
66. Saera-Vila A, Benedito-Palos L, Sitja-Bobadilla A *et al.* (2009) Assessment of the health and antioxidant trade-off in gilthead sea bream (*Sparus aurata* L.) fed alternative diets with low levels of contaminants. *Aquaculture* **296**, 87-95.
67. Fang Y-Z, Yang S & Wu G (2002) Free radicals, antioxidants, and nutrition. *Nutrition* **18**, 872-879.
68. Wu GY, Fang YZ, Yang S *et al.* (2004) Glutathione metabolism and its implications for health. *J Nutr* **134**, 489-492.
69. Liemburg-Apers DC, Willems PHGM, Koopman WJH *et al.* (2015) Interactions between mitochondrial reactive oxygen species and cellular glucose metabolism. *Arch Toxicol* **89**, 1209-1226.
70. Castro C, Corraze G, Pérez-Jiménez A *et al.* (2015) Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Br J Nutr* **114**, 1143-1156.
71. Zhou C, Liu B, Ge X *et al.* (2013) Effect of dietary carbohydrate on the growth performance, immune response, hepatic antioxidant abilities and heat shock protein 70 expression of Wuchang bream, *Megalobrama amblycephala*. *J Appl Ichthyol* **29**, 1348-1356.
72. Castro C, Perez-Jimenez A, Guerreiro I *et al.* (2012) Effects of temperature and dietary protein level on hepatic oxidative status of Senegalese sole juveniles (*Solea senegalensis*). *Comp Biochem Physiol A* **163**, 372-378.
73. Borges P, Valente LMP, Véron V *et al.* (2014) High Dietary Lipid Level Is Associated with Persistent Hyperglycaemia and Downregulation of Muscle Akt-mTOR Pathway in Senegalese Sole (*Solea senegalensis*). *PLoS ONE* **9**, e102196.

74. Pérez-Jiménez A, Abellán E, Arizcun M *et al.* (2015) Nutritional and metabolic responses in common dentex (*Dentex dentex*) fed on different types and levels of carbohydrates. *Comp Biochem Physiol A* **184**, 56-64.

Chapter 9-

General conclusions and final considerations

9.1 General conclusions

The following conclusions can be drawn from the results presented in this thesis:

Dietary replacement of up to 70% FO by a blend of VO (rapeseed, linseed, palm; 20:50:30) and dietary inclusion of 20% gelatinized starch led to small effects on growth performance and feed efficiency in European sea bass and gilthead sea bream.

Reduction of dietary protein from 60 to 45 % in European sea bass or from 65 to 50% in gilthead sea bream by replacing it with gelatinized starch increased protein retention (% protein intake) supporting a protein-sparing effect of dietary starch in both species.

Although the ADC of starch is high both in European sea bass and gilthead sea bream, intestine seems to be poorly responsive to increased glucose influx, as expression of glycolytic and gluconeogenesis enzymes was not affected by dietary carbohydrate.

European sea bass and gilthead seabream were able to restore plasma glucose levels within 12h after feeding starch rich-diets.

Both species stored excess glucose as glycogen but seem to have poor control over gluconeogenesis at molecular level, corroborating previous evidences on this issue.

Dietary starch also increased lipogenesis in both species, which in turn caused an increase in liver and muscle SFA levels and a decrease of PUFA levels.

Short-term exposure to VO did not markedly affect lipid digestion, absorption, and transport, or lipid metabolite kinetics in European sea bass and gilthead sea bream.

Dietary starch did not affect lipid digestibility in gilthead sea beam but seems to modulate transcriptional mechanisms involved in lipid absorption and transport at intestinal level, probably by interfering with TAG availability and by delaying lipid absorption.

Nutritional modulation of FADS2 (the rate-limiting enzyme involved in LC-PUFA biosynthesis) by VO was demonstrated in both species at molecular level. This induction of FADS2 was evident in European sea bass at 6h post-feeding but not at 18h post-feeding, while in gilthead sea bream gene induction occurred at 18h after feeding.

Liver and muscle FA profile markedly reflected the FA composition of dietary lipid sources, and dietary starch increased tissue SFA content. Despite the induction of FADS2 by both dietary starch and VO this did not seem to have promoted deposition of n-3 LC-PUFA in liver and muscle.

In European sea bass dietary carbohydrate induced at a molecular level, cholesterol biosynthesis capacity. Up-regulation of cholesterol biosynthesis at molecular level seems to constitute a metabolic adaption to VO diets in European sea bass but not in gilthead sea bream.

Interactions between dietary carbohydrates and VO were mainly observed in gilthead sea bream, and were reflected in modifications in cholesterol body pool content and glycogen deposition. Starch intake, when coupled with dietary VO, seems to enhance the hypocholesterolemic effects of dietary VO. On the other hand, the stimulatory effect of dietary starch on muscle glycogen deposition did not occur in fish fed the VO diet.

Interactions between dietary carbohydrates and FO were mainly observed in whole-body and liver lipid deposition in gilthead sea bream. Indeed, the positive contribution of dietary carbohydrates to the overall lipid deposition was not observed in the liver or whole body of fish fed FO diet, which suggest a higher lipid mobilization, transport and/or utilization in these fish.

Liver and intestine oxidative status of both species were affected by dietary starch content and lipid source in a tissue and species specific manners.

Dietary starch seemed to improve liver oxidative status both in European sea bass and gilthead sea bream, probably by increasing the production of reducing power in the form of NADPH through the pentose phosphate pathway (increased G6PD activity). Differently, also in both species, at intestinal level dietary carbohydrate did not induce alterations in lipid oxidative damage nor substantial modifications in enzymatic antioxidant defense. Dissimilarities between liver and intestine susceptibility to lipid oxidative damage may reflect differences in glucose and glutathione metabolism in the two tissues.

Dietary VO contributed to reduce lipid oxidative damage in gilthead sea bream liver and intestine, but in European sea bass it only reduced lipid oxidative damage in the intestine. Enzymes that were responsive to dietary VO were mainly those linked to glutathione metabolism (increased GR and GPX activities). The potential protective role of VO against liver and intestine lipid oxidative damage in both species was achieved by means of an enhancement of glutathione metabolism or due to the lower FA unsaturation index of the VO diets.

9.2 Final considerations

As pointed out before, preservation of n-3 LC-PUFA content in fish is desirable from fish health and human consumption perspectives. In the context of sustainable aquafeeds, which tend to include increasing amounts of plant feedstuffs and VO, multiple strategies have been exploited to overcome the FA modification of farmed fish that are fed with these alternative formulations. Among these strategies are:

- a) use alternative sources of n-3 LC-PUFA, such as single-cell oils (provided by several microbial taxa at the base of the marine food chain such as thraustochytrids, diatoms, other microalgae, and some marine bacteria) that proved to be suitable alternatives to FO. However, their current high production cost and reliance as feed resource limits their immediate wider use in aquafeeds (Turchini et al., 2009; Miller et al., 2010; Tocher et al., 2015).
- b) use a finishing period based on a FO diet (wash-out or finishing diet) to restore the original FA profile of fish previously fed alternative lipid sources. This proved to be a valuable approach in several fish species including European sea bass and gilthead sea bream (Izquierdo et al. 2005; Montero et al. 2005; Turchini et al., 2009).
- c) the production of strains of farmed animals with enhanced capacity to biosynthesise or deposit n-3 LC-PUFA, or production of genetically modified n-3 LC-PUFA rich vegetable crops. These have been pointed out as promising approaches. However, the use of transgenic technology in food or feed production is not well accepted by consumers and therefore several socio-political and environmental issues need to be overcome before becoming commercially viable (Turchini et al., 2009; Leaver et al., 2011; Betancor et al., 2015; Tocher, 2015).
- d) use nutritional or metabolic programming strategy. This concept started to be applied very recently in fish nutrition and is based on the observation that an acute nutritional stimulus during critical developmental stages during early life may induce long-term changes in nutrient metabolism in later stages. Using this approach, the possibility to induce FA desaturation enzymes involved in n-3 LC-PUFA synthesis was tested in European seabass larvae challenged with a restricted n-3 LC-PUFA diet (0.5 or 0.7 % EPA+ DHA) (Vagner et al., 2009). The early nutritional conditioning induced FADS2 expression and this increased FAD expression was retained in pre-conditioned juveniles fed an n-3 LC-PUFA-restricted diet (0.3 % EPA+ DHA), after a transient feeding period with a n-3 LC-PUFA rich diet. Although this nutritional conditioning had no noticeable

influence on FA composition and growth performance of juveniles (Vagner et al., 2009), and the long-term prevalence of this stimulus on n-3 LC-PUFA metabolism of adult fish remains unclear, this demonstrate the possibility of nutritional programming European sea bass for a better use of VO.

As stated by a number of reviews on this topic, namely those of Turchini et al. (2009, 2010) and Tocher (2015), the benefits of the different approaches to overcome the FA modification that may occur in farmed fish fed VO-rich diets are numerous. However, broad application of such strategies appears unfeasible, at least in the short to medium term, and therefore solutions to the issue of reduced n-3 LC-PUFA in farmed fish still remains to be studied. Thus, it is acknowledged that nutritional strategies and genetic selection approaches aiming to optimise n-3 LC-PUFA biosynthesis and retention in fish should be thoroughly evaluated, as potential benefits can arise from this increased knowledge. In this context, although we were unable to demonstrate a positive VO-carbohydrate interaction effect on n-3 LC-PUFA biosynthesis in gilthead sea bream and European sea bass, we provided for the first time evidence of a transcriptional induction of LC-PUFA biosynthesis pathway by dietary carbohydrates, in addition to VO, in gilthead sea bream. Although a nutritional regulation of FADS2 gene expression by VO was previously demonstrated in European sea bass and gilthead sea bream (Seiliez et al., 2003; González-Rovira et al., 2009), this thesis demonstrated that in each species the response of FADS2 gene expression to a nutritional stimulus might be conditioned by other factors, such as time after feeding. In this sense, this should also be taken in consideration in future studies on the overall n-3 LC-PUFA metabolism in marine fish. Nevertheless, although the induction of genes related to n-3 LC-PUFA biosynthesis apparently had no benefits in tissue n-3 LC-PUFA content, our data provides new perspectives on the use of nutritional strategies for inducing LC-PUFA biosynthesis in marine fish.

In light of the present knowledge that individually VO and carbohydrates enhance key FADS2 gene expression in gilthead sea bream, it would be interesting to test, in the future, the combined effect of genetically select lines of gilthead sea bream, exhibiting a different capacity to biosynthesize or deposit n-3 LC-PUFA, with dietary carbohydrate, when given as part of VO based diet, on FA bioconversion and n-3 LC-PUFA content of the flesh. The possible application of this approach and of the metabolic programming concept in marine carnivorous species of high value to aquaculture (such as meagre and Senegalese sole) will be relevant, as they may provide new insights on n-3 LC-PUFA metabolism in fish and possibly improve the use of VO in aquafeeds.

At the same time, relevant information arises from the present study regarding cholesterol metabolism and highlighted several points that need to be taken into account when using plant feedstuffs and VO-rich aquafeeds. Indeed, this thesis revealed species-specific differences in the dietary regulation of cholesterol biosynthesis pathway by VO and differences in species sensitivities to cholesterol availability in alternative lipid sources. Considering that dietary supply of cholesterol will be also limited in aquafeeds with the replacement of FM and FO by plant feedstuffs and VO, species differences in cholesterol metabolism should be taken into consideration, as specific adjustments of feeding strategies may be required, including the need of dietary cholesterol supplementation. Furthermore, the findings of a relation between dietary carbohydrate and cholesterol biosynthetic pathway in European sea bass makes it essential that future studies be carried out to clarify the mechanisms of regulation of cholesterol metabolism by dietary carbohydrates.

Chapter 10- References

- Agaba, M.K., Tocher, D.R., Dickson, C.A., Zheng, X., Dick, J.R., Teale, A.J. 2005. Cloning and functional characterisation of polyunsaturated fatty acid elongases from marine and freshwater teleost fish. *Comp. Biochem. Physiol. B* 142: 342–352.
- Agulleiro, M.J., André, M., Morais, S., Cerdà, J., Babin, P.J. 2007. High transcript level of fatty acid-binding protein 11 but not of very low-density lipoprotein receptor is correlated to ovarian follicle atresia in a teleost fish (*Solea senegalensis*). *Biol. Reprod.* 77: 504–516.
- Aksnes, A. 1995. Growth, feed efficiency and slaughter quality of salmon, *Salmo salar* L., given feeds with different ratios of carbohydrate and protein. *Aquacult. Nutr.* 1: 241–248.
- Alimuddin, G. Y., Kiron, V., Satoh, S., Takeuchi, T. 2005. Enhancement of EPA and DHA biosynthesis by over-expression of masu salmon $\Delta 6$ -desaturase-like gene in zebrafish. *Transgenic Res.* 14: 159–165.
- Alimuddin, G.Y., Kiron, V., Satoh, S., Takeuchi, T. 2007. Expression of masu salmon $\Delta 5$ -desaturase-like gene elevated EPA and DHA biosynthesis in zebrafish. *Mar. Biotechnol.* 9: 92–100.
- Alimuddin, G.Y., Kiron, V., Satoh, S., Takeuchi, T., Yoshizaki, G. 2008. Cloning and over-expression of a masu salmon (*Oncorhynchus masou*) fatty acid elongase-like gene in zebrafish. *Aquaculture* 282: 13–18.
- Alliot, E., Febvre, A., Metailler, R. and Pastoureaud, A. 1974. Besoins nutritifs du bar (*Dicentrarchus labrax* L.). Etude du taux de protéine et du taux de lipid dans le régime. *Actes de Colloque CNEXO* 1: 215–228.
- Almaida-Pagán, P.F., Hernández, M.D., García García, B., Madrid, J.A., De Costa, J., Mendiola, P. 2007. Effects of total replacement of fish oil by vegetable oils on n-3 and n-6 polyunsaturated fatty acid desaturation and elongation in sharpsnout seabream (*Diplodus puntazzo*) hepatocytes and enterocytes. *Aquaculture* 272: 589–598.
- Álvarez, M.J., Díez, A., López-Bote, C., Gallego, M., Bautista, J.M. 2000. Short-term modulation of lipogenesis by macronutrients in rainbow trout (*Oncorhynchus mykiss*) hepatocytes. *Br. J. Nutr.* 84: 619–628.
- Álvarez, M.J., López-Bote, C.J., Díez, A., Corraze, G., Arzel, J., Dias, J., Kaushik, S.J., Bautista, J.M. 1998. Dietary fish oil and digestible protein modify susceptibility to lipid

peroxidation in the muscle of rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*). Br. J. Nutr. 80: 281-289.

Álvarez, M.L., López-Bote, C.J., Díez, A., Corraze, G., Arzel, J., Dias, J., Kaushik, S.J., Bautista, J.M. 1999. The partial substitution of digestible protein with gelatinized starch as an energy source reduces susceptibility to lipid oxidation in rainbow trout (*Oncorhynchus mykiss*) and sea bass (*Dicentrarchus labrax*) muscle. J. Anim. Sci. 77:3322–3329.

Amirkolaie, A.K., Verreth, J.A.J., Schrama, J.W. 2006. Effect of gelatinisation degree and inclusion level in dietary starch on the characteristics of digesta and faeces in Nile tilapia (*Oreochromis niloticus* (L)). Aquaculture 260:194–205.

Arnesen, P., Krogdahl, Å., Kristiansen, I.Ø. 1993. Lipogenic enzyme activities in liver of Atlantic salmon (*Salmo salar* L.). Comp. Biochem. Physiol. B 105: 541–546.

Babin, P.J. and Vernier, J.M. 1989. Plasma lipoproteins in fish. J. Lipid Res. 30:467–489.

Bakke, A.M., Glover, C., Krogdahl, Å. 2010. Feeding, digestion and absorption of nutrients. In: Fish Physiology (Grosell, M., Farrell, A.P., Brauner, C.J. eds), pp. 57– 110. Academic Press, USA.

Ballestrazzi, R., Lanari, D., Dagaro, E., Mion, A. 1994. The effect of dietary protein level and source on growth, body composition, total ammonia and reactive phosphate excretion of growing sea bass (*Dicentrarchus labrax*). Aquaculture 127: 197-206.

Bañuelos-Vargas I, López, L.M., Pérez-Jiménez, A., Peres, H. 2014. Effect of fishmeal replacement by soy protein concentrate with taurine supplementation on hepatic intermediary metabolism and antioxidant status of totoaba juveniles (*Totoaba macdonaldi*). Comp. Biochem. Physiol. B 170:18–25.

Barroso, J.B., Peragón, J., García-Salguero, L., de la Higuera, M., Lupiáñez, J.A. 2001. Carbohydrate deprivation reduces NADPH-production in fish liver but not in adipose tissue. Int. J. Biochem. Cell Biol. 33: 785–796.

Bell, J.G. and Koppe, W. 2010. Lipids in Aquafeeds. In: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (Turchini, G.M., Ng, W.K., Tocher, D.R. eds), pp. 21–59. Taylor & Francis, CRC Press, Boca Raton.

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

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

Betancor, M. B., Sprague, M., Usher, S., Sayanova, O., Campbell, P. J., Napier, J. A., Tocher, D. R. 2015. A nutritionally-enhanced oil from transgenic *Camelina sativa* effectively replaces fish oil as a source of eicosapentaenoic acid for fish. Scientific reports 5.

Black, D.D. 2007. Development and physiological regulation of intestinal lipid absorption. I. Development of intestinal lipid absorption: cellular events in chylomicron assembly and secretion. Am. J. Physiol. 293: G519 –G524.

Bogevik, A.S., Tocher, D.R., Langmyhr, E., Waagbø, R., Olsen, R.E. 2009. Atlantic salmon (*Salmo salar*) postsmolts adapt lipid digestion according to elevated dietary wax esters from *Calanus finmarchicus*. Aquacult. Nutr. 15: 94–103.

Bonamusa, L., García de Frutos, P., Fernandez, F., Baanante, I. V. 1992. Nutritional effects on key glycolytic-gluconeogenic enzyme activities and metabolite levels in the liver of the teleost fish *Sparus aurata*. Mol. Mar. Biol. Biotechnol. 1: 113-125.

Borges, P., Médale, F., Véron, V., Pires, M. dos A., Dias, J., Valente, L.M.P. 2013. Lipid digestion, absorption and uptake in *Solea senegalensis*. Comp. Biochem. Physiol. A 166: 26–35.

Borges, P., Valente, L.M., Véron, V., Dias, K., Panserat, S., Médale, F. 2014b. High dietary lipid level is associated with persistent hyperglycaemia and downregulation of muscle Akt-mTOR pathway in Senegalese sole (*Solea senegalensis*). PLoS One 9:e102196.

Borges, P., Reis, B., Fernandes, T. J., Palmas, Â., Castro-Cunha, M., Médale, F., Oliveira, M.B.PP., Valente, L. M. 2014a. Senegalese sole juveniles can cope with diets

devoid of supplemental fish oil while preserving flesh nutritional value. *Aquaculture* 418: 116-125.

Borrebaek, B. and Christophersen, B. 2000. Hepatic glucose phosphorylating activities in perch (*Perca fluviatilis*) after different dietary treatments. *Comp. Biochem. Physiol. B* 125: 387–393.

Bou, M., Todorčević, M., Fontanillas, R., Capilla, E., Gutiérrez, J., Navarro, I. 2014. Adipose tissue and liver metabolic responses to different levels of dietary carbohydrates in gilthead sea bream (*Sparus aurata*). *Comp. Biochem. Physiol. A* 175: 72-81.

Boujard, T., Gelineau, A., Coves, D., Corraze, G., Dutto, G., Gasset, E., Kaushik, S. 2004. Regulation of feed intake, growth, nutrient and energy utilisation in European sea bass (*Dicentrarchus labrax*) fed high fat diets. *Aquaculture* 231: 529-545.

Boyd, C.E. 2015. Overview of aquaculture feeds: global impact of ingredient use. In: Feed and Feeding Practices in Aquaculture (Davis, A. ed.), pp. 3-25. Woodhead Publishing, Cambridge, UK.

Brauge, C., Médale, F., Corraze, G. 1994. Effect of dietary carbohydrate levels on growth, body composition and glycemia in rainbow trout, *Oncorhynchus mykiss*, reared in seawater. *Aquaculture* 123:109 –120.

Caballero, M.J., Izquierdo, M.S., Kjørsvik, E., Fernández, E., Rosenlund, G. 2004. Histological Iterations in the liver of sea bream, *Sparus aurata* L., caused by short- or long-term feeding with vegetable oils. Recovery of normal morphology after feeding fish oil as the sole lipid source. *J. Fish Dis.* 27:531–541.

Caballero, M.J., Torstensen, B.E., Robaina, L., Montero, D., Izquierdo, M. 2006b. Vegetable oils affect the composition of lipoproteins in sea bream (*Sparus aurata*). *Br. J. Nutr.* 96: 830–839.

Caballero, M.J., Obach, A., Rosenlund, G., Montero,D., Gisvold, M., Izquierdo, M.S. 2002. Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* 214: 253-271.

- Caballero, M.J., Gallardo, G., Robaina, L., Montero, D., Fernández, A., Izquierdo, M. 2006a. Vegetable lipid sources affect in vitro biosynthesis of triacylglycerols and phospholipids in the intestine of sea bream (*Sparus aurata*). Br. J. Nutr. 95: 448–454.
- Caballero, M. J., Izquierdo, M. S., Kjorsvik, E., Montero, D., Socorro, J., Fernández, A. J., Rosenlund, G. 2003. Morphological aspects of intestinal cells from gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225: 325-340.
- Capilla, E., Díaz, M., Gutiérrez, J., Planas, J. V. 2002. Physiological regulation of the expression of a GLUT4 homolog in fish skeletal muscle. Am. J. Physiol. Endocrinol. Metab. 283: E44-E49.
- Capilla, E., Díaz, M., Albalat, A., Navarro, I., Pessin, J. E., Keller, K., Planas, J. V. 2004a. Functional characterization of an insulin-responsive glucose transporter (GLUT4) from fish adipose tissue. Am. J. Physiol. Endocrinol. Metab. 287: E348-E357.
- Capilla, E., Médale, F., Panserat, S., Vachot, C., Rema, P., Gomes, E., Kaushik, S., Navarro, I., Gutiérrez, J. 2004b. Response of hexokinase enzymes and the insulin system to dietary carbohydrates in the common carp, *Cyprinus carpio*. Reprod. Nutr. Dev. 44: 233–242.
- Carmona-Antoñanzas, G., Tocher, D. R., Taggart, J. B., Leaver, M. J. 2013. An evolutionary perspective on Elovl5 fatty acid elongase: comparison of Northern pike and duplicated paralogs from Atlantic salmon. BMC Evol. Biol. 13: 85.
- Carmona-Antoñanzas, G., Monroig, Ó., Dick, J.R., Davie, A., Tocher D.R. 2011. Biosynthesis of very longchain fatty acids (C > 24) in Atlantic salmon: Cloning, functional characterisation, and tissue distribution of an Elovl4 elongase. Comp. Biochem. Physiol. B 159:122-129.
- Caseras, A., Metón, I., Vives, C., Egea, M., Fernández, F., Baanante,I.V. 2002. Nutritional regulation of glucose-6-phosphatase gene expression in liver of the gilthead sea bream (*Sparus aurata*). Br. J. Nutr. 88:607–614.
- Castro, L.F.C., Monroig, Ó., Leaver, M.J., Wilson, J., Cunha, I., Tocher, D.R. 2012. Functional desaturase Fads1 ($\Delta 5$) and Fads2 ($\Delta 6$) orthologues evolved before the origin of jawed vertebrates. Public Libr. Sci. PLoS ONE 7: e31950.
- Castro, C., Corraze, G., Panserat, S., Oliva-Teles, A. 2015b. Effects of fish oil replacement by a vegetable oil blend on digestibility, postprandial serum metabolite

profile, lipid and glucose metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Aquacult. Nutr.* 21: 592-603.

Castro, C., Pérez-Jiménez, A., Coutinho, F., Pousão-Ferreira, P., Brandão, T.M., Oliva-Teles, A., Peres, H. 2013. Digestive enzymes of meagre (*Argyrosomus regius*) and white seabream (*Diplodus sargus*). Effects of dietary brewer's spent yeast supplementation. *Aquaculture* 416–417:322–327.

Castro, C., Corraze, G., Pérez-Jiménez, A., Larroquet, L., Cluzeaud, M., Panserat, S., Oliva-Teles, A. 2015a. Dietary carbohydrate and lipid source affect cholesterol metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Br. J. Nutr.* 114: 1143-1156.

Castro, C., Peréz-Jiménez, A., Coutinho, F., Díaz-Rosales, P., Serra, C.A., Panserat, S., Corraze, G., Peres, H., Oliva-Teles, A. 2015c. Dietary carbohydrate and lipid sources affect differently the oxidative status of European sea bass (*Dicentrarchus labrax*) juveniles. *Br J Nutr.* 114:1584-1593.

Castro, C., Couto, A., Pérez-Jiménez, A., Serra, C.R., Díaz-Rosales, P., Fernandes, R., Corraze, G., Panserat, S., Oliva-Teles, A. 2016. Effects of fish oil replacement by vegetable oil blend on digestive enzymes and tissue histomorphology of European sea bass (*Dicentrarchus labrax*) juveniles. *Fish Physiol. Biochem.* 42:203-217.

Chakrabarti, I., Gani, M.A., Chaki, K., Sur, R., Misra, K. 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. *Comp. Biochem. Physiol. A* 112: 167–177.

Chapman, M.J. 1980. Animal lipoproteins: chemistry, structure, and comparative aspects. *J. Lipid Res.* 21:789–853.

Chen, X.W., Jiang, S., Shi, Z.Y. 2012. Identification and expression analysis of fabp2 gene from common carp *Cyprinus carpio*. *J. Fish Biol.* 80: 679–691.

Cook, H.W. and McMaster, R.C.R. 2004. Fatty acid desaturation and chain elongation in eukaryotes. In: *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D.E. and Vance, J.E. eds), pp. 181–204. Elsevier, Amsterdam, Netherlands.

Corraze, G. 2001. Lipid Nutrition. In: *Nutrition and Feeding of Fish and Crustaceans* (Guillaume, J., Kaushik, S., Bergot, P. and Métailler, K. eds), pp. 111–130. Springer Prarois, Chichester.

Couto, A., Enes, P., Peres, H., Oliva-Teles, A. 2012. Temperature and dietary starch level affected protein but not starch digestibility in gilthead sea bream juveniles. *Fish Physiol. Biochem.* 38: 595–601.

Couto, A., Peres, H., Oliva-Teles, A., Enes, P. 2016. Screening of nutrient digestibility, glycaemic response and gut morphology alterations in gilthead seabream (*Sparus aurata*) fed whole cereal meals. *Aquaculture* 450: 31–37.

Coutteau, P., Vanstappen, G., Sorgeloos, P. 1996. A standard experimental diet for the study of fatty acid requirements of weaning and first ongrowing stages of the European sea bass *Dicentrarchus labrax* L - comparison of extruded and extruded/coated diets. *Arch. Anim. Nut.* 49: 49-59.

Darias, M.J., Mazurais, D., Koumoundouros, G., Glynatsi, N., Christodouloupolou, S., Huelvan, C., Desbruyeres, E., Le Gall, M.M., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L. 2010. Dietary vitamin D₃ affects digestive system ontogenesis and ossification in European sea bass (*Dicentrarchus labrax*, Linnaeus, 1758). *Aquaculture*. 298: 300-307.

De Francesco, M., Parisi, G., Pérez-Sánchez, J., Gómez-Réqueni, P., Médale, F., Kaushik, S. J., Mecatti, M., Poli, B. M. 2007. Effect of high-level fish meal replacement by plant proteins in gilthead sea bream (*Sparus aurata*) on growth and body/fillet quality traits. *Aquacult. Nutr.* 13: 361–372.

De la Parra, A.M., Rosas, A., Lazo, J.P., Viana, A.T. 2007. Partial characterization of the digestive enzymes of Pacific bluefin tuna *Thunnus orientalis* under culture conditions. *Fish Physiol. Biochem.* 33: 223–231.

Degani, G., Viola, S., Yehuda, Y. 1997. Apparent digestibility of protein and carbohydrate in feed ingredients for adult tilapia (*Oreochromis aureus* × *O. niloticus*). *Isr. J. Aquac.-Bamidgeh* 49: 115-123.

Denstadli, V., Vegusdal, A., Krogdahl, A., Bakke-McKellep, A. M., Berge, G. M., Holm, H., Hillestad, M., Ruyter, B. 2004. Lipid absorption in different segments of the gastrointestinal tract of Atlantic salmon (*Salmo salar* L.). *Aquaculture* 240: 385–398.

Dias, J., Rueda-Jasso, R., Panserat, S., Conceição, L. E. d., Gomes, E. F., Dinis, M. T. 2004. Effect of dietary carbohydrate-to-lipid ratios on growth, lipid deposition and metabolic hepatic enzymes in juvenile Senegalese sole (*Solea senegalensis*, Kaup). *Aquac. Res.* 35: 1122-1130.

- Dias, J., Álvarez, M.J., Díez, A., Arzel, J., Corraze, G., Bautista, J.M., Kaushik, S.J. 1998. Regulation of hepatic lipogenesis by dietary protein/energy in juvenile European seabass (*Dicentrarchus labrax*). Aquaculture 161: 169-186.
- Diaz, J. P., Guyot, E., Vigier, S., and Connes, R. 1997. First events in lipid absorption during post-embryonic development of the anterior intestine in gilt-head sea bream. J. Fish Biol. 51: 180–192.
- Ekmann, K.S., Dalsgaard, J., Holm, J., Campbell, P.J., Skov, P.V. 2013. Glycogenesis and de novo lipid synthesis from dietary starch in juvenile gilthead sea bream (*Sparus aurata*) quantified with stable isotopes. Br. J. Nutr. 109-. 2135–2146.
- Eliason, E.J., Djordjevic, B., Trattner, S., Pickova, J., Karlsson, A., Farrell, A.P., Kiessling, A.K. 2010. The effect of hepatic passage on postprandial plasma lipid profile of rainbow trout (*Oncorhynchus mykiss*) after a single meal. Aquacult. Nutr. 16: 536-543.
- Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. 2006a. Effect of normal and waxy maize starch on growth, food utilization and hepatic glucose metabolism in European sea bass (*Dicentrarchus labrax*) juveniles. Comp. Biochem. Physiol. A 143:89–96.
- Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. 2006b. Rapid metabolic adaptation in European sea bass (*Dicentrarchus labrax*) juveniles fed different carbohydrate sources after heat shock stress. Comp. Biochem..Physiol. A 145: 73–81.
- Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. 2008a. Growth performance and metabolic utilization of diets with native and waxy maize starch by gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture 274:101–108.
- Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. 2008b. Hepatic glucokinase and glucose-6-phosphatase responses to dietary glucose and starch in gilthead sea bream (*Sparus aurata*) juveniles reared at two temperatures. Comp. Biochem. Physiol. A 149:80–86.
- Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. 2009. Nutritional regulation of hepatic glucose metabolism in fish. Fish Physiol. Biochem. 35: 519-539.
- Enes, P., Peres, H., Couto, A., Oliva-Teles, A. 2010. Growth performance and metabolic utilization of diets including starch, dextrin, maltose or glucose as carbohydrate source by gilthead sea bream (*Sparus aurata*) juveniles. Fish Physiol. Biochem. 36: 903-910.

Enes, P., Panserat, S., Kaushik, S., Oliva-Teles, A. 2011. Dietary carbohydrate utilization by European sea bass (*Dicentrarchus labrax* L.) and gilthead sea bream (*Sparus aurata* L.) juveniles. Rev. Fish. Sci. 19: 201-215.

Engelking, L.R. 2010. Textbook of Veterinary Physiological Chemistry, updated 2nd ed., Academic Press (Elsevier), 596 pp., Amsterdam, Netherlands.

EUFOMA. 2014. European Market Observatory for fisheries and aquaculture 2014. The EU fish market 2014 Edition European Commission, Directorate-General for Maritime Affairs and Fisheries, Director-General (Ed.), 61pp., Brussels.

FAO. 2014. The State of World Fisheries and Aquaculture 2014. Food and Agriculture Organization of the United Nations (Ed.), 223 pp., Rome.

FAO. 2015a. Available on: <http://www.fao.org/fishery/statistics/en> (accessed on 9th November 2015).

FAO. 2015b. Available on: <http://faostat3.fao.org> (accessed on 9th November 2015).

FAO. 2015c. Available on: <http://www.fao.org/fishery/affris/species-profiles/european-seabass/european-seabass-home/en/> (accessed on 9th November 2015).

FAO, 2015d. Available on: <http://www.fao.org/fishery/affris/species-profiles/gilthead-seabream/gilthead-seabream-home/en/> (accessed on 9th November 2015).

FEAP. 2015. Federation of European Aquaculture Producers 2015. European aquaculture production report 2005-2014., 54 pp, Belgium.

Feedipedia. 2015. Available on: <http://www.feedipedia.org/> (accessed on 9th November 2015).

Fernández, F., Miquel, A.G., Cordoba, M., Varas, M., Metón, I., Caseras, A., Baanante, I.V. 2007. Effects of diets with distinct protein-to-carbohydrate ratios on nutrient digestibility, growth performance, body composition and liver intermediary enzyme activities in gilthead seabream (*Sparus aurata*, L.) fingerlings. J. Exp. Mar. Biol. Ecol. 343:1–10.

Figueiredo-Silva, A., Corraze, G., Borges, P., Valente, L. 2010. Dietary protein/lipid level and protein source effects on growth, tissue composition and lipid metabolism of blackspot seabream (*Pagellus bogaraveo*). Aquac. Nutr. 16: 173–187.

Figueiredo-Silva, A.C., Panserat, S., Kaushik, S., Geurden, I., Polakof, S. 2012. High levels of dietary fat impair glucose homeostasis in rainbow trout. *J. Exp. Biol.* 215: 169–178.

Figueiredo-Silva, A. C., Saravanan, S., Schrama, J. W., Panserat, S., Kaushik, S., Geurden, I. 2013. A comparative study of the metabolic response in rainbow trout and Nile tilapia to changes in dietary macronutrient composition. *Br. J. Nutr.* 109: 816-826.

Figueiredo-Silva, A., Rocha, E., Dias, J., Silva, P., Rema, P., Gomes, E., Valente, L.M.P. 2005. Partial replacement of fish oil by soybean oil on lipid distribution and liver histology in European sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) juveniles. *Aquacult. Nutr.* 11:147–155.

Fountoulaki, E., Alexis, M. N., Nengas, I., Venou, B. 2005. Effect of diet composition on nutrient digestibility and digestive enzyme levels of gilthead sea bream (*Sparus aurata* L.). *Aquac. Res.* 36: 1243-1251.

Fountoulaki, E., Morgane, H., Rigos, G., Vasilaki, A., Mente, E., Sweetman, J., Nengas, I. 2010. Evaluation of zinc supplementation in European seabass (*Dicentrarchus labrax*) juvenile diets. *Aquac. Res.* 41: 208–216.

Fountoulaki E., Vasilaki A., Hurtado R., Grigorakis K., Karacostas I., Nengas I., Rigos G., Kotzamanis Y., Venou B., Alexis M.N. 2009. Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. *Aquaculture* 289:317–326.

Francis, D.S., Turchini, G.M., Jones, P.L., De Silva, S.S. 2007 Effects of fish oil substitution with a mix blend vegetable oil on nutrient digestibility in Murray cod, *Maccullochella peelii* peelii. *Aquaculture* 269: 447–455.

Fujikawa, Y., Shimokawa, M., Satoh, F., Satoh, O., Yoshioka, D., Aida, S., Uematsu, K., Iijima, N. 2012. Ontogeny of gene expression of group IB phospholipase A2 isoforms in the red sea bream, *Pagrus (Chrysophrys) major*. *Comp. Biochem. Physiol. A* 161:185-92.

Fynn-Aikins, K., Hung, S.S.O., Liu, W., Li, H. 1992. Growth, lipogenesis and liver composition of juvenile white sturgeon fed different levels of D-glucose. Aquaculture 105: 61-72.

Ganga, R., Montero, D., Bell, J.G., Atalah, E., Ganuza, E., Vega-Orellana, O., Tort, L., Acerete, L., Afonso, J.M., Benitez-Sanatana, T., Vaquero, A.F., Izquierdo, M. 2011. Stress response in sea bream (*Sparus aurata*) held under crowded conditions and fed diets containing linseed and/or soybean oil. Aquaculture 311: 215–223.

Gao, J., Koshio, S., Ishikawa, M., Yokoyama, S., Ren, T., Komilus, C. F., Han, Y. 2012. Effects of dietary palm oil supplements with oxidized and non-oxidized fish oil on growth performances and fatty acid compositions of juvenile Japanese sea bass, *Lateolabrax japonicus*. Aquaculture 324: 97-103.

Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W., Herman, E., Hu, G.S., Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealey, W., Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtele, E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38: 551e579.

Geay, F., Ferrarese, S., Zambonino-Infante, J. L., Bargelloni, L., Quentel, C., Vandeputte, M., Kaushik, S., Cahu, C. L., Mazurais, D. 2011. Effects of the total replacement of fish-based diet with plant-based diet on the hepatic transcriptome of two European sea bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with the plant-based diet. BMC Genomics 12.

Gélineau, A., Corraze, G., Boujard, T., Larroquet, L., Kaushik, S. 2001. Relation between dietary lipid level and voluntary feed intake, growth, nutrient gain, lipid deposition and hepatic lipogenesis in rainbow trout. Reprod. Nutr. Dev. 41: 487–503.

Geurden, I., Kaushik, S., Corraze, G. 2008. Dietary phosphatidylcholine affects postprandial plasma levels and digestibility of lipid in common carp (*Cyprinus carpio*). Br. J. Nutr. 100: 512-517.

Geurden I., Jutfelt F., Olsen R., Sundell K. 2009. A vegetable oil feeding history affects digestibility and intestinal fatty acid uptake in juvenile rainbow trout *Oncorhynchus mykiss*. Comp. Biochem. Physiol. A 152:552-559.

Glencross, B.D. 2009. Exploring the nutritional demand for essential fatty acids by aquaculture species. Rev. Aquacult. 1: 71–124.

- Gómez-Requeni, P., Mingarro, M., Caldúch-Giner, J. A., Médale, F., Martín, S. A. M., Houlihan, D. F., Kaushik, S., Pérez-Sánchez, J. 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (*Sparus aurata*). Aquaculture 232: 493-510.
- González-Rovira, A., Mourente, G., Zheng, X., Tocher, D.R., Pendón, C. 2009. Molecular and functional characterization and expression analysis of a $\Delta 6$ fatty acyl desaturase cDNA of European sea bass (*Dicentrarchus labrax* L.). Aquaculture 298: 90–100.
- Gouveia, A. and Davies, S.J. 1998. Preliminary nutritional evaluation of pea seed meal (*Pisum sativum*) for juvenile European sea bass (*Dicentrarchus labrax*). Aquaculture 166: 311–320.
- Gouveia, A. and Davies, S.J. 2000. Inclusion of an extruded dehulled pea seed meal in diets for juvenile European sea bass (*Dicentrarchus labrax*). Aquaculture 182: 183–193.
- Gregory, M., See, V.H.L., Gibson, R.A., Shuller, K.A. 2010. Cloning and functional characterisation of a fatty acyl elongase from southern bluefin tuna (*Thunnus maccoyii*). Comp. Biochem. Physiol. B 155: 178-185.
- Grim, J.M., Miles, D.R., Crockett, E.L. 2010. Temperature acclimation alters oxidative capacities and composition of membrane lipids without influencing activities of enzymatic antioxidants or susceptibility to lipid peroxidation in fish muscle. J. Exp. Biol. 213: 445–452.
- Grisdale-Helland, B. and Helland, S.J. 1997. Replacement of protein by fat and carbohydrate in diets for Atlantic salmon *Salmo salar* at the end of the freshwater stage. Aquaculture 152: 167–180.
- Grisdale-Helland, B. and Helland, S. J. 1998. Macronutrient utilization by Atlantic halibut (*Hippoglossus hippoglossus*): diet digestibility and growth of 1 kg fish. Aquaculture 166: 57-65.
- Gu, M., Kortner, T. M., Penn, M., Hansen, A. K., Krogdahl, Å. 2014. Effects of dietary plant meal and soya-saponin supplementation on intestinal and hepatic lipid droplet accumulation and lipoprotein and sterol metabolism in Atlantic salmon (*Salmo salar* L.). Br J Nutr. 111: 432-444.

- Hall, J. R., Short, C. E. Driedzic, W. R. 2006. Sequence of Atlantic cod (*Gadus morhua*) GLUT4, GLUT2 and GPDH: developmental stage expression, tissue expression and relationship to starvation-induced changes in blood glucose. *J. Exp. Biol.* 209: 4490-4502.
- Hall, J. R., MacCormack, T. J., Barry, C. A. Driedzic, W. R. 2004. Sequence and expression of a constitutive, facilitated glucose transporter (GLUT1) in Atlantic cod *Gadus morhua*. *J. Exp. Biol.* 207: 4697-4706.
- Hall, J. R., Richards, R. C., MacCormack, T. J., Ewart, K. V., Driedzic, W. R. 2005. Cloning of GLUT3 cDNA from Atlantic cod (*Gadus morhua*) and expression of GLUT1 and GLUT3 in response to hypoxia. *Biochim. Biophys. Acta* 1730: 245-252.
- Halliwell, B. and Chirico, S. 1993. Lipid peroxidation: its mechanism, measurement, and significance. *Am. J. Clin. Nutr.* 57:715S-725S.
- Halliwell, B. and Gutteridge, J.M. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. *Method. Enzymol.* 186:1-85.
- Halliwell, B. and Gutteridge, J.M.C. 2015. Free Radicals in Biology and Medicine, 5th Ed., Oxford University Press, pp. 944, Croydon, UK.
- Hansen, J.Ø., Berge, G.M., Hillestad, M., Krogdahl, Å., Galloway, T., Holm, H., Holm, J., Ruyter, B. 2008. Apparent digestion and apparent retention of lipid and fatty acids in Atlantic cod (*Gadus morhua*) fed increasing dietary lipid levels. *Aquaculture* 284, 159–166.
- Hastings, N., Agaba, M., Tocher, D.R., Leaver, M.J., Dick, J.R., Sargent, J.R., Teale A.J. 2001. A vertebrate fatty acid desaturase with Δ5 and Δ6 activities. *Proc. Natl. Acad. Sci. U.S.A.* 98: 14304-14309.
- Hastings, N., Agaba, M.K., Tocher, D.R., Zheng, X., Dickson, C.A., Dick, J.R., Teale, A.J. 2005. Molecular cloning and functional characterization of fatty acyl desaturase and elongase cDNAs involved in the production of eicosapentaenoic and docosahexaenoic acids from α-linolenic acid in Atlantic salmon (*Salmo salar*). *Mar. Biotechnol.* 6: 463-474.
- Hemre, G-I., Mommsen, T.P., Krogdahl, A. 2002a. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquacult Nutr.* 8:175–194.

Hemre, G.-I., Sandnes, K., Lie, Ø., Torrisen, O., Waagbø, R. 1995. Carbohydrate nutrition in Atlantic salmon, *Salmo salar* L.: growth and feed utilization. Aquac. Res. 26: 149–154.

Hemre, G. I., Bjørnevik, M., Beattie, C., Bjørnson, B. T., Hansen, T. 2002b. Growth and salt-water tolerance of juvenile Atlantic salmon, *Salmo salar*, reared under different combinations of dietary carbohydrate and photoperiod regime. Aquacult. Nutr. 8: 23-32.

Henderson, R.J. and Sargent, J.R. 1985. Fatty acid metabolism in fish. In: Nutrition and Feeding in Fish (Cowey, C.B. ed), pp. 349-363. Academic Press, New York.

Henderson, R.J. 1996. Fatty acid metabolism in freshwater fish with particular reference to polyunsaturated fatty acids. Arch. Tierernahr. 49:5-22.

Hernandez-Blazquez, F. J., Guerra, R. R., Kfouri, J. R., Bombonato, P. P., Cogliati, B., da Silva, J. R. M. C. 2006. Fat absorptive processes in the intestine of the Antarctic fish *Notothenia coriiceps* (Richardson, 1844). Polar Biol. 29: 831–836.

Hidalgo, F. and Alliot, E. 1988. Influence of water temperature on protein requirement and protein utilization in juvenile sea bass, *Dicentrarchus labrax*. Aquaculture 72: 115-129.

Hilton, J.W. and Atkinson, J.L. 1982. Response of rainbow trout (*Salmo gairdneri*) to increased levels of available carbohydrate in practical trout diets. Br. J. Nutr. 47: 597.

Hrytsenko, O., Pohajdak, B., Xu, B.-Y., Morrison, C., vanTol, B., Wright Jr, J. R. 2010. Cloning and molecular characterization of the glucose transporter 1 in tilapia (*Oreochromis niloticus*). Gen. Comp. Endocrinol. 165: 293-303.

Hung, S.S.O., Flynn-Aikins, F.K., Lutes, P.B., Xu, R. 1989. Ability of Juvenile White Sturgeon (*Acipenser transmontanus*) to utilize different carbohydrate sources. J. Nutr. 119: 727-733.

Ibeas C., Izquierdo M.S., Lorenzo A. 1994. Effect of different levels of n-3 highly unsaturated fatty acids on growth and fatty acid composition of juvenile gilthead seabream (*Sparus aurata*). Aquaculture 127: 177-188.

Ibeas C., Cejas J.R., Fores R., Badia P., Gomez T., Lorenzo A., Hernandez A. 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream (*Sparus aurata*) juveniles. Aquaculture 150: 91 - 102.

Iijima, N., Gotou, T., Kayama, M. 1995. Isolation and characterization of serum lipoproteins in red + sea bream. Fish. Sci. 61: 297–303.

Iijima, N., Kayama, M., Okazaki, M., Hara, I. 1985. Time course changes of lipid distribution in carp plasma lipoprotein after force-feeding with soybean oil. Nippon Suisan Gakkaishi 51: 467-471.

Iijima N, Aida S, Mankura M, Kayama M. 1990. Intestinal absorption and plasma transport of dietary triglyceride and phosphatidylcholine in the carp (*Cyprinus carpio*). Comp. Biochem. Physiol. A 96: 45– 55.

Izquierdo, M.S. and Henderson, R.J. 1998. The determination of lipase and phospholipase activities in gut contents of turbot (*Scophthalmus maximus*) by fluorescence-based assays. Fish Physiol. Biochem. 19: 153–162.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L.E., Rosenlund, G. 2003. Dietary lipid sources for seabream and sea bass: growth performance, tissue composition and flesh quality. Aquacult. Nutr. 9:397-407.

Izquierdo, M.S., Montero, D., Robaina. L.E., Caballero, M.J., Rosenlund, G., Ginés, R. 2005. Alteration in filled fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long period. Recovery of fatty acid profiles by fish oil feeding. Aquaculture 250:431-44.

Jaya-Ram, A., Ishak, S.D., Enyu, Y-L., Kuah, M-K., Wong, K-L., Shu-Chien, A.C. 2011. Molecular cloning and ontogenic mRNA expression of fatty acid desaturase in the carnivorous striped snakehead fish (*Channa striata*). Comp. Biochem. Physiol. A 158: 415–422.

Jin, J., Médale, F., Kamalam, B.S., Aguirre, P., Véron, V., Panserat, S. 2014. Comparison of glucose and lipid metabolic gene expressions between fat and lean lines of rainbow trout after a glucose load. PLOS One 9: e105548.

Jordal, A.-E.O., Lie, Ø., Torstensen, B.E. 2007. Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (*Salmo salar* L.). Aquacult. Nutr. 13: 114–130.

Jordal, A-E.O., Hordvik, I., Pelsers, M., Bernlohr, D.A., Torstensen, B.E. 2006. FABP3 and FABP10 in Atlantic salmon (*Salmo salar* L.): general effects of dietary fatty acid composition, and life cycle variations. Comp. Biochem. Physiol. B 145: 147–158.

Jutfelt, F., Olsen, R.E., Björnsson, B.T.h., Sundell, K. 2007. Parr-smolt transformation and dietary vegetable lipids affect intestinal nutrient uptake, barrier function and plasma cortisol levels in Atlantic salmon. *Aquaculture* 273: 298–311.

Kalogeropoulos N., Alexis M.N., Henderson R.J. 1992. Effects of dietary soybean and cod-liver oil levels on growth and body composition of gilthead bream (*Sparus aurata*). *Aquaculture* 104: 293-308.

Kamalam, B.S., Médale, F., Larroquet, L., Corraze, G., Panserat, S. 2013b. Metabolism and fatty acid profile in fat and lean rainbow trout lines fed with vegetable oil: effect of carbohydrates. *PloS one* 8: e76570.

Kamalam, B. S., Médale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S. Panserat, S. 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. *J. Exp. Biol.* 215: 2567-2578.

Kamalam, B. S., Panserat, S., Aguirre, P., Geurden, I., Fontagné-Dicharry, S., Médale, F. 2013a. Selection for high muscle fat in rainbow trout induces potentially higher chylomicron synthesis and PUFA biosynthesis in the intestine. *Comp. Biochem. Physiol. A* 164: 417-427.

Kaushik, S. J. 1998a. Whole body amino acid composition of European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*) with an estimation of their IAA requirement profiles. *Aquat. Living Resour.* 11: 355-358.

Kaushik, S.J., Gouillou-Coustans, M.F., Cho, C.Y. 1998b. Application of the recommendations on vitamin requirements of finfish by NRC (1993) to salmonids and sea bass using practical and purified diets. *Aquaculture* 161: 463–474.

Kaushik, S. J., Coves, D., Dutto, G., Blanc, D. 2004. Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture* 230: 391-404.

Kirchner, S., Panserat, S., Lim, P.L., Kaushik, S., Ferraris, R.P. 2008. The role of hepatic, renal and intestinal gluconeogenic enzymes in glucose homeostasis of juvenile rainbow trout. *J. Comp. Physiol. B. Biochem. Syst. Environ. Physiol.* 178:429–438.

Kissil, G. W. and Lupatsch, I. 2004. Successful replacement of fishmeal by plant proteins in diets for the gilthead seabream, *Sparus aurata* L. *Isr. J. Aquac. Bamidgeh* 56: 188-199.

Kissil, G. W., Cowey, C. B., Adron, J. W., Richards, R. H. 1981. Pyridoxine requirements of the gilthead bream, *Sparus aurata*. Aquaculture 23: 243-255.

Kissil, G. W., Lupatsch, I., Higgs, D. A., Hardy, R. W. 1997. Preliminary evaluation of rapeseed protein concentrate as an alternative to fish meal in diets for gilthead seabream (*Sparus aurata*). Isr. J. Aquac. Bamidgeh 49: 135–143.

Kjaer, M.A., Vegusdal, A., Gjoen, T., Rustan, A.C., Todorcevic, M., Ruyter, B. 2008. Effect of rapeseed oil and dietary n-3 fatty acids on triacylglycerol synthesis and secretion in Atlantic salmon hepatocytes. Biochim. Biophys. Acta 1781:112–122.

Kjær, M. A., Aursnes, I. A., Berge, G. M., Sørensen, M., Marchenko, Y., Gjøen, T., Ruyter, B. 2014. The influence of different dietary oil qualities on growth rate, feed utilization and oxidative stress in Atlantic cod. Aquacult Nutr. 20:192–204.

Klinger, D. and Naylor, R. 2012. Searching for solutions in aquaculture: charting a sustainable course. Annu. Rev. Environ. Resour. 37: 247-276.

Kokou, F., Rigos, G., Henry, M., Kentouri, M., Alexis, M. 2012. Growth performance, feed utilization and non-specific immune response of gilthead sea bream (*Sparus aurata* L.) fed graded levels of a bioprocessed soybean meal. Aquaculture 364: 74-81.

Kortner, T.M., Gu, J., Krogdahl, A., Bakke, A.M. 2013. Transcriptional regulation of cholesterol and bile acid metabolism after dietary soybean meal treatment in Atlantic salmon, *Salmo salar* L. Br. J. Nutr. 109: 593 – 604.

Kortner, T.M., Björkhem, I., Krasnov, A., Timmerhaus, G., Krogdahl, Å. 2014. Dietary cholesterol supplementation to a plant-based diet suppresses the complete pathway of cholesterol synthesis and induces bile acid production in Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 111:2089-103.

Koven, W.M., Henderson, R.J., Sargent, J.R. 1994. Lipid digestion in turbot (*Scophthalmus maximus*): I. Lipid class and fatty acid composition of digesta from different segments of the digestive tract. Fish Physiol. Biochem. 13: 69–79.

Krogdahl, A., Hemre, G. I., Mommsen, T. P. 2005. Carbohydrates in fish nutrition: Digestion and absorption in postlarval stages. Aquacult. Nutr. 11:103–122.

Kurtovic, I., Marshall, S.N., Zhao, X., Simpson, B.K. 2009. Lipases from Mammals and Fishes. Rev. Fish Sci. 17:18-40.

- Lanari, D. and D'Agaro, E. 2005. Alternative plant protein sources in sea bass diets. *Ital. J. Anim. Sci.* 4: 365-374.
- Lanari, D., Poli, B. M., Ballestrazzi, R., Lupi, P., D'Agaro, E., Mecatti, M. 1999. The effects of dietary fat and NFE levels on growing European sea bass (*Dicentrarchus labrax* L.). Growth rate, body and fillet composition, carcass traits and nutrient retention efficiency. *Aquaculture* 179: 351-364.
- Leaver, M.J., Bautista, J.M., Björnsson, T., Jönsson, E., Krey, G., Tocher, D.R., Torstensen, B.E. 2008a. Towards fish lipid nutrigenomics: current state and prospects for fin-fish. *Rev. Fisheries Sci.* 16: 71–92.
- Leaver, M.J., Villeneuve, L.A., Obach, A., Jensen, L., Bron, J.E., Tocher, D.R., Taggart, J.B. 2008b. Functional genomics reveals increases in cholesterol biosynthetic genes and highly unsaturated fatty acid biosynthesis after dietary substitution of fish oil with vegetable oils in Atlantic salmon (*Salmo salar*). *BMC Genomics* 9:299.
- Leaver, M. J., Taggart, J. B., Villeneuve, L., Bron, J. E., Guy, D. R., Bishop, S. C., Houston, R. D., Matika, O., Tocher, D. R. 2011. Heritability and mechanisms of n-3 long chain polyunsaturated fatty acid deposition in the flesh of Atlantic salmon. *Comp. Biochem. Physiol. D* 6: 62-69.
- Lee, S.-M. and Lee, J.H. 2004. Effect of dietary glucose, dextrin and starch on growth and body composition of juvenile starry flounder *Platichthys stellatus*. *Fish. Sci.* 70: 53–58.
- Leger, C., Bauchart, D., Flanzy, J. 1977. Some properties of pancreatic lipase in *Salmo gairdnerii* Rich.: Km, effects of bile salts and Ca²⁺, gel filtrations. *Comp. Biochem. Physiol. B* 57:359–363.
- Leung, L.Y. and Woo, N.Y. 2012. Influence of dietary carbohydrate level on endocrine status and hepatic carbohydrate metabolism in the marine fish *Sparus sarba*. *Fish Physiol. Biochem.* 38: 543–554.
- Li, Y., Monroig, Ó., Zhang, L., Wang, S., Zheng, X., Dick, J.R., You, C., Tocher, D.R. 2010. Vertebrate fatty acyl desaturase with Δ4 activity. *Proc. Natl. Acad. Sci. U. S. A.* 107: 16840–16845.
- Li, Y. Y., Hu, C. B., Zheng, Y. J., Xia, X. A., Xu, W. J., Wang, S. Q., Chen, W.Z., Sun, Z.W., Huang, J.H. 2008. The effects of dietary fatty acids on liver fatty acid composition

and delta 6-desaturase expression differ with ambient salinities in *Siganus canaliculatus*. Comp. Biochem. Physiol. B: 151:183–190.

Liang, X.F., Ogata, H.Y., Oku, H. 2002. Effect of dietary fatty acids on lipoprotein lipase gene expression in the liver and visceral adipose tissue of fed and starved red sea bream *Pagrus major*. Comp. Biochem. Physiol. A 132: 913–919.

Librán-Pérez, M., Figueiredo-Silva, A. C., Panserat, S., Geurden, I., Míguez, J. M., Polakof, S., Soengas, J. L. 2013. Response of hepatic lipid and glucose metabolism to a mixture or single fatty acids: possible presence of fatty acid-sensing mechanisms. Comp. Biochem. Physiol. A 164: 241-248.

Lie, Ø., Sandvin, A., Waagbø, R. 1993. Influence of dietary fatty acids on the lipid composition of lipoproteins in farmed Atlantic salmon (*Salmo salar*). Fish Physiol. Biochem. 12: 249–260.

Likimani T.A. and Wilson R.P. 1982 Effects of diet on lipogenic enzyme activities in channel catfish hepatic and adipose tissue. J. Nutr. 112: 112-117.

Liland, N.S., Espe, M., Rosenlund, G., Waagbø, R., Hjelle, J.I., Lie, Ø., Fontanillas, R., Torstensen, B.E. 2013. High levels of dietary phytosterols affect lipid metabolism and increase liver and plasma TAG in Atlantic salmon (*Salmo salar* L.). Br. J. Nutr. 110: 1958–1967.

Lin, Y.H. and Shiau, S.Y. 2007. Effects of dietary blend of fish oil with corn oil on growth and non-specific immune responses of grouper, *Epinephelus malabaricus*. Aquacult. Nutr. 13:137–144.

Liscum, L. 2008. Cholesterol biosynthesis. In: Biochemistry of Lipids, Lipoproteins and Membranes (Vance, D.E. and Vance, J.E. eds), pp. 399–420. 5th edition, Elsevier, Oxford.

Luquet, P. and Sabaut, J.J. 1974. Nutrition azotée et croissance chez la daurade et la truite. Colloque sur l'aquaculture, Actes des colloques(ed. CNEXO), no. 1.

Luo, Z., Tan, X. Y., Li, X. D., Yin, G. J. 2012. Effect of dietary arachidonic acid levels on growth performance, hepatic fatty acid profile, intermediary metabolism and antioxidant responses for juvenile *Synechogobius hasta*. Aquacult. Nutr. 18: 340-348.

- Luo, L., Xue, M., Vachot, C., Geurden, I., Kaushik, S. 2014. Dietary medium chain fatty acids from coconut oil have little effects on postprandial plasma metabolite profiles in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 420-421: 24-31.
- Lygren, B. and, Hemre, G.I. 2001. Influence of dietary carbohydrate on antioxidant enzyme activities in liver of Atlantic salmon (*Salmo salar* L.). Aquacult. Int. 9: 421427.
- Mansbach II, C.M. and Gorelick, F. 2007. Development and physiological regulation of intestinal lipid absorption. II. Dietary lipid absorption, complex lipid synthesis, and the intracellular packaging and secretion of chylomicrons. Am. J. Physiol. 293: 645 – 650.
- Marcouli, P. A., Alexis, M. N., Andriopoulou, A., Iliopoulou-Georgudaki, J. 2006. Dietary lysine requirement of juvenile gilthead seabream *Sparus aurata* L. Aquacult. Nutr. 12: 25-33.
- Martínez-Álvarez, R., Morales, A., Sanz, A. 2005. Antioxidant defenses in fish: biotic and abiotic factors. Rev. Fish Biol. Fish. 15: 75–88.
- Martínez-Llorens, S., Vidal, A. T., Moñino, A. V., Torres, M. P., Cerdá, M. J. 2007b. Effects of dietary soybean oil concentration on growth, nutrient utilization and muscle fatty acid composition of gilthead sea bream (*Sparus aurata* L.). Aquac. Res 38: 76–81.
- Martínez-Llorens, S., Moñino, A. V., Vidal, A. T., Salvador, V. J. M., Torres, M. P., Cerdá, M. J. 2007a. Soybean meal as a protein source in gilthead sea bream (*Sparus aurata* L.) diets: effects on growth and nutrient utilization. Aquac. Res. 38: 82-90.
- Martins, D.A., Valente, L.M.P., Lall, S.P. 2009. Apparent digestibility of lipid and fatty acids in fish oil, poultry fat and vegetable oil diets by Atlantic halibut, *Hippoglossus hippoglossus* L. Aquaculture 294: 132-137.
- Mazurais, D., Glynatsi, N., Darias, M.J., Christodouloupolou, S., Cahu, C., Zambonino-Infante, J.-L., Koumoundouros, G. 2009. Optimal levels of vitamin A for reduced deformity incidence during development of European seabass larvae (*Dicentrarchus labrax*) depend on malformation type. Aquaculture 294: 262–270.
- Mehta, S.K. and Gowder, S.J.T. 2015. Members of antioxidant machinery and their functions. In: Basic principles and clinical significance of oxidative stress (Gowder, S.J.T. ed), pp 59 -85. InTech, Rijeka, Croatia.

Menoyo, D., López-Bote, C.J., Bautista, J.M., Obach, A. 2003. Growth, digestibility and fatty acid utilization in large Atlantic salmon (*Salmo salar*) fed varying levels of n-3 and saturated fatty acids. *Aquaculture* 225: 295–307.

Menoyo, D., Izquierdo, M.S., Robaina, L., Gines, R., López-Bote, C.J., Bautista, J.M. 2004. Adaptation of lipid metabolism, tissue composition and flesh quality in gilthead sea bream (*Sparus aurata*) to the replacement of dietary fish oil by linseed and soyabean oils. *Br. J. Nutr.* 92: 41–52.

Menoyo, D., Diez, A., López-Bote, C. J., Casado, S., Obach, A., Bautista, J. M. 2006. Dietary fat type affects lipid metabolism in Atlantic salmon (*Salmo salar* L.) and differentially regulates glucose transporter GLUT4 expression in muscle. *Aquaculture* 261: 294-304.

Merchie, G., Lavens, P., Starch, V., Abel, U., Nelis, H., De Leenheer, A., Sorgeloos, P. 1996. Influence of dietary vitamin C dosage on turbot (*Scophthalmus maximus*) and European sea bass (*Dicentrarchus labrax*) nursery stages. *Comp. Biochem. Physiol. A* 114: 123–133.

Messina, M., Piccolo, G., Tulli, F., Messina, C. M., Cardinaletti, G., Tibaldi, E. 2013. Lipid composition and metabolism of European sea bass (*Dicentrarchus labrax* L.) fed diets containing wheat gluten and legume meals as substitutes for fish meal. *Aquaculture* 376:6-14.

Metailler, R., Aldrin, J. F., Messager, J. L., Mevel, G., Stephan, G. 1981. Feeding of European sea bass *Dicentrarchus labrax*: role of protein and energy source. *J. World Maricul. Soc.* 12:117-118.

Metón, I., Mediavilla, D., Caseras, A., Canto, E., Fernández, F., Baanante, I.V. 1999. Effect of diet composition and ration size on key enzyme activities of glycolysis-gluconeogenesis, the pentose phosphate pathway and amino acid metabolism in liver of gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* 82:223–232.

Miller, M.R., Nichols, P.D., Carter, C.G. 2010. New alternative n-3 long chain polyunsaturated fatty acid-rich oil sources. In: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (Turchini, G.M., Ng, W.K., Tocher, D.R. eds), pp. 325–349. Taylor & Francis, CRC Press, Boca Raton.

Mithieux, G. 2005. The new functions of the gut in the control of glucose homeostasis. *Curr. Opin. Clin. Nutr. Metab. Care* 8: 445.

Mohd-Yusof, N.Y., Monroig, Ó., Mohd-Adnan, A., Wan, K.-L., Tocher, D.R. 2010. Investigation of highly unsaturated fatty acid metabolism in the Asian sea bass, *Lates calcarifer*. Fish Physiol. Biochem. 36: 827-843.

Monroig, Ó., Navarro, J.C., Tocher, D.R. 2011a. Long-chain polyunsaturated fatty acids in fish: recent advances on desaturases and elongases involved in their biosynthesis. In: Proceedings of the XI International Symposium on Aquaculture Nutrition (Cruz-Suarez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D.A., Gamboa-Delgado, J., Hernández-Hernández, L.H. eds.), pp. 257–282. Universidad Autónoma de Nuevo León, Monterrey, Nuevo León.

Monroig, Ó., Li, Y., Tocher, D.R. 2011b. Delta-8 desaturation activity varies among fatty acyl desaturases of teleost fish: high activity in delta-6 desaturases of marine species. Comp. Biochem. Physiol. B 159: 206–213.

Monroig, Ó., Tocher, D.R., Navarro, J.C. 2013. Biosynthesis of polyunsaturated fatty acids in marine invertebrates: molecular studies in cephalopods. Mar. Drugs 11: 3998–4018.

Monroig, Ó., Rotllant, J., Sánchez, E., Cerdá-Reverter, J.M., Tocher, D.R. 2009. Expression of long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis genes during zebrafish *Danio rerio* early embryogenesis. Biochim. Biophys. Acta 1791: 1093–1101.

Monroig, Ó., Webb, K., Ibarra-Castro, L., Holt, G.J., Tocher, D.R. 2011c. Biosynthesis of long-chain polyunsaturated fatty acids in marine fish: Characterization of an Elovl4-like elongase from cobia *Rachycentron canadum* and activation of the pathway during early life stages. Aquaculture 312: 145–153.

Monroig, Ó., Zheng, X., Morais, S., Leaver, M.J., Taggart, J.B., Tocher, D.R. 2010a. Multiple genes for functional Δ6 fatty acyl desaturases (Fad) in Atlantic salmon (*Salmo salar* L.): Gene and cDNA characterization, functional expression, tissue distribution and nutritional regulation. Biochim. Biophys. Acta 1801: 1072–1081.

Monroig, Ó., Rotllant, J., Cerdá-Reverter, J.M., Dick, J.R., Figueras, A., Tocher, D.R. 2010b. Expression and role of Elovl4 elongases in biosynthesis of very long-chain fatty acids during zebrafish *Danio rerio* early embryonic development. Biochim. Biophys. Acta 1801: 1145-1154.

Monroig, Ó., Navarro, J.C., Hontoria, F., Tocher, D.R., Martínez-Palacios, C., Fonseca-Madrigal, J. 2014. Diversification of substrate specificities in teleostei Fads2 fatty acyl desaturases: cloning and functional characterisation of $\Delta 4$ and bifunctional $\Delta 6\Delta 5$ desaturases in the freshwater atherinopsid *Chirostoma estor*. *J. Lipid Res.* 55:1408–1419.

Montero, D., Robaina, L., Caballero, R., Ginés, R., Izquierdo, M.S. 2005 Growth, feed utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: a time-course study on the effect of a re-feeding period with a 100% fish oil diet. *Aquaculture* 248: 121–134.

Montero D., Kalinowski T., Obach A., Robaina L., Tort L., Caballero, M.J., Izquierdo M.S. 2003. Vegetable lipid sources for gilthead sea bream (*Sparus aurata*): effects on fish health. *Aquaculture* 225: 353–370.

Montero, D., Grasso, V., Izquierdo, M. S., Ganga, R., Real, F., Tort, L., Caballero, M.J., Acosta, F. 2008. Total substitution of fish oil by vegetable oils in gilthead seabream (*Sparus aurata*) diets: effects on hepatic Mx expression and some immune parameters. *Fish Shellfish. Immunol.* 24:147e55.

Montero, D., Mathlouthi, F., Tort, L., Afonso, J. M., Torrecillas, S., Fernández-Vaquero, A., Negrín, D., Izquierdo, M. S. 2010. Replacement of dietary fish oil by vegetable oils affects humoral immunity and expression of pro-inflammatory cytokines genes in gilthead sea bream *Sparus aurata*. *Fish Shellfish. Immun.* 29: 1073-1081.

Morais, S., Edvardsen, R.B., Bell, J.G., Tocher, D.R. 2012b. Transcriptomic analyses of genes of lipid metabolism in intestine of Atlantic cod (*Gadus morhua*) fed diets with increasing proportions of Camelina oil as replacement for fish oil. *Comp. Biochem. Physiol. B* 161: 283–293.

Morais S, Monroig O, Zheng X, Leaver MJ, Tocher DR 2009. Highly unsaturated fatty acid synthesis in Atlantic salmon: characterization of ELOVL5- and ELOVL2-like elongases. *Mar Biotechnol.* 1: 627–639.

Morais, S., Mourente, G., Ortega, A., Tocher, J.A., Tocher, D.R. 2011a. Expression of fatty acyl desaturase and elongase genes, and evolution of DHA:EPA ratio during development of Atlantic bluefin tuna (*Thunnus thynnus* L.). *Aquaculture* 313: 129-139.

Morais, S., Castanheira, F., Martínez-Rubio, L., Conceição, L.E.C., Tocher, D.R. 2012a. Long chain polyunsaturated fatty acid synthesis in a marine vertebrate: ontogenetic and

nutritional regulation of a fatty acyl desaturase with Δ4 activity. *Biochim. Biophys. Acta* 1821: 660–671.

Morais, S., Pratoomyot, J., Torstensen, B.E., Taggart, J.B., Guy, D.R., Bell, J.G., Tocher, D.R. 2011b. Diet x genotype interactions in hepatic cholesterol and lipoprotein metabolism in Atlantic salmon (*Salmo salar*) in response to replacement of dietary fish oil with vegetable oil. *Br. J. Nutr.* 106: 1457–1469.

Morais, S., Pratoomyot, J., Taggart, J.B., Bron, J.E., Guy, D.R., Bell, J.G., Tocher, D.R. 2011c. Genotype-specific responses in Atlantic salmon (*Salmo salar*) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genomics* 12: 255.

Morais, S., Silva, T., Carneiro, O., Rodrigues, P., Guy, D.R., Bron, J.E., Taggart, J.B., Bell, J.G., Tocher, D.R. 2012c. Effects of genotype and dietary fish oil replacement with vegetable oil on the intestinal transcriptome and proteome of Atlantic salmon (*Salmo salar*). *BMC Genomics* 13: 448 -444.

Morales, A.E., Pérez-Jiménez, A., Hidalgo, M.C., Abellán, E., Cardenete, G. 2004. Oxidative stress and antioxidant defenses after prolonged starvation in *Dentex dentex* liver. *Comp. Biochem. Physiol. C* 139: 153–161.

Moreira, I., Peres, H., Couto, A., Enes, P. and Oliva-Teles, A. 2008. Temperature and dietary carbohydrate level effects on performance and metabolic utilisation of diets in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* 274: 153-160.

Morris, P. C. and Davies, S. J. 1995a. The Requirement of the Gilthead Seabream (*Sparus aurata* L) for Nicotinic Acid. *Anim. Sci.* 61: 437-443.

Morris, P. C. and Davies, S. J. 1995b. Thiamin supplementation of diets containing varied lipid - carbohydrate ratio given to gilthead seabream (*Sparus aurata* L). *Anim. Sci.* 61: 597-603.

Mourente, G. and Tocher, D.R. 1993. Incorporation and metabolism of C-14-labeled polyunsaturated fatty-acids in juvenile gilthead sea bream *Sparus aurata* in vivo. *Fish Physiol. Biochem.* 10: 443–453.

Mourente, G. and Dick, J.R. 2002. Influence of partial substitution of dietary fish oil by vegetable oils on the metabolism (desaturation and β-oxidation) of [1-¹⁴C] 18:3n-3 in isolated hepatocytes of European sea bass (*Dicentrarchus labrax* L.). *Fish Physiol. Biochem.* 26: 297–308.

Mourente, G., Good, J.E., Bell, J.G. 2005b. Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass (*Dicentrarchus labrax* L.): effects on flesh fatty acid composition, plasma prostaglandins E2 and F2a, immune function and effectiveness of a fish oil finishing diet. *Aquacult. Nutr.* 11:25-40.

Mourente, G., Bell, J.G., Tocher, D.R. 2007a. Does dietary tocopherol level affect fatty acid metabolism in fish? *Fish Physiol. Biochem.* 33: 269–280.

Mourente, G., Díaz-Salvago, E., Bell, J.G., Tocher, D.R. 2002. Increased activities of hepatic antioxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. *Aquaculture* 214: 343–361.

Mourente, G., Dick, J.R., Bell, J.G., Tocher, D.R. 2005a. Effect of partial substitution of dietary fish oil by vegetable oils on desaturation and oxidation of [1-¹⁴C]18:3 n-3 and [1-¹⁴C]20:5 n-3 in hepatocytes and enterocytes of European sea bass (*Dicentrarchus labrax* L.). *Aquaculture* 248: 173–186.

Mourente, G., Good, J. E., Thompson, K. D., Bell, J. G. 2007b. Effects of partial substitution of dietary fish oil with blends of vegetable oils, on blood leucocyte fatty acid compositions, immune function and histology in European sea bass (*Dicentrarchus labrax* L.). *Br. J. Nutr.* 98: 770-779.

Mu, H. and Hoy, C.E. 2004. The digestion of dietary triacylglycerols. *Prog. Lipid Res.* 43: 105–133.

Nanton, D.A., Mcniven, M.A., Lall, S.P. 2006. Serum lipoproteins in haddock, *Melanogrammus aeglefinus* L. *Aquacult. Nutr.* 12: 363–371.

Nanton, D.A., Lall, S.P., Ross, N.W., McNiven, M.A. 2003. Effect of dietary lipid level on fatty acidb-oxidation and lipid composition in various tissues of haddock, *Melanogrammus aeglefinus* L. *Comp. Biochem. Physiol.* 135: 95–108.

Natarajan, M., Ross, B., Ross, L. 1992. Susceptibility of carp and tilapia α -amylase to purified wheat amylase inhibitor. *Aquaculture* 102: 265-274.

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., Nichols, P.D. 2009. Feeding aquaculture in an era of finite resources. *Proc. Natl. Acad. Sci.* 106: 15103-15110.

Nelson, D. L. and Cox, M. M. 2005. Lehninger's Principles of Biochemistry, 4th Ed., WH Freeman and Company, 1126pp., New York.

Ng, W.K., Sigholt, T., Bell, J.G. 2004. The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. *Aquac. Res.* 35: 1228–1237.

Ng, W.-K., Campbell, P.J., Dick, J.R., Bell, J.G. 2003. Interactive effects of dietary palm oil concentration and water temperature on lipid digestibility in rainbow trout, *Oncorhynchus mykiss*. *Lipids* 38: 1031-1038.

Ng, W. K., Chong, C. Y., Wang, Y., Romano, N. 2013. Effects of dietary fish and vegetable oils on the growth, tissue fatty acid composition, oxidative stability and vitamin E content of red hybrid tilapia and efficacy of using fish oil finishing diets. *Aquaculture* 372: 97-110.

Noori, S. 2012. An overview of oxidative stress and antioxidant defensive system. *Open Access Sci. Rep.* 1: 413.

Nordrum, S., Krogdahl, A., Røsjø, C., Olli, J. J., Holm, H. 2000. Effects of methionine, cysteine and medium chain tryglycerides on nutrient digestibility, absorption of aminoacids along the intestinal tract and nutrient retention in Atlantic salmon (*Salmo salar* L.) under pair-feeding regime. *Aquaculture* 186: 341–360.

NRC (National Research Council).1993. Nutrient Requirements of Fish. The National Academy Press, 114 pp., Washington D.C.

NRC (National Research Council). 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, 376pp., Washington DC

Oliva-Teles, A. 2000. Recent advances in European sea bass and gilthead sea bream nutrition. *Aquacult. Int.* 8: 477–492.

Oliva-Teles, A. and Pimentel-Rodrigues, A. 2004. Phosphorus requirement of European seabass (*Dicentrarchus labrax* L.) juveniles. *Aquac. Res.* 35: 636–642.

Oliva-Teles, A., Enes, P., Peres, H. 2015. Replacing fishmeal and fish oil in industrial aquafeeds for carnivorous fish. In: Feed and Feeding Practices in Aquaculture (Davis, A., ed.), pp. 203-233.Woodhead Publishing, Cambridge, UK.

Olsen, R. E., and Ringø, E. 1997. Lipid digestibility in fish: a review. *Recent Res. Dev. Lipids Res.* 1:199–265.

Olsen, R.L. and Hasan, M.R. 2012. A limited supply of fishmeal: impact on future increases in global aquaculture production. *Trends Food Sci. Technol.* 27: 120-e128.

Olsen, R.E., Henderson, R.J., Ringo, E. 1998. Digestion and selective absorption of dietary fatty acids in Arctic charr, *Salvelinus alpinus*. *Aquacult. Nutr.* 4: 13–21.

Olsen, R. E., Myklebust, R., Kaino, T., Ringø, E. 1999. Lipid digestibility and ultrastructural changes in the enterocytes of Arctic char *Salvelinus alpinus* L. fed linseed oil and soybean lecithin. *Fish Physiol. Biochem.* 21: 35–44.

Olsen, R. E., Waagbø, R., Melle, W., Ringø, E., Lall, S. P. 2010. Alternative marine resources. In: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (Turchini, G.M., Ng, W.K., Tocher, D.R. eds), pp. 267-324. Taylor & Francis, CRC Press, Boca Raton.

Olsvik, P.A., Torstensen, B.E., Berntssen, M.H.G. 2007. Effects of complete replacement of fish oil with plant oil on gastrointestinal cell death, proliferation and transcription of eight genes encoding proteins responding to cellular stress in Atlantic salmon *Salmo salar* L. *J. Fish Biol.* 71: 550–568.

Østbye, T.K., Kjær, M. A., Rørå, A. M. B., Torstensen, B., Ruyter, B. 2011. High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. *Aquacult. Nutr.* 17: 177–190.

Oxley, A., Torstensen, B.E., Rustan, A.C., Olsen, R.E. 2005. Enzyme activities of intestinal triacylglycerol and phosphatidylcholine biosynthesis in Atlantic salmon (*Salmo salar* L.). *Comp. Biochem. Physiol. B* 141: 77–87.

Oxley, A., Jutfelt, F., Sundell, K., Olsen, R.E. 2007. Sn-2-monoacylglycerol, not glycerol, is preferentially utilised for triacylglycerol and phosphatidylcholine biosynthesis in Atlantic salmon (*Salmo salar* L.) intestine. *Comp. Biochem. Physiol. B* 146:115-23.

Ozcan, A. and Ogun, M. 2015. Biochemistry of Reactive Oxygen and Nitrogen Species. In: Basic principles and clinical significance of oxidative stress (Gowder, S.J.T. ed), pp 37 -58. InTech, Rijeka, Croatia.

Panserat, S., Kaushik, S., Médale, F. 2013. Rainbow trout as a model for nutrition and nutrient metabolism studies. In: Trout: from physiology to conservation (Polakof, S. and Moon, T. W. eds.), Nova Science Publishers, pp. 131-153.

- Panserat, S., Plagnes-Juan, E., Kaushik, S. 2002a. Gluconeogenic enzyme gene expression is decreased by dietary carbohydrates in common carp (*Cyprinus carpio*) and gilthead seabream (*Sparus aurata*). *Biochim. Biophys. Acta.* 1579: 35-42.
- Panserat, S., Perrin, A., Kaushik, S. 2002b. High dietary lipids induce liver glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). *J. Nutr.* 132: 137-141.
- Panserat, S., Plagnes-Juan, E., Brèque, J., Kaushik, S. 2001. Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* 204: 359-365.
- Panserat, S., Kolditz, C., Richard, N., Plagnes-Juan, E., Piumi, F., Esquerré, D., Médale, F., Corraze, G., Kaushik, S. 2008. Hepatic gene expression profiles in juvenile rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or fish oil-free diets. *Br. J. Nutr.* 100: 953-967.
- Panserat, S., Skiba-Cassy, S., Seiliez, I., Lansard, M., Plagnes-Juan, E., Vachot, C., Aguirre, P., Larroquet, L., Chavergnac, G., Médale, F., Corraze, G., Kaushik, S., Moon, T.W. 2009. Metformin improves postprandial glucose homeostasis in rainbow trout fed dietary carbohydrates: a link with the induction of hepatic lipogenic capacities? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 297: R707-R715.
- Peng, S., Chen, L., Qin, J. G., Hou, J., Yu, N., Long, Z., Ye, J., Sun, X. 2008. Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, *Acanthopagrus schlegeli*. *Aquaculture* 276: 154-161.
- Peng, M., Xu, W., Mai, K., Zhou, H., Zhang, Y., Liufu, Z., Zhang, K., Ai, Q. 2014. Growth performance, lipid deposition and hepatic lipid metabolism related gene expression in juvenile turbot (*Scophthalmus maximus* L.) fed diets with various fish oil substitution levels by soybean oil. *Aquaculture* 433:442-449.
- Pereira, T.G. and Oliva-Teles, A. 2002. Preliminary evaluation of pea seed meal in diets for gilthead sea bream (*Sparus aurata*) juveniles. *Aquac. Res.* 33: 1183-1189.
- Pereira, T.G. and Oliva-Teles, A. 2003. Evaluation of corn gluten meal as a protein source in diets for gilthead sea bream (*Sparus aurata* L.) juveniles. *Aquac. Res.* 34: 1111-1117.

- Pereira, T.G. and Oliva-Teles, A. 2004. Evaluation of micronized lupin seed meal as an alternative protein source in diets for gilthead sea bream *Sparus aurata* L. juveniles. *Aquac. Res.* 35: 828–835.
- Peres, M. H. and Oliva-Teles, A. 1999a. Influence of temperature on protein utilization in juvenile European seabass (*Dicentrarchus labrax*). *Aquaculture* 170: 337-348.
- Peres, M. H. and Oliva-Teles, A. 1999b. Effect of dietary lipid level on growth performance and feed utilization by European sea bass juveniles (*Dicentrarchus labrax*). *Aquaculture* 179: 325-334.
- Peres, H. and Oliva-Teles, A. 2002. Utilization of raw and gelatinized starch by European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture* 205:287–299.
- Peres, H. and Oliva-Teles, A. 2009. The optimum dietary essential amino acid profile for gilthead seabream (*Sparus aurata*) juveniles. *Aquaculture* 296: 81-86.
- Pérez, L., Gonzalez, H., Jover, M., Fernández-Carmona, J. 1997. Growth of European sea bass fingerlings (*Dicentrarchus labrax*) fed extruded diets containing varying levels of protein, lipid and carbohydrate. *Aquaculture* 156: 183-193.
- Pérez-Jiménez, A., Peres, H., Rubio, V.C., Oliva-Teles, A. 2012. The effect of dietary methionine and white tea on oxidative status of gilthead sea bream (*Sparus aurata*). *Br. J. Nutr.* 108: 1202 - 1209.
- Pérez-Jiménez, A., Hidalgo, M.C., Morales, A.E., Arizcun, M., Abellan, E., Cardenete, G. 2009. Antioxidant enzymatic defenses and oxidative damage in *Dentex dentex* fed on different dietary macronutrient levels. *Comp. Biochem. Physiol. C* 150: 537–545.
- Pimentel-Rodrigues, A.M. and Oliva-Teles, A. 2001. Phosphorus requirements of gilthead sea bream (*Sparus aurata* L.) juveniles. *Aquac. Res.* 32: 157–161.
- Planas, J. V., Capilla, E., Gutiérrez, J. 2000. Molecular identification of a glucose transporter from fish muscle1. *FEBS Lett.* 481: 266-270.
- Polakof, S., Álvarez, R., Soengas, J.L. 2010. Gut glucose metabolism in rainbow trout: implications in glucose homeostasis and glucosensing capacity. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 299: 19–32.
- Polakof, S., Panserat, S., Soengas, J. L., Moon, T. W. 2012. Glucose metabolism in fish: a review. *J. Comp. Physiol. B* 182: 1015-1045.

Puangkaew, J., Kiron, V., Satoh, S., Watanabe, T. 2005. Antioxidant defense of rainbow trout (*Oncorhynchus mykiss*) in relation to dietary n-3 highly unsaturated fatty acids and vitamin E contents. *Comp. Biochem. Physiol. C* 140: 187–196.

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

Richard, N., Mourente, G., Kaushik, S., Corraze, G. 2006b. Replacement of a large portion of fish oil by vegetable oils does not affect lipogenesis, lipid transport and tissue lipid uptake in European seabass (*Dicentrarchus labrax* L.). *Aquaculture* 261:1077–1087.

Richard, N., Kaushik, S., Larroquet, L., Panserat, S., Corraze, G. 2006a. Replacing dietary fish oil by vegetable oils has little effect on lipogenesis, lipid transport and tissue lipid uptake in rainbow trout (*Oncorhynchus mykiss*). *Br. J. Nutr.* 96: 299–309.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M. T., Panserat, S. 2015. Glucose metabolism and gene expression in juvenile zebrafish (*Danio rerio*) challenged with a high carbohydrate diet: effects of an acute glucose stimulus during late embryonic life. *Br. J. Nutr.* 113: 403-413.

Sabaut, J.J. and Luquet, P. 1973. Nutritional requirements of the gilthead bream *Chrysophrys aurata*. Quantitative protein requirements. *Marine Biology* 18: 50–54.

Sæle, O., Nordgreen, A., Olsvik, P.A., Hamre, K. 2011. Characterisation and expression of secretory phospholipase A2 group IB during ontogeny of Atlantic cod (*Gadus morhua*). *Br. J. Nutr.* 105: 228–237.

Sagone, A.L., Greenwald, J., Kraut, E.H., Bianchine, J., Singh, D. 1983. Glucose: a role as a free radical scavenger in biological systems. *J. Lab. Clin. Med.* 101: 97–103.

Santigosa, E., Sánchez, J., Médale, F., Kaushik, S., Pérez-Sánchez, J., Gallardo, M. A. 2008. Modifications of digestive enzymes in trout (*Oncorhynchus mykiss*) and sea bream (*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. *Aquaculture* 282: 68–74.

Santigosa, E., García-Meilán, I., María Valentín, J., Navarro, I., Pérez-Sánchez, J., Ángeles Gallardo, M. 2011b. Plant oils' inclusion in high fish meal-substituted diets:

effect on digestion and nutrient absorption in gilthead sea bream (*Sparus aurata* L.). Aquac. Res. 42: 962-974.

Santigosa, E., Geay, F., Tonon, T., Le Delliou, H., Kuhl, H., Reinhardt, R., Corcos, L., Cahu, C., Zambonino-Infante, J.L., Mazurais, D. 2011a. Cloning, tissue expression analysis, and functional characterization of two $\Delta 6$ -desaturase variants of sea bass (*Dicentrarchus labrax* L.). Mar. Biootechnol. 13: 22-31.

Santinha, P. J. M., Gomes, E. F. S., Coimbra, J. O. 1996. Effects of protein level of the diet on digestibility and growth of gilthead sea bream, *Sparus auratus* L. Aquacult. Nutr. 2: 81-87.

Santinha, P. J. M., Médale, F., Corraze, G., Gomes, E. F. S. 1999. Effects of the dietary protein: lipid ratio on growth and nutrient utilization in gilthead seabream (*Sparus aurata* L.). Aquacult. Nutr. 5: 147-156.

Santulli, A., Curatolo, A., Modica, A., D'Amelio, V. 1988. Time-course changes of plasma lipid levels and lipoprotein pattern after feeding in cultured sea bass, *Dicentrarchus labrax* L. J. Fish Biol. 32: 859-867.

Santulli, A., Messina, C., Modica, A., Curatolo, A., D`Amelio, V. 1996. Lipid and apolipoprotein composition of lipoproteins of European sea bass (*Dicentrarchus labrax* L.). Comp. Biochem. Physiol. B 114: 321–326.

Sargent, J.R., Tocher, D.R., Bell, J.G. 2002. The lipids. In: Fish Nutrition (Halver, J.E., Hardy, R.W., eds.), pp. 181–257. 3rd edition, Academic Press, San Diego.

Sarker, M.A-A., Yamamoto, Y., Haga, Y., Sarker, M.S.A., Miwa, M., Yoshizaki, G., Satoh, S. 2011. Influences of low salinity and dietary fatty acids on fatty acid composition and fatty acid desaturase and elongase expression in red sea bream *Pagrus major*. Fish. Sci. 77: 385–396.

Seiliez, I., Panserat, S., Kaushik, S., Bergot, P. 2001. Cloning, tissue distribution and nutritional regulation of a $\Delta 6$ -desaturase-like enzyme in rainbow trout. Comp. Biochem. Physiol. B 130: 83–93.

Seiliez, I., Panserat, S., Corraze, G., Kaushik, S., Bergot, P. 2003. Cloning and nutritional regulation of a $\Delta 6$ -desaturase-like enzyme in the marine teleost gilthead sea bream (*Sparus aurata*). Comp. Biochem. Physiol. B 135: 449–460.

- Sheridan, M.A.1988. Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. Comp. Biochem. Physiol. B 90: 679 –690.
- Shi, Y. and Cheng, D. 2009. Beyond triglyceride synthesis: the dynamic functional roles of MGAT and DGAT enzymes in energy metabolism. Am. J. Physiol. Endocrinol. Metab. 297:10-18.
- Shikata, T. and Shimeno, S. 1994. Regulation of Carbohydrate Metabolism in Fish-XX. Metabolic Response to Dietary Stearic Acid, Linoleic Acid, and Highly Unsaturated Fatty Acid in Carp. Fish. Sci. 60: 735-739.
- Shikata, T., Iwanaga, S., Shimeno, S.1994. Effects of dietary glucose, fructose, and galactose on hepatopancreatic enzyme activities and body composition in carp. Fish. Sci. 60: 613-617.
- Shimeno, S., Ming, D.-C., Takeda, M. 1993. Metabolic response to dietary carbohydrate to lipid ratios in *Oreochromis niloticus*. Nippon Suisan Gakkaishi 59: 827–833.
- Singh, R. P., and Nose, T. 1967. Digestibility of carbohydrates in young rainbow trout. Bull. Freshwater Fish. Res. Lab: 17: 21-25.
- Skalli, A. and Robin, J. H. 2004. Requirement of n-3 long chain polyunsaturated fatty acids for European sea bass (*Dicentrarchus labrax*) juveniles: growth and fatty acid composition. Aquaculture 240: 399-415.
- Skiba-Cassy, S., Panserat, S., Larquier, M., Dias, K., Surget, A., Plagnes-Juan, E., Kaushik, S., Seiliez, I. 2013. Apparent low ability of liver and muscle to adapt to variation of dietary carbohydrate: protein ratio in rainbow trout (*Oncorhynchus mykiss*). Lipids 1500: 5.
- Soengas, J.L. and Moon, T.W. 1998. Transport and metabolism of glucose in isolated enterocytes of the black bullhead *Ictalurus melas*: effects of diet and hormones. J. Exp. Biol. 201: 3263–3273.
- Sprecher, H. 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim. Biophys. Acta 1486: 219–231.
- Stéphan, G., Guillaume, J., Lamour, F. 1995. Lipid peroxidation in turbot (*Scophthalmus maximus*) tissue: effect of dietary vitamin E and dietary n-6 or n-3 polyunsaturated fatty acids. Aquaculture 130: 251–268.

- Sitjà-Bobadilla, A., Pena-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sánchez, J. 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus aurata*). Aquaculture 249: 387-400.
- Stone, D. A. J. 2003. Dietary carbohydrate utilization by fish. Rev. Fish. Sci. 11:337–369.
- Storch, J. and Thumser, A. E. 2010. Tissue-specific functions in the fatty acid-binding protein family. J. Biol. Chem. 285: 32679-32683.
- Storebakken, T., Shearer, K.D., Refstie, S., Lagocki, S., McCool, J. 1998. Interactions between salinity, dietary carbohydrate concentration on the digestibility of macronutrients and energy in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 163: 347-359.
- Storey, K.B. 1996. Oxidative stress: animal adaptations in nature. Braz. J. Med. Biol. Res. 29: 1715–1733.
- Stubhaug, I., Frøyland, L., Torstensen, B.E. 2005b. β -Oxidation capacity of red and white muscle and liver in Atlantic salmon (*Salmo salar* L.)—effects of increasing dietary levels of rapeseed oil (0–100%) and olive oil (50%) to replace capelin oil. Lipids 40: 39–47.
- Stubhaug, I., Lie, Ø., Torstensen, B.E. 2006. β -oxidation capacity in liver increases during parr-smolt transformation of Atlantic salmon (*Salmo salar* L.) fed vegetable and fish oil. J. Fish. Biol. 69: 504–517.
- Stubhaug, I., Lie, Ø., Torstensen, B.E. 2007. Fatty acid productive value and β -oxidation capacity in Atlantic salmon tissues (*Salmo salar* L.) fed on different lipid sources along the whole growth period. Aquacult. Nutr. 13: 145–155.
- Stubhaug, I., Tocher, D.R., Bell, J.G., Dick, J.R., Torstensen, B.E. 2005a. Fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) hepatocytes and influence of dietary vegetable oil. Biochim. Biophys. Acta 1734: 277–288.
- Tacon, A.G.J., Metian, M., Hasan, M.R. 2009. Feed ingredients and fertilizers for farmed aquatic animals: sources and composition. In: FAO Fisheries and Aquaculture Technical Paper. No. 540. FAO, 209pp., Rome.
- Tacon, A.G.J., Hasan, M.R., Metian, M. 2011. Demand and supply of feed ingredients for farmed fish and crustaceans. Trends and prospects. In: FAO Fisheries and Aquaculture Technical Paper. No. 564. FAO, 87 pp., Rome.

Tan, Q., Xie, S., Zhu, X., Lei, W., Yang, Y. 2006. Effect of dietary carbohydrate sources on growth performance and utilization for gibel carp (*Carassius auratus gibelio*) and Chinese long snout catfish (*Leiocassis longirostris* Günther). *Aquacult. Nutr.* 12:61–70.

Teerijoki, H., Krasnov, A., Pitkänen, T. I., Mölsä, H. 2000. Cloning and characterization of glucose transporter in teleost fish rainbow trout (*Oncorhynchus mykiss*). *Biochim. Biophys. Acta* 1494: 290-294.

Tibaldi, E. and Lanari, D. 1991. Optimal dietary lysine levels for growth and protein utilisation of fingerling sea bass (*Dicentrarchus labrax* L.) fed semipurified diets. *Aquaculture* 95: 297–304.

Tibaldi, E., Tulli, F., Lanari, D. 1994. Arginine requirement and effect of different dietary arginine and lysine levels for fingerlings sea bass (*Dicentrarchus labrax*). *Aquaculture* 127: 207–218.

Tibaldi, E. and Tulli, F. 1999. Dietary threonine requirement of juvenile sea bass (*Dicentrarchus labrax*). *Aquaculture* 175: 155–166.

Tibaldi, E., Lanari, D., Ballestrazzi, R., Tulli, F., Pinosa, M. 1993. Preliminary evaluation of the arginine requirement of fingerling seabass (*Dicentrarchus labrax*). *Rivista Italiana Acquacoltura* 28: 105–115.

Tocher, D.R. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.* 11: 107–184.

Tocher, D.R. 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquac. Res.* 41: 717-732.

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

Tocher, D., Francis, D., Coupland, K. 2010. N-3 polyunsaturated fatty acid-rich vegetable oils and blends. In: In: *Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds* (Turchini, G.M., Ng, W.K., Tocher, D.R. eds), pp. 209-244. Taylor & Francis, CRC Press, Boca Raton.

Tocher, D.R., Bendiksen, E.A., Campbell, P.J., Bell, J.G. 2008. The role of phospholipids in nutrition and metabolism of teleost fish. *Aquaculture* 280: 21–34.

Tocher, D.R., Zheng, X., Schlechtriem, C., Hastings, N., Dick, J.R., Teale, A.J. 2006. Highly unsaturated fatty acid synthesis in marine fish: cloning, functional

characterization, and nutritional regulation of fatty acyl Δ6 desaturase of Atlantic cod (*Gadus morhua* L.). *Lipids* 41:1003–1016.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Díaz, E., Bell, J.G., Geurden,I., Lavens, P., Olsen, Y. 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). *Aquacult. Nutr.* 8: 195–207.

Torstensen, B.E. and Stubhaug, I. 2004. Beta-oxidation of 18:3 n-3 in Atlantic salmon (*Salmo salar* L.) hepatocytes treated with different fatty acids. *Lipids* 39: 1–8.

Torstensen, B.E. and Tocher, D.R. 2010.The effects of fish oil replacement on lipid metabolism of fish In: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds (Turchini, G.M., Ng, W.K., Tocher, D.R. eds), pp. 405-437. Taylor & Francis, CRC Press, Boca Raton.

Torstensen, B.E., Lie, O., Froyland, L. 2000. Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.) — effects of capelin oil, palm oil, and oleic acid enriched sunflower oil as dietary lipid sources. *Lipids* 35: 653–664.

Torstensen, B.E., Frøyland, L., Lie, Ø. 2004. Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil – effects on Atlantic salmon (*Salmo salar*) tissue and lipoprotein composition and lipogenic enzyme activities. *Aquacult. Nutr.* 10:175–192.

Torstensen, B.E., Nanton, D., Olsvik, P.A., Sundvold, H., Stubhaug, I. 2009. Gene expression of fatty acid binding proteins (FABPs), fatty acid transport proteins (cd36 and FATP) and b-oxidation related genes in Atlantic salmon (*Salmo salar* L.) fed fish oil or vegetable oil. *Aquacult. Nutr.*15:440 –445.

Tranulis, M.A., Dregni, O., Christophersen, B., Krogdahl, A., Borrebaek, B. 1996. A glucokinase-like enzyme in the liver of Atlantic salmon (*Salmo salar*). *Comp. Biochem. Physiol. B* 114:35–39.

Turchini, G.M., Torstensen, B.E., Ng, W.-K. 2009. Fish oil replacement infinfish nutrition. *Rev. Aquacult.* 1: 10-57.

Turchini, G.M., Ng, W.-K., Tocher, D.R. 2010. Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. Taylor & Francis, CRC Press, pp. 533, Boca Raton.

Vagner, M. and Santigosa, E. 2011. Characterization and modulation of gene expression and enzymatic activity of delta-6 desaturase in teleosts: a review. *Aquaculture* 315:131–143.

Vagner, M., Robin, J.H., Tocher, D.R., Zambonino Infante, J.L., Person-Le Ruyet, J., 2009. Ontogenetic effects of early feeding of sea bass (*Dicentrarchus labrax*) larvae with a range of dietary n-3 HUFA levels on the functioning of polyunsaturated fatty acid desaturation pathways. *Br. J. Nutr.* 101: 1452–1462.

van Greevenbroek, M.M. and de Bruin, T.W. 1998. Chylomicron synthesis by intestinal cells in vitro and in vivo. *Atherosclerosis* 141:S9–S16.

Vance, J.E. and Adeli, K. 2008. Assembly and secretion of triacylglycerol-rich lipoproteins. In: *Biochemistry of Lipids, Lipoproteins and Membranes*, 5th edn (Vance, D.E. and Vance, J.E. eds), pp. 507–532, Elsevier, Oxford.

Venold, F. F., Penn, M. H., Thorsen, J., Gu, J., Kortner, T. M., Krogdahl, Å., Bakke, A. M. 2013. Intestinal fatty acid binding protein (fabp2) in Atlantic salmon (*Salmo salar*): localization and alteration of expression during development of diet induced enteritis. *Comp. Biochem. Physiol. A* 164: 229-240.

Venou, B., Alexis, M.N., Fountoulaki, E., Nengas, I., Apostolopoulou, M., Castritsi-Cathariou, I. 2003. Effect of extrusion of wheat and corn on gilthead seabream (*Sparus aurata*) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. *Aquaculture* 225: 207–223.

Vergara, J. M. and Jauncey, K. 1993. Studies on the use of dietary energy by gilthead sea bream (*Sparus aurata* L.) juveniles. *Fish Nutrition in Practice. Les Colloques, INRA.* 453-458.

Vergara, J. M., Robaina, L., Izquierdo, M., Delahiguera, M. 1996. Protein sparing effect of lipids in diets for fingerlings of Gilthead sea bream. *Fish. Sci.* 62: 624-628.

Vergara, J. M., Lopez-Calero, G., Robaina, L., Caballero, M. J., Montero, D., Izquierdo, M. S., Aksnes, A. 1999. Growth, feed utilization and body lipid content of gilthead seabream (*Sparus aurata*) fed increasing lipid levels and fish meals of different quality. *Aquaculture* 179: 35-44.

Viegas, I., Mendes, V. M., Leston, S., Jarak, I., Carvalho, R. A., Pardal, M. Â., Manadas, B., Jones, J.G. 2011. Analysis of glucose metabolism in farmed European sea bass

(*Dicentrarchus labrax* L.) using deuterated water. Comp. Biochem. Physiol. A 160: 341–347.

Villeneuve, L., Gisbert, E., Le Delliou, H., Cahu, C.L., Zambonino-Infante, J.L. 2005a. Dietary levels of all-trans retinol affect retinoid nuclear receptor expression and skeletal development in European sea bass larvae. Br. J. Nutr. 93: 791–801.

Wallaert, C. and Babin, P. J. 1994. Effects of temperature variations on dietary lipid absorption and plasma lipoprotein concentrations in trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. B 109: 473-487.

Walzem, R.L., Storebakken, T., Hung, S.S.O., Hansen, R.J. 1991. Relationship between Growth and Selected Liver Enzyme Activities of Individual Rainbow Trout. J. Nutr. 121: 1090-1098.

Wassem, E.A., Wahby, O.M., Sakr, E.M. 2007. Effect of dietary vegetable oils on health and liver histology of gilthead sea bream (*Sparus aurata*) growers. Aquac. Res. 38:852–861.

Watson, A. M., Barrows, F. T., Place, A. R. 2013. Taurine supplemented plant protein based diets with alternative lipid sources for juvenile gilthead sea bream, *Sparus aurata*. J. Fish. Aquac. 4: 59-66.

Webb, K.A., Rawlinson, L.T., Holt, G.J. 2010. Effects of dietary starches and protein to energy ratio on growth and feed efficiency of juvenile cobia, *Rachycentron canadum*. Aquacult. Nutr. 16:447–456.

Wilson, R.P. 1994. Utilization of dietary carbohydrate by fish. Aquaculture 124: 67-80.

Yamamoto, Y., Kabeya, N., Takeuchi, Y., Alimuddin, Haga, Y., Satoh, S., Takeuchi, T., Yoshizaki, G. 2010. Cloning and nutritional regulation of polyunsaturated fatty acid desaturase and elongase of a marine teleost, the nibe croaker *Nibea mitsukurii*. Fish. Sci. 76: 463–472.

Xiao, C., Hsieh, J., Adeli, K., Lewis, G.F. 2011 Gut–liver interaction in triglyceride-rich lipoprotein metabolism. Am. J. Physiol. Endocrinol. Metab. 301: E429–E446.

Xue, X., Feng, C.Y., Hixson, S.M., Johnstone, K., Anderson, D.M., Parrish, C.C., Rise, M.L. 2014. Characterization of the fatty acyl elongase (elovl) gene family, and hepatic elovl and delta-6 fatty acyl desaturase transcript expression and fatty acid responses to

diets containing camelina oil in Atlantic cod (*Gadus morhua*) Comp. Biochem. Physiol. B 175:9-22.

Zhang, Z., Wu, R. S., Mok, H. O., Wang, Y., Poon, W. W., Cheng, S. H., Kong, R. Y. 2003. Isolation, characterization and expression analysis of a hypoxia-responsive glucose transporter gene from the grass carp, *Ctenopharyngodon idellus*. Eur. J. Biochem. 270: 3010-3017.

Zheng, X., Tocher, D.R., Dickson, C.A., Bell, J.G., Teale, A.J. 2004b. Effects of diets containing vegetable oil on expression of genes involved in highly unsaturated fatty acid biosynthesis in liver of Atlantic salmon (*Salmo salar*). Aquaculture 236: 467-483.

Zheng, X., Tocher, D.R., Dickson, C.A., Dick, J.R., Bell, J.G., Teale, A.J. 2005a. Highly unsaturated fatty acid synthesis in vertebrates: new insights with the cloning and characterisation of a $\Delta 6$ desaturase of Atlantic salmon. Lipids 40: 13-24.

Zheng, X., Torstensen, B.E., Tocher, D.R., Dick, J.R., Henderson, R.J., Bell, J.G. 2005b. Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturases and elongase genes in liver of Atlantic salmon (*Salmo salar*). Biochim. Biophys. Acta 1734: 13–24.

Zheng, X., Ding, Z., Xu, Y., Monroig, O., Morais, S., Tocher, D.R. 2009. Physiological roles of fatty acyl desaturase and elongase in marine fish: Characterisation of cDNAs of fatty acyl $\Delta 6$ desaturase and Elovl5 elongase of cobia (*Rachycentron canadum*). Aquaculture 290: 122-131.

Zheng, X., Seiliez, I., Hastings, N., Tocher, D.R., Panserat, S., Dickson, C.A., Bergot, P., Teale A.J. 2004a. Characterization and comparison of fatty acyl $\Delta 6$ desaturase cDNAs from freshwater and marine teleost fish species. Comp. Biochem. Physiol. B 139: 269-279.

