ASSESSING EPA + DHA REQUIREMENTS OF *SPARUS AURATA* AND *DICENTRARCHUS LABRAX*: IMPACTS ON GROWTH, COMPOSITION AND LIPID METABOLISM

THESIS SUBMITTED TO THE UNIVERSITY OF STIRLING FOR THE

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Sam James Silver Houston

BSc (Hons), MSc

INSTITUTE OF AQUACULTURE, UNIVERSITY OF STIRLING,

UNITED KINGDOM

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To Hilary Shaw, who passed while I was doing this.

Declaration

This thesis and the work contained within it, was my own work. Where I have made use of others' work it is appropriately cited. This thesis has not been submitted for any other qualification.

Abstract

The gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) require n-3 long-chain polyunsaturated fatty acids (LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid DHA, for optimal growth and health. Due to the rapid growth of global aquaculture the quantity of marine oils used in aquafeeds has been limited, yet the overall quantity of oil in an aquafeed has increased by the addition of vegetable oil (VO) to supply dietary energy. For aquaculture to continue to grow more fish must be produced with less marine ingredients, yet EPA and DHA must be maintained at levels above fish requirements. This project set out to re-evaluate the requirement for EPA and DHA in gilthead seabream and European seabass. Two dose-response studies were designed and executed where juvenile seabream and seabass were fed one of six levels of EPA+DHA (0.2 – 3.2 % as fed). Biometric data were collected and analysed to determine new requirement estimates for EPA+DHA for fish of two weight ranges (24 – 80 g and 80 – 200 g). The effects of the dietary LC-PUFA gradient on lipid composition and metabolism were also considered. This project found that the requirement for EPA+DHA declines with fish weight and that the current published EFA requirements are too low for both species when fed modern diet formulations. At a size range of 24 – 80 g, the period when a 3 mm pellet is consumed, the optimum requirement for growth is 1.3 – 1.5 % EPA+DHA, for both species. Beyond ~80 g (4.5 mm pellet) seabream require 1.20 – 1.25 % EPA+DHA, whereas seabass require 1.10 – 1.20 % EPA+DHA. Previous studies in both species, indicated that juveniles require approximately 1% LC-PUFA in their diets. In both species the addition of VO to the diet increased the level of lipid in the liver. Fatty acid and gene expression data showed that LC-PUFA biosynthesis was stimulated in key tissues, liver and

mid-intestine, as FO was replaced by VO. The expression of lipogenic genes was also upregulated in the mid-intestine of both species but in liver only in seabream. The implications of this project are that EPA and DHA need to be supplied at a higher level when fish are < 80 g (3 mm pellet) and then in larger pellet sizes dietary FO can be reduced, whence optimizing the application of this commodity.

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Abbreviations

AGR – Absolute growth rate (g day^{-1})

AIC – Akaike information criterion

ALA – Alpha linolenic acid

ANOVA – Analysis of variance

ARA – Arachidonic acid

ATP – Adenosine triphosphate

AWERB – Animal Welfare and Ethical Review Body

BHT – Butylated hydroxytoluene

BLAST – Basic Local Alignment Search Tool

cDNA – Complementary Deoxyribonucleic Acid

CDS – Coding DNA sequence

COX - Cyclooxygenase

CRAN – Comprehensive R Archive Network

Ct – Threshold cycle

DGI – Daily growth index

DHA – Docosahexaenoic acid

DPA – Docosapentaenoic acid

EFA – Essential fatty acid

ELOVL – Elongation of very long-chain fatty acids protein

EPA – Eicosapentaenoic acid

EST – Expressed sequence tag

FADH – Flavin adenine dinucleotide

FAME – Fatty acid methyl ester

FAS – Fatty acid synthase

FCR – Feed conversion ratio

FER – Feed efficiency ratio

FM – Fishmeal

FO – Fish oil

FOB – Free on board

FPL – Four parameter logistic function

FTU – Feed Trial Unit

GC – Gas chromatography

HPLC – High performance liquid chromatography

HSD – Honest significant differences

HSI – Hepatosomatic index

IGF – Insulin-like growth factor

IRLS - Iteratively reweighted least squares

LC-PUFA – Long-chain polyunsaturated fatty acid

LOA – Linoleic acid

LOX – Lipooxygenase

MUFA – Monounsaturated fatty acid

NADH – Nicotinamide adenine dinucleotide

NCBI – National Center for Biotechnology Information

ND – Not determined/detected

NLS – Nonlinear least squares

NRC – National Research Council

NTC – None template control

OFN – Oxygen free nitrogen

OLS – Ordinary least squares

OV – Overall (the whole experimental period)

PC – Phosphatidylcholines

PCA – Principal components analysis

PE – Phosphatidylethanolamine

PI – Phosphatidylinositol

PO – Palm oil

PS – Phosphatidylserine

PUFA – Polyunsaturated fatty acid

RAS – Recirculation aquaculture system

RNA – Ribonucleic acid

RO – Rapeseed oil

RSS – Residual sum of the squares

RT-qPCR - Real-time quantitative polymerase chain reaction

SCD – Steroyl CoA desaturase

SD – Standard deviation

SFA – Saturated fatty acid

SGR – Specific growth rate

TAG – Triacylglycerol

VO – Vegetable oil

WG – Weight gain

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I. General Introduction

I.1 Introduction

Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) represent the principal farmed finfish species in the Mediterranean region. Both are essentially marine species, although the European seabass is known to enter estuarine waters. In 2015, global aquaculture production of gilthead seabream and European seabass was 166,794 and 162,399 tonnes, respectively (Faostat 2015). Gilthead seabream and European seabass are typically produced in intensive inshore submerged net cages, where fish are fed extruded aquafeeds (Rosa, Marques et al. 2014). They are mainly carnivorous species that obtain energy from the protein and lipid components of their diets, and make poor use of dietary carbohydrates (Oliva-Teles 2000). Modern diets for finfish species have reduced fishmeal (FM) and fish oil (FO) contents, which is driven by two key incentives: 1) these raw materials are expensive, and 2) their supply is limited by the productivity of marine fisheries (Fisheries 2016). Aquaculture is a growing sector with a vital role to play in increasing world food supply. However, if it is to be a net producer of fish it must seek to minimise its use of capture fisheries production by seeking alternative sources of protein and lipid to support its expansion (Tacon, Hasan et al. 2006, Merino, Barange et al. 2012, Shepherd, Jackson 2013).

Living organisms have requirements for specific nutrients. In its simplest terms, a requirement can be defined as the quantity of a given nutrient required for growth and reproduction (Molina-Poveda 2016). Traditionally, in nutritional studies growth is used as the response from which to define a requirement as this is the primary concern of aquaculturists (National Research Council 2011). The definition of requirement becomes more complex when different responses such as health or the desired composition (or

even colour) of the product are considered. Furthermore, a diet is supplied to the organism as a mixture of nutrients that may have interactions with one another and may contain antinutrients that affect ingredient digestibility (Krogdahl, Penn et al. 2010). Organisms must acquire essential nutrients from their diets; a nutrient is essential to an organism if it cannot be synthesised *de novo*, or in sufficient quantities to meet physiological demand (Molina-Poveda 2016). Essential nutrients, therefore, have quantitative requirements, which are usually expressed as a proportion of the diet (National Research Council 2011). Therefore, essential fatty acids (EFA) must be supplied in feeds for farmed fish (aquafeeds) to ensure growth and normal physiological functions. There are two fatty acids that cannot be synthesised by vertebrates, namely the C_{18} polyunsaturated fatty acids (PUFA) α-linolenic acid (18:3n-3, ALA) and linoleic acid (18:2n-6, LOA), due to the absence of enzymes that produce them from oleic acid (18:1n-9) (Castro, Tocher et al. 2016). However, particularly in marine species, long-chain ($C \geq 20$) polyunsaturated fatty acids (LC-PUFA) are also required preformed in the diet as the activities of elongation and desaturation enzymes are insufficient to supply LC-PUFA from C_{18} PUFA precursors (Glencross 2009, Sargent, Tocher et al. 2002). Consequently, three LC-PUFA are regarded as EFA for marine species with low LC-PUFA biosynthetic capability, namely the n-3 fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and the n-6 arachidonic acid (ARA, 20:4n-6) (see Section I.2.1.2) (Sargent et al. 2002).

EPA and DHA are abundant in marine ingredients, particularly FO, and aquafeeds formulated with these raw materials can satisfy the physiological demands of fish. However, oils derived from terrestrial crops, herein referred to as "vegetable oils" (VO) (e.g. soya, linseed, rapeseed or palm oil) used to replace FO are devoid of LC-PUFA and

instead, are rich in oleic acid, ALA and LOA. As a dietary ingredient, VO is an effective means of supplying dietary energy but the requirements for LC-PUFA must be met with FO or an alternative source. This is important to ensure good growth, health, welfare and fillet EFA content of farmed fish (Sargent et al. 2002, Tocher 2003, National Research Council 2011).

There has been a move towards higher energy diet formulations, by the addition of oil such as VO to promote fast growth and to "spare" dietary protein (Sargent et al. 2002), and this may affect the nutrient demands of the fish (Glencross 2009). The requirements for n-3 LC-PUFA in gilthead seabream and European seabass are based on very few studies and so further data would contribute towards diet formulations while the pressure on limited marine materials is growing. Due to the increased lipid levels of today's diet formulations there is a need to revisit requirement levels for LC-PUFA, particularly in the diets of marine fish where oil content (typically as VO) has increased while FM content has been reduced (National Research Council 2011, Glencross 2009).

I.2 Lipids, structure, function and metabolism

Lipids and fatty acids are a diverse group of organic molecules unified by their solubility in organic solvents. Most lipids are found in biological samples as lipid classes, such as triacylglycerols (TAG), phospholipids or sphingolipids, many lipid classes include fatty acids conjugated with ester bonds (Christie 2003). This section will give a brief introduction to aspects of lipid metabolism that are relevant to EFA requirement research. The reader should appreciate that most of the data presented in this section are derived from studies on mammals, whose lipid metabolism is operated, at least

qualitatively, in a similar way to fish. The contents of this section are generally applicable to all vertebrates.

I.2.1 Lipid structure and nomenclature

I.2.1.1 Lipid classes

Analytically, lipid classes are separated by their polarity and can thus be classified as non-polar (or neutral) lipids and polar lipids. The molecule TAG, is composed of three fatty acids esterified to glycerol hydroxyl groups. The three positions of the fatty acids are denoted: *sn-1*, *sn-2* and *sn-3*. All animal tissues contain TAG but there are tissues used as lipid depots (e.g. adipose tissue) where TAG will dominate the lipid class composition. The primary function of TAG is to store energy. TAG fatty acid compositions are highly susceptible to dietary fatty acid compositions (Christie 2003).

The sterols are planar tetracyclic carbon compounds. The main sterol in animal tissues is cholesterol, which has a single hydroxyl group at one end and a flexible octyl side chain at the other end. The structure of cholesterol allows the rigid aliphatic rings to embed into the acyl chains of animal membranes and its polar hydroxyl group interacts with the polar head groups of fatty acids. Cholesterol is also the precursor molecule for the steroid hormones. The hydroxyl group of cholesterol may also be esterified to a fatty acid creating a sterol ester (Christie 2003).

Polar lipids include phospholipids and sphingolipids. The term phospholipid (or glycerophospholipid) refers to the major lipid constituent of membranes and typically they consist of two fatty acids esterified to glycerol and a hydrophilic phosphate group. Phospholipids are amphipathic compounds with a polar head group (choline, inositol,

ethanolamine or serine) and two aliphatic acyl chains esterified to glycerol in the *sn-1* and *sn-2* positions. The diversity of fatty acids gives rise to a great diversity of chemical species, but generally a PUFA is esterified to the *sn-2* position and a saturated fatty acid (SFA) to the *sn-1* position (Sargent et al. 2002). The principal phospholipids are phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC). The amphipathic properties of the phospholipids are central in their main role in the formation of bilayer cellular membranes (Christie 2003). Sphingolipids are another important lipid class associated with the polar fraction. They are formed from a long chain fatty acid linked to a sphingosine base (an 18-carbon amino alcohol) by an amine bond to form a ceramide. A vast range of molecules can be attached to one of the hydroxyl groups (carbohydrates and polar head groups) making sphingolipids important in cell recognition and signalling. Sphingomyelin is a ceramide with a polar head group (choline, ethanolamine) and interacts with cholesterol in the outer leaflet of cellular membranes to form microdomains (lipid rafts) (Christie 2003).

I.2.1.2 Fatty acids

Fatty acids consist of a chain of carbon and hydrogen atoms, called the acyl chain, with one end having a methyl group (CH_3) and the other a carboxylic acid group (COOH). The acyl chain can be any number of carbons but in animal lipids mostly even numbers of carbons are found. The acyl chain is also classified by varying degrees of saturation. A SFA has all the carbon atoms occupied by two hydrogen atoms and three for the methyl end. Unsaturations (or double bonds) occur in fatty acyl chains when two hydrogens are removed from two adjacent carbons forming an ethylene group (RHC=CHR), natural ethylene groups are invariably in the *cis* configuration and introduce a bend to the acyl

chain. Unsaturation increases the fluidity (i.e. reduces the melting point) of the fatty acid. If there is one ethylene group the fatty acid is a monounsaturated fatty acid (MUFA) or "monoene", when two or more are present it is a polyunsaturated fatty acid (PUFA) or "polyene". The nomenclature for classifying PUFA used in the nutrition literature is the n (or ω) series, which denotes the position of the first ethylene group relative to the methyl terminal of the acyl chain. This nomenclature is adopted here. Series of PUFA occurring in animal samples include n-3, n-6, n-9 and n-11 series. The Δ nomenclature denotes the position of the first ethylene group relative to the carboxyl group but is not widely used by nutritionists. As an example, 18:1n-9 (*cis* Δ^9 octadecanoic acid, 18:1 Δ^9) has 18 carbons, with one single ethylene bond (i.e. MUFA) on the 9th carbon from the methyl group and termed with the trivial name of oleic acid. Representative fatty acids from the SFA, MUFA, and n-3 and n-6 PUFA are given in Figure I.1. (Christie 2003, Tocher 2003, Sargent et al. 2002).

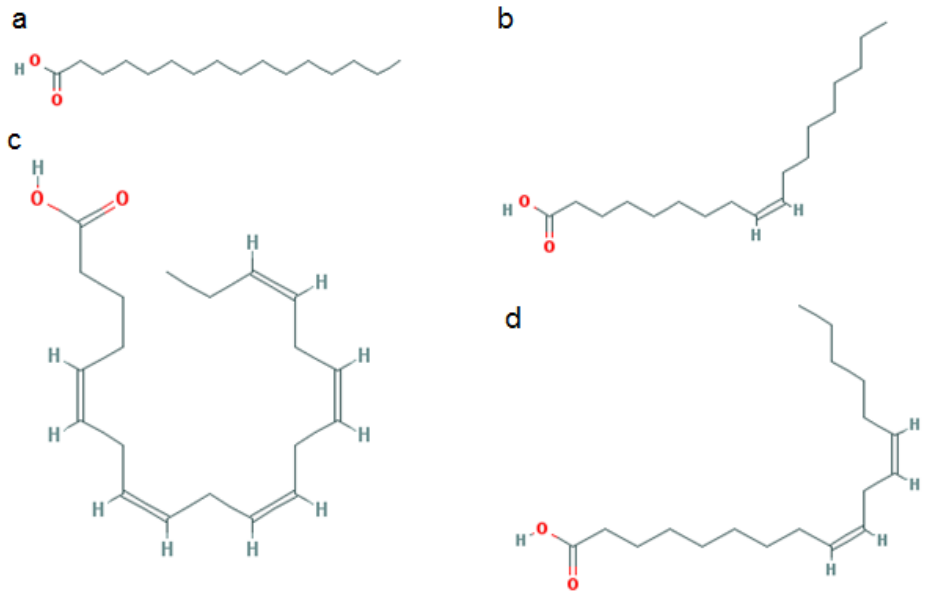

Figure I.1. A representative from each fatty acid group: a saturated fatty acid, palmitic acid (a); a monounsaturated fatty acid, oleic acid (b); an n-3 polyunsaturated fatty acid, eicosapentaenoic acid (c) and an n-6 polyunsaturated fatty acid, linoleic acid (d).

I.2.2 Fatty acid and LC-PUFA biosynthesis

Vertebrate animals have varying capacities for the *de novo* synthesis and modification of fatty acids (Monroig, Navarro et al. 2011). There are several key enzymes involved in the synthesis and modification of fatty acids, these including the fatty acid synthase complex, fatty acid elongases and desaturases (Guillou, Zadravec et al. 2010).

I.2.2.1 Synthesis of saturated and monounsaturated fatty acids

In animals, the SFA palmitic acid (16:0) and stearic acid (18:0) can be produced by a cytosolic enzyme complex termed the type I fatty acid synthase (FAS I). This complex is formed of two identical monomers arranged in an anti-parallel manner, a feature which is crucial to its function (Chirala, Wakil 2004). The first substrate is acetyl-CoA ($COCH_3$-CoA). Two carbons are sequentially added in a cyclic series of reactions sourcing carbons from the substrate malonyl-CoA (a 3-carbon unit, one lost as CO_2), which involves condensation, keto-reduction, dehydration and enoyl-reduction. The overall reaction is summarised as:

$$Acetyl - CoA + 7\ Malonyl - CoA + 14\ NADPH + 14\ H^+$$
$$\rightarrow Palmitic\ acid + 7\ CO_2 + 8\ CoA + 14\ NADP + 6\ H_2O$$

Although the primary products of FAS I are palmitic acid (16:0) and stearic acid (18:0), the FAS I complex is not completely substrate specific and other products include shorter chain length SFA such as lauric acid (12:0) and myristic acid (14:0), and even branched chain fatty acids (Smith 1994, Chirala, Wakil 2004). FAS I activity in marine carnivorous fish is not likely to be of high importance since these fish have high lipid diets and therefore have little need to synthesise lipids *de novo* (Tocher 2003).

The biosynthesis of MUFA is achieved by the action of the stearoyl CoA desaturase-1 (SCD), an enzyme with Δ9 desaturation activity (Guillou et al. 2010). Consequently, SCD can desaturate palmitic (16:0) and stearic (18:0) acids to palmitoleic (16:1n-7) and oleic (18:1n-9) acids, respectively.

I.2.2.2 LC-PUFA biosynthesis in vertebrates

LC-PUFA biosynthesis (Figure I.2) is the alternate elongation and desaturation reaction sequence of PUFA by elongase and desaturase enzymes, respectively. The protein/gene names for elongases and desaturases are Elovl/*Elovl* and Fads/*Fads*, respectively (Castro et al. 2016). It is an organism's repertoire of functional elongases and desaturases that determines whether the LC-PUFA (EPA, ARA and DHA) are essential in the diet (Castro et al. 2016). Vertebrates do not possess enzymes capable of the Δ12 or Δ15 desaturations that are required to desaturate oleic acid (18:1n-9) to n-6 LOA or n-3 ALA, respectively, and therefore LOA and ALA have a dietary requirement (Wallis, Watts et al. 2002). Many marine fish cannot elongate and or desaturate LOA and ALA so therefore LC-PUFA are essential (Castro et al. 2016).

The elongases, or elongation of very long-chain fatty acid (ELOVL) proteins, are a family of enzymes that elongate fatty acids in the endoplasmic reticulum. In mammalian models, Guillou et al. (2010) describes seven elongases (ELOVL1-7), these being divided into two groups according to their substrate specificities: 1) ELOVL1, 3, 6 and 7, which elongate SFA and MUFA; and 2) ELOVL2, 4 and 5, which primarily elongate PUFA. However, this is a simplistic generalisation as ELOVL4 has also been shown to be involved in the elongation of SFA to produce very long-chain SFA (VLC-SFA) of C_{28-30} (Agbaga, Brush et al. 2008). The elongases use acyl-CoA fatty acids as substrates and

condense them with malonyl-CoA moieties, therefore extending the acyl chain by two carbons per interaction with the elongase. ELOVL1, 5 and 6 are ubiquitously expressed in tissues, whereas, ELOVL2, 3, 4 and 7 show tissue specific expression (Guillou et al. 2010). Studies in murine models indicate that tissue specific expression of these enzymes is related to the respective tissues demand for very long chain (VLC, $>C_{24}$) fatty acids (Guillou et al. 2010, Jakobsson, Westerberg et al. 2006).

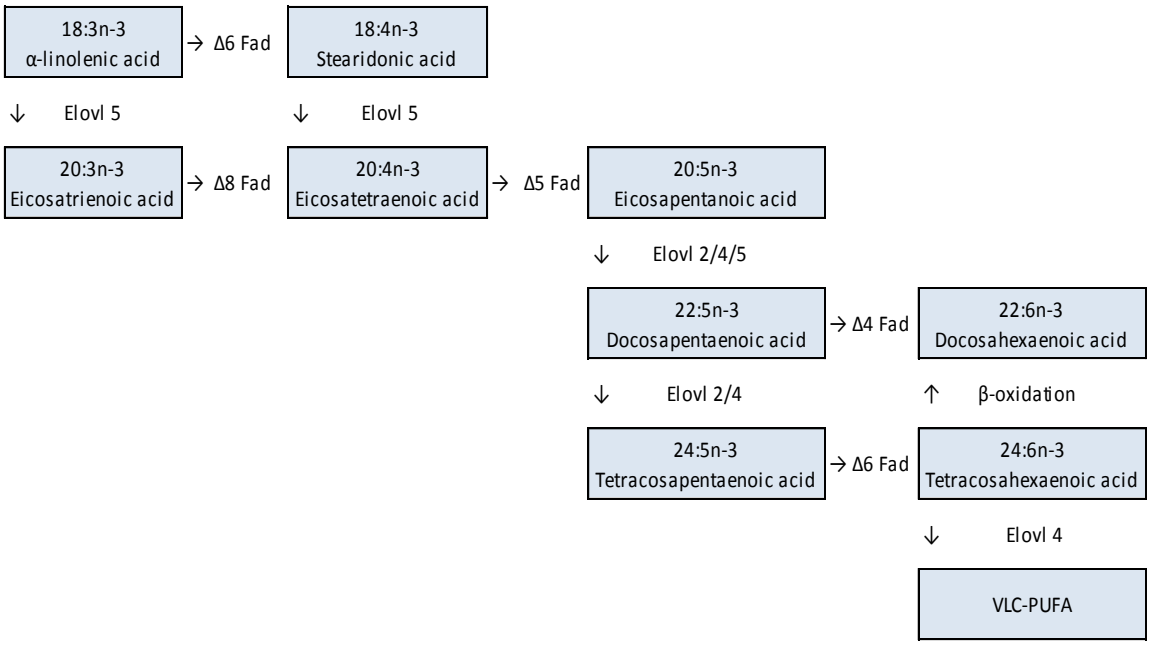

Figure I.2. Pathways of n-3 LC-PUFA biosynthesis (Castro, Monroig et al. 2012). Desaturations performed by Fads enzymes proceed from left to right and elongations performed by elongases proceed from top to bottom. Note: 1. The same enzymes modify the n-6 PUFA. 2. The critical position of Δ5 Fads.

The Fads insert double bonds into acyl chains at specific positions, denoted from the Δx carbon. As with the Elovl enzymes, the Fads are localised in the endoplasmic reticulum. The same enzymes operate on n-3 and n-6 equivalent substrates. ARA and EPA can be produced by the action of a Δ6/8 Fads and elongation by ELOVL5.

ELOVL2/4/5 can further elongate ARA or EPA to docosapentaenoic acid (DPA, 22:5n-3) and a Δ4 Fads is required to produce DHA, however most vertebrates (except some fish) do not possess a Δ4 Fads. Therefore, DHA is produced via the Sprecher pathway, DPA is elongated to 24:5n-3, which can be desaturated by a Δ6 Fads to 24:6n-3, this fatty acid being subsequently shortened by a single round peroxisomal β-oxidation to produce DHA (Voss, Reinhart et al. 1991). As a result, all LC-PUFA can be produced with Δ5 and Δ6 Fads enzymes (i.e. no Δ4 activity required). However, some Δ8 activity has also been demonstrated towards 20:2n-6 and 20:3n-3 using a baboon (*Papio anubis*) *FADS2* sequence (Park, Kothapalli et al. 2009). In mammals there are three Fads genes, *FADS1*, *FADS2* and *FADS3*. *FADS1* and *FADS2* encode proteins with Δ5 and Δ6 activity, respectively (Guillou et al. 2010, Zhang, Qin et al. 2014). *FADS3* has recently been shown to be important in regulating hepatic DHA synthesis in new-born mice (Zhang, Qin et al. 2017). Due to the presence of this pathway only LOA and ALA are EFA in many mammals as they can be used to synthesise LC-PUFA.

I.2.2.3 LC-PUFA biosynthesis in fish

As mentioned, the ability to produce LC-PUFA is determined by the complement of Elovl and Fads enzymes expressed by an organism and their regulatory mechanisms (Sargent, Bell et al. 1999, Monroig et al. 2011). The inability of marine fish such as gilthead seabream and European seabass, to operate LC-PUFA biosynthesis pathways is largely related to the absence or inefficient activity of Δ5 Fads and absence of an Elovl2 (Castro et al. 2016).

The presence of functional teleost elongases has been demonstrated in yeast expression systems (Castro et al. 2016). No Elovl2 (elongates C_{20}-C_{22} PUFA) enzymes

have been found in any marine farmed fish species (Morais, Monroig et al. 2009, Castro et al. 2016), but Elovl2 has been found in Atlantic salmon (Morais et al. 2009) and zebrafish (*Danio rerio*) (Monroig, Rotllant et al. 2009). As in mammals, the teleost Elovl5 has activity on C_{18} and C_{20} substrates, but also with some activity towards C_{22} substrates in gilthead seabream, Atlantic salmon (*Salmo salar*) and turbot (*Psetta maxima*) (Agaba, Tocher et al. 2005). Furthermore, the meagre (*Argyrosomus regius*) has been shown to possess an Elovl5 with activity towards C_{16} - C_{20} substrates (Monroig, Tocher et al. 2013). Thus, it can be concluded that Elovl5 is more functionally diverse in marine teleosts than mammals (Castro et al. 2016). There are two Elovl4 isoforms in fish (Elovl4a and Elovl4b) in zebrafish, both were shown to have capacity to elongate SFA up to C_{36} but the Elovl4b also showed activity against PUFA (Monroig et al. 2009). Elovl4b orthologues have been found to be active in Cobia (*Rachycentrum canadum*) (Monroig, Webb et al. 2011) Atlantic salmon (Carmona-Antoñanzas, Monroig et al. 2011), rabbitfish (*Siganus canaliculatus*) (Monroig et al. 2011) and Nibe croaker (Kabeya, Yamamoto et al. 2015). The evidence suggests that this elongase can perform the elongation of 22:5n-3 to 24:5n-3, which can reach DHA via Δ6 desaturation and β-oxidation. Whence, in some marine species, Elovl4b may perform some elongations previously thought to be limited to Elovl2 (Castro et al. 2016).

Teleost Fads are all orthologues of mammalian *FADS2* with mostly Δ6 activities (Monroig et al. 2011). However, in zebrafish (*Danio rerio*), a bifunctional Fads was discovered with Δ5 and Δ6 activities (Hastings, Agaba et al. 2001) and in rabbitfish (Li, Monroig et al. 2010) a bifunctional Fads enzyme has been described that possesses Δ5 and Δ6 activities. Subsequently, the Atlantic salmon was found to have a monofunctional Δ5 Fads (Hastings, Agaba et al. 2004), an enzyme also described in rainbow trout (Hamid,

Carmona-Antoñanzas et al. 2016). Interestingly, the rabbitfish (Li et al. 2010), Senagalese sole (*Solea senegalensis*) (Morais, Castanheira et al. 2012), medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*) also possesses a Fads with Δ4 activity required to desaturate DPA (22:5n-3) to DHA (22:6n-3) (Oboh, Kabeya et al. 2017). Therefore, there are two pathways in fish that could potentially produce DHA, the Sprecher pathway and the Δ4 pathway (Oboh et al. 2017, Castro et al. 2016).

It has been demonstrated in Atlantic salmon that the pathways for LC-PUFA synthesis are influenced by levels of EPA and DHA in the diet; activities of the relevant enzymes can be increased in the absence of dietary EPA and DHA (Zheng, Tocher et al. 2005). Due to this ability, Atlantic salmon have a lower requirement for EPA and DHA. However, the expression of the pathway is tissue specific with highest activities reported in the intestine, liver and neural tissues (Zheng et al. 2005). Therefore, despite an ability to synthesise LC-PUFA, it is still desirable to supply Atlantic salmon with LC-PUFA as muscle EPA and DHA will be low (Bell, Tocher et al. 2003, Tocher 2015). The point to emphasise with this example is that an organism with the capacity to synthesise LC-PUFA will do so in the tissues where LC-PUFA is required and not necessarily in the edible muscle.

In agreement with the marine species described above, the herein studied species gilthead seabream and European seabass have inefficient LC-PUFA biosynthetic pathways. In gilthead seabream, a Δ6 Fads2 with minimal Δ5 desaturase activity has been demonstrated in the brain, eye and liver by injection of C_{14}-labelled linolenate and recovery of radioactive DHA, albeit low quantities (Mourente, Tocher 1998). Further work using molecular techniques has shown that a Δ6 desaturase is expressed in the

viscera but not a Δ5. Furthermore, Δ6 desaturation products such as 18:3n-6 and Iso 18:2n, are known to accumulate in tissues when gilthead seabream is fed a diet deficient in LC-PUFA (Torrecillas, Robaina et al. 2017). According to a recent study, the gilthead seabream Fads2 is active towards C_{24} substrates, which could be shortened to DHA via peroxisomal β-oxidation, providing adequate dietary precursors (i.e. EPA or DPA) are present (Oboh et al. 2017). Like other marine species, incomplete LC-PUFA synthesis in gilthead seabream is due to absence of Δ5 desaturase and elongase activity towards C_{20} PUFA (Elovl2). European seabass has been shown to possess Fads with Δ6 activity, but no Δ5 activity and Δ6 activity was unaffected by VO inclusion (Mourente, Dick et al. 2005, Mourente, Dick 2002). Genetic characterisation has demonstrated that this is a *fads2* homologue with higher expression patterns in the brain and intestine (Santigosa, Geay et al. 2011, González-Rovira, Mourente et al. 2009, Geay, Santigosa et al. 2010). However, when expressed in yeast one study found Fads2 to show n-3 substrate preferences (González-Rovira et al. 2009) and a later study found no preference for n-3 or n-6 substrates (Santigosa et al. 2011). Importantly, biochemical and polymerase chain reaction (PCR) based approaches have generated coherent data. Like other marine species, LC-PUFA synthesis in European seabass is primarily due to absence of Δ5 desaturase and elongase activity towards C_{20} PUFA (Elovl2).

In summary, gilthead seabream and European seabass do not have the ability to produce LC-PUFA from C_{18} precursor fatty acids, and so, these must be supplied in the diet, as is the case with most marine carnivorous fish (Sargent et al. 2002). The presence or absence of an active pathway for LC-PUFA biosynthesis is most likely a result of the animal's diet, but this interpretation may also be complicated by the phylogenetic history of the lineage (Glencross 2009, Monroig et al. 2011, Castro et al. 2016).

I.2.3 Fatty acid oxidation

Chemical energy from the carbon bonds of a fatty acid can be accessed by the β-oxidation pathway. β-oxidation occurs primarily in mitochondria although some branched chain fatty acids or LC-PUFA must be catabolised by peroxisomal β-oxidation. The role of mitochondrial β-oxidation is to produce acetyl-CoA for the tricarboxylic acid cycle and the reducing agents nicotidinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FADH2) that participate in oxidative phosphorylation. β-oxidation is a cyclical process and two carbon acetyl are removed from the carboxyl end of the acyl-CoA each cycle (Halver, Hardy 2002). β-oxidation involves five reactions:

1. Formation of the acyl-CoA, consuming 2ATP (cytosol), the acyl-CoA is transferred into mitochondria via the carnitine shuttle
2. Dehydration to form β-dehyroacyl-CoA
3. Hydration to form β-hyroxyacyl-CoA
4. Oxidation to β-ketoacyl-CoA
5. Lysis (after the β carbon) to form an acyl-CoA and an acetyl-CoA

Using palmitate as an example:

$$Palmitoyl\ CoA + 2ATP + 7NAD + 7FAD$$
$$\rightarrow 8\ Acetyl\ CoA\ (96ATP) + 7NADH\ (21ATP) + 7FADH_2\ (14ATP)$$

The acetyl-CoA molecules are available for the tricarboxylic acid cycle and NADH and $FADH_2$ are electron transporters in oxidative phosphorylation. In the mitochondria, β-oxidation is coupled to oxidative phosphorylation and provides energy to the organism in the form of adenine triphosphate (ATP) (Halver, Hardy 2002, Houten, Wanders 2010).

The oxidation of fatty acids can have an impact on the fatty acid composition, particularly in the TAG fraction. The fatty acids, 22:1n-11, 20:1n-9, 18:1n-9, 16:1n-7, 16:0 and 18:0 are good substrates for β-oxidation in fish (Tocher 2003, Henderson, Sargent 1985).

In mammals, long chain fatty acids (> C_{20}) and branched chain fatty acids cannot be handled by mitochondrial β-oxidation and are oxidised within peroxisomes, which employ a different subset of enzymes (Wanders, Poll-The 2017). This appears to be different in fish, at least in *Notothenia gibberifrons*, where C_{20} PUFA were demonstrated to be adequate substrates for mitochondrial β-oxidation (Crockett, Sidell 1993).

The chemical energy stored in the carbon bonds of fatty acids is accessed via mitochondrial or peroxisomal oxidation (Houten, Wanders 2010). The purpose of peroxisomal oxidation appear to be to process fatty acids (branched or LC-PUFA) that cannot be handled by mitochondrial oxidation. The energy yield of peroxisomal β-oxidation is approximately half of the yield of mitochondrial β-oxidation (Mannaerts, van Veldhoven 1996). It should be noted that vertebrates must catabolise or store carbon compounds.

I.2.4 Membranes and DHA

Membrane lipid bilayers are formed of a complex mixture of lipid classes, mainly the glycerophospholipids, cholesterol and sphingolipids. Membranes are not homogenous and their lipid compositions vary between the leaflets, laterally, between different cellular organelles or even between basolateral and apical membranes in epithelial cells. DHA has specialised functions within membranes, particularly in retina and neural tissues (Bari, Gaburro et al. 2017, Jeffrey, Weisinger et al. 2001). Such organisation and diversity

rely on complex control mechanisms to regulate the synthesis of lipids and ship them to different cellular locations. To this effect ~5% of the transcribed mammal genome is involved in lipid biosynthesis and regulation (Van Meer, Voelker et al. 2008). The role of the fatty acids within the membrane is to provide the hydrophobic barrier and the properties of the membrane are a function of its fatty acid composition (Van Meer et al. 2008).

Phospholipids have two fatty acid chains and, as previously noted, a PUFA often occupies the *sn-2* position of glycerol and a SFA, usually stearic or palmitic acids, occupies the *sn-1* position. The functional significance of this is to keep the membrane in a fluid phase at physiological temperatures. DHA forms 30 – 50% of total fatty acids in vertebrate nervous tissues (Salem Jr, Litman et al. 2001). Although the precise functions of the molecule within these membranes are not entirely clear, studies of molecular state of DHA phospholipids reveal that this molecule is highly flexible due to the C-C bonds in between each of its C=C (Gawrisch, Eldho et al. 2003). It has been postulated that DHA containing phospholipids accommodates the conformational changes that proteins undergo that are critical to their functions (Gawrisch et al. 2003). Furthermore, DHA plasmaethanolamine is thought to accelerate membrane fusion events that are critical to release neurotransmitters into synaptic junctions (Bari et al. 2017, Géraldine, Stéphanie et al. 2010). The effect of DHA rich glycerophospholipids has also been studied in the context of lipid-lipid interactions and the presence of this fatty acid is suggested to facilitate lateral heterogeneity across the membrane by excluding cholesterol and sphingomyelin rich domains (lipid rafts) (Stillwell, Shaikh et al. 2005, Van Meer et al. 2008). The neurological tissues exhibit pathologies when DHA is absent and therefore DHA plays a special role in these tissues (Salem Jr et al. 2001). From the reviews cited

above, DHA has unique properties that appear to enhance the properties of membranes that are essential to their function and performance.

I.2.5 Fatty acid derivatives

LC-PUFA are precursor molecules for secondary metabolites (lipid mediators) that play roles as retrograde mediators of inflammation. Eicosanoids and docosanoids are produced by the cleavage of LC-PUFA of the n-3 or n-6 series from membrane phospholipids. Lipid mediator classes include the eicosanoids (derived from C_{20} fatty acids), prostanoids (prostaglandins and prostacyclins), thromboxanes, leukotrienes, lipoxins, hydroxyl and epoxy fatty acids and the docosanoids (derived from DHA): resolvins, protectins and maresins. It is important to emphasise that the fatty acid composition of membrane phospholipids influences the production of lipid mediators and thus the resolution or stimulation of inflammation (Tocher, Glencross 2015).

Lipid mediators are potent molecules with short half-lives (10s to 10mins depending on metabolite and species) exerting their effects locally in inflamed tissues. Inflammation is a natural response to infection or injury, and its primary purpose is to make the local tissue more accessible to the host immune system. In vertebrates, symptoms of inflammation include swelling, pain and redness due to vasodilation. Leukocytes of the host are attracted to inflammatory sites by a range of cytokines (e.g. Tumor-necrosis-factor 1α, Interleukin 1, 6) and the production of some of these cytokines can be amplified or dampened by pro- or anti-inflammatory eicosanoids (e.g. Prostaglandin E_2, F_2). Acute inflammation helps resolve infection and recover from injury, but chronic inflammation can be the cause of inflammatory diseases, therefore it is imperative that the host can modulate inflammation (Calder 2006).

After LC-PUFA are cleaved from phospholipids by phospholipiase A_2, lipid mediators are produced by the action of lipoxygenase (LOX) and cyclooxygenase (COX) enzymes (except the epoxyeicosatetranoic acids which involve cytochrome P450 epoxygenase). The COX and LOX enzymes oxygenate and restructure acyl chains inserting ring structures, hydroxyl and epoxy groups (Calder 2006). Lipid mediators have an extensive range of physiological functions, such as vasodilation or constriction, platelet aggregation, sensitise neurones (leading to pain), regulation of hormones, tissue regeneration and apoptosis. Functional diversity is derived not only from molecular diversity but also receptor diversity (Calder 2006, O'Connor, Manigrasso et al. 2014). As C_{20} lipid mediators are derived from C_{20} fatty acids of the n-3 or n-6 series, the ratio between the n-3 and n-6 fatty acids determine the moieties of prostaglandins, thromboxins or leukotrienes that will be produced by substrate competition for the COX or LOX pathways. Generally, autocoids (hormone-like molecules that exert their action locally) from n-3 fatty acid such as EPA and eicosatetraenoic acid (20:4n-3) have anti-inflammatory effects, whereas the n-6 autocoids have pro-inflammatory effects. Therefore, the fatty acid composition of polar lipid classes has consequences on the organism's response to disease or trauma, particularly those involving chronic inflammation (Schmitz, Ecker 2008). However, prostaglandin-E_2 exerts both pro- and anti-inflammatory actions complicating the generalisation between n-3 and n-6 derived lipid mediators (Calder 2009). Compared to mammals, lipid mediators are not well understood in fish, but the available data suggest that their metabolism is broadly similar (Ghioni, Porter et al. 2002). Lipid mediators are essential to vertebrates and these cannot be produced in the absence of dietary n-3 or n-6 fatty acids.

I.2.6 Conclusions

Animals need lipids for energy and to build cell membranes. Animals also need specific n-3 or n-6 fatty acids for their properties within membranes but also as autocrine/paracrine messenger molecules. Organisms have the machinery required to apply complex organisation to their lipids. Marine carnivores have limited capacity to biosynthesise LC-PUFA due to the absence of enzymatic abilities, which is driven by evolutionary forces, particularly diets rich in preformed LC-PUFA.

I.3 Methods for defining EFA requirements

Two possible methods for defining nutrient requirements are the factorial method and empirical method. In the factorial method, nutrient demand is modelled using data on nutrient intake, endogenous losses and requirements for tissue synthesis (Shearer 1995, Hauschild, Pomar et al. 2010). In the empirical method, the response of an animal is measured after delivery of a gradient of the nutrient in question, the response is typically some metric of growth, for example weight gain (WG) or daily growth index (DGI) but other responses maybe considered, for instance feed conversion ratio (FCR) (National Research Council 2011). The empirical method is the most frequently applied in fish studies and is the method presented here.

The empirical method to assess nutrient requirements involves dose-response studies and analysis of the data is performed using regression techniques (Mambrini, Guillaume 1999). Statistically, it is necessary to have five or more treatments to allow sufficiently robust regression analysis (Shearer 2000). Deducing requirements from studies that do not conform to this is questionable, particularly if other dietary parameters are

manipulated. A measurable response (or endpoint) is used to define the requirement, and this is usually growth. The requirement can then be determined using regression analysis to establish the response as a function of the supply of the nutrient in question. Low levels of the nutrient will suppress growth, increasing levels will cause gains in growth until a threshold level is reached and gains in growth diminish, this nutrient level is the requirement. This is the traditional approach to define a requirement and is still very practical and commonly used (National Research Council 2011). However, other responses could be considered such as health or welfare, but they must be measurable by suitable and established biomarkers. This is not widely employed because growth is a robust parameter to gauge health and welfare, and further parameters considerably complicate the task of defining a requirement.

Several points are important to consider when assessing EFA requirements. The dietary lipid level should be established as the EFA requirements can be increased with the lipid content of the diet (National Research Council 2011). As mentioned, current diets are richer in oil for supplying dietary energy to rapidly growing farmed fish and sparing protein for growth (Sargent et al. 2002, Corraze 2001). Growth in fish is not linear and small fish have relatively higher growth rates. Furthermore, the partitioning of dietary components between growth and maintenance changes as the fish grow, with the maintenance components increasing as a function of fish size as the relative growth rate decreases (Lupatsch, Kissil et al. 2003a, Lupatsch 2005). Lipids are added to fish diets primarily as a source of energy but protein, and carbohydrates to a lesser extent, can also supply energy, so there is not an absolute requirement for lipid, rather a range of lipid levels that can be tolerated by a species (National Research Council 2011). The range tolerated by a species is dependent on its natural diet and typically carnivores prefer to

obtain energy via protein and lipid, whereas herbivores make a more efficient use of carbohydrates (Halver, Hardy 2002). The requirements for any nutrient should be determined under conditions (environmental and nutritional), that allows the species to grow at its maximum rate, and only the nutrients to which the research pertains should be restricted with all the others being at levels that satisfy the known physiological demands for that species. Failure in this regard is likely to result in underestimations of the requirements.

The researcher may still have to make trade-offs. For instance, does the researcher use realistic sources of dietary nutrients that are commercially available or, instead, purified components such as purified amino acids or fatty acid methyl ester derivatives that may have higher digestibility and performance characteristics, but will never be used in the practical diets of farmed fish? Trade-offs in EFA nutrition research are paramount as fatty acids are supplied as a complex mixture and there is strong evidence that there are interactions between these components that must be understood prior to designing a diet (Izquierdo 2005, Sargent et al. 2002, Watanabe 1982). It is nearly impossible to design an experiment that perfectly defines the requirement for each of the EFA, and perhaps meaningless, as this level is affected by its ratio with other fatty acids. Most studies group the EFA together and express them as EPA+DHA or n-3 LC-PUFA (previously as "n-3 HUFA", standing for "n-3 highly unsaturated fatty acids"). Furthermore, what is the best way to express the requirement value? The studies cited above seem to indicate that expressing fatty acids as a fraction of total fatty acids is most meaningful as it is dependent on the lipid level of the diet (Watanabe 1982). However, in terms of practicality expressing EFA requirements as a fraction of diet makes more sense so long as the effect of dietary lipid level is understood. This is the approach taken in

Chapters III and IV and the EFA are expressed as the sum of EPA and DHA (EPA+DHA% of diet). Practically, the key question is: How much EFA are required by a species and what is the most practical (and economical) way to supply it?

I.4 Current n-3 LC-PUFA requirements of gilthead seabream and European seabass

This section will review the current understanding of the LC-PUFA requirements of gilthead seabream and European seabass existing prior to this study. Initial requirement levels will be given as referenced by the NRC (2011). Then the studies from which these data are derived will be evaluated.

I.4.1 Gilthead seabream

According to the NRC (2011), juvenile gilthead seabream have a dietary requirement for LC-PUFA of 0.9-1.9 % with DHA:EPA ratios of 0.5-1. Kalogeropoulos et al. (1992) used six diets with 13% crude lipid formulated with soya oil and cod liver oil to yield EPA + DHA values of 0.3-1.9% of diet, the DHA:EPA ratio was approximately 1. The initial weight of the fish was 1.2 g and the experimental groups reached 10-12 g in weight over five months feeding. The SGR values were not reported, but assuming 150 d growth, they can be estimated to be 1.39 – 1.54 $\ln(g).d^{-1}$. The authors concluded that the requirements for n-3 LC-PUFA were 0.9% of dry diet. Subsequently, Ibeas et al. (1994) reported a requirement value of 1.9% for n-3 LC-PUFA, with diets with DHA:EPA of 0.5%, albeit in larger fish (43 g) than the former study. This result was somewhat surprising as many studies have shown that LC-PUFA requirements (relative to fish mass) are higher in larval/juvenile fish due to increased provision of these compounds to fulfil

demands of neural tissues forming during early life-cycle stages (Sargent, McEvoy et al. 1997). Two later studies, by the same authors, found that the best growth rates with 11.5 g gilthead seabream were achieved at n-3 LC-PUFA levels of 1.1% when the DHA:EPA ratio was 0.5 (Ibeas, Cejas et al. 1996, Ibeas, Cejas et al. 1997). The discrepancy in EFA requirement estimations between the earlier publication (Ibeas et al. 1994) and the later publications (Ibeas et al. 1996, Ibeas et al. 1997) is most likely related to the four treatment levels of n-3 LC-PUFA used in the former publication (0.76%; 1.87%; 2.87%; 2.94%), which meant that the lower treatment was just below requirements but leading the authors to conclude that the requirement was 1.9%. Furthermore, larval gilthead seabream require a higher DHA:EPA ratio than juveniles, suggesting that larval gilthead seabream have a higher requirement for DHA than juveniles (Rodriguez, Pérez et al. 1998).

Therefore, the two values provided in the NRC Report are valid guides, but they should be qualified. In the first study the fish were relatively small (1.2 grams) and the higher DHA:EPA value of 1 may have been more appropriate as the gilthead seabream grow from larvae to juvenile (Kalogeropoulos et al. 1992). However, the value 1.9% n-3 LC-PUFA (Ibeas et al. 1994) study is likely to be an overestimation as later good growth was reported by the same authors with n-3 LC-PUFA levels of ~1% of dry diet with smaller fish (Ibeas et al. 1996). Therefore, some conflicting results have been attained and these can be related to the size of the fish, the levels of EFA supply and the DHA:EPA ratio chosen by the authors.

I.4.2 European seabass

The NRC (2011) recommends that European seabass diets should contain 1% dry weight EPA and DHA, with at least 0.5% DHA. This value was derived from a study with only two LC-PUFA levels, 1 and 2.5 % dry diet, which are not a sufficient number of treatment levels to define a requirement, and the data were not compared to fish fed on deficient diets (Coutteau, Van Stappen et al. 1996). The study that could have been considered by the NRC was published by Skali and Robin (2004). The latter study was designed to specifically determine the n-3 LC-PUFA requirement of European seabass juveniles (14-35 g). There were six levels of dietary LC-PUFA, 0.23-1.89% of dry diet, achieved using blends of rapeseed oil (RO) and FO. The crude protein and lipid levels were 54% and 18%, respectively, using 12% FM and 3% fish hydrolysate as the marine sources of protein. Using regression analysis of daily growth index (DGI) as a function of dietary LC-PUFA, it was concluded that the minimum n-3 LC-PUFA requirements were 0.7% on a dry diet basis. The DGI values attained in this study were not optimal (0.97 – 1.09), subsequent authors have reported much higher growth rates in juvenile European seabass (Yildiz, Şener 2004, Torrecillas et al. 2017). Therefore, the NRC value of 1% n-3 LC-PUFA remains as a valid conservative value for which to design diets for juvenile European seabass with ~20% crude lipids. Two reviews have reported that this value is too low and that growth performance of European seabass can be further enhanced by increasing LC-PUFA to 3.5 % of diet (Izquierdo 2005, Kousoulaki, Sæther et al. 2015). The origins of this value can be traced to two studies. The first, was an investigation into the use of fatty acid salts as experimental diets (Lanari, Ballestrazzi et al. 1993) and the second, examining the effects of increased lipid and starch in the diet (Lanari, Poli et al. 1999). The statistical analysis in the latter paper demonstrates that the

source of increased growth is clearly the level of lipid in the diet. Work into FO replacement has shown that up to 60% of dietary FO can be replaced in European seabass diets with no impact to growth (Montero, Robaina et al. 2005, Mourente et al. 2005). Therefore, further study of the n-3 LC-PUFA requirements of European seabass and gilthead seabream would increase the knowledge of the requirements of these species

I.5 Effects of dietary fatty acid composition on body composition of gilthead seabream and European seabass

In order to support the continued growth of aquaculture the content of FM and FO needs to be reduced due to the limited production of these marine ingredients mostly derived from capture fisheries (Tacon, Metian 2015, Glencross 2009, Merino et al. 2012). This will rely heavily on clear knowledge of EFA requirements. The fatty acid composition of fish is mainly dependent up on the fatty acid composition of the diet (Tocher 2015), which in turn is dependent on the lipid sources used to formulate the feed. When gilthead seabream or European seabass are fed diets including VO, whole body or fillet fatty acid compositions are altered. There is an increase the proportion of C_{18} PUFA and MUFA, particularly ALA, LOA and oleic acid, but LC-PUFA (both n-3 and n-6) decrease as these fatty acids are not found in VO (Izquierdo, Montero et al. 2005, Mourente et al. 2005, Turchini, Ng et al. 2010, Yildiz, Şener 2004). DHA is more efficiently accumulated than EPA in tissues demonstrating positive retention of this fatty acid (Glencross 2009). The composition of neutral lipids is more sensitive to dietary modification than the polar lipid fraction (Mourente, Bell 2006). Gilthead seabream and European seabass have lean fillets with lipid levels of 2-6% and both species deposit perivisceral lipids (not consumed). Other species of fish, for example Atlantic salmon,

are therefore better consumed as sources of LC-PUFA for humans (Mourente, Bell 2006). The detrimental effects of dietary VO on muscle fatty acid composition can be partially restored by several weeks feeding with FO "finishing" diets in gilthead seabream and European seabass (Mourente, Bell 2006, Izquierdo et al. 2005). However, this technique was not as efficient as in Atlantic salmon, which stores fat reserves in the edible muscle (Bell et al. 2003). To the author's knowledge this technique is not used in commercial rearing of gilthead seabream and European seabass as the production cycle is much shorter than Atlantic salmon. The response to FO substitution is very similar in gilthead seabream and European seabass as other fish species because the diet is the main factor that influences fatty acid composition (Turchini et al. 2010, Glencross 2009).

I.6 Project aims

The major objective of the present project was to re-define the requirements of gilthead seabream and European seabass for EPA and DHA using modern diet formulations (20% crude fat, 12.5% FM), as recommended by the NRC (2011). The strategy to achieve this aim involved two feeding trials to deliver a gradient of EPA and DHA to juvenile gilthead seabream and European seabass. Furthermore, the effects of this gradient on the composition, fatty acid content and gene expression of key metabolic tissues were explored. As the two trial designs were nearly identical, the species' responses to EFA gradient were discussed comparatively. This thesis contains four result chapters (Chapters III - VI) that have been prepared as stand-alone manuscripts. Finally, a General Discussion (Chapter VII) will serve to bring the findings of the preceding four chapters together.

The results chapters:

III) **A comparison of regression models for defining EPA+DHA requirements using the gilthead seabream (*Sparus aurata*) as a model species**

A range of modelling strategies for defining the requirements for EPA and DHA are described and illustrated in this chapter. The regression strategies are presented in more detail in this chapter than in Chapter IV (EFA requirements of European seabass) and the advantages and drawbacks addressed in the discussion. Requirements for EPA+ DHA are presented for seabream of two different weight ranges. Evidence for a dynamic requirement for LC-PUFA is presented and discussed. The economic considerations of the diets are also considered in a hypothetical cost-benefit analysis.

The main hypothesis addressed in this chapter is a re-evaluation of EPA+DHA requirements for juvenile gilthead seabream. However, the chapter also explores the range of analyses that can be applied to biometric data and, furthermore, analyses the data over two pellet sizes.

IV) **Application of robust nonlinear regression to determine the requirements of juvenile European seabass (*Dicentrarchus labrax*) for eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids**

Like Chapter III, this chapter will advance the EPA and DHA requirement estimates for European seabass. However, as the foundations have been laid, this chapter also serves to confirm that conclusions drawn from Chapter III apply to other marine carnivorous fish species.

The main hypothesis in this chapter is the reassessment of EPA+DHA requirements in juvenile European seabass.

V) **The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary fish oil and associated n-3 long-chain polyunsaturated fatty acid content**

This chapter addresses the dietary effect on composition, tissue fatty acids and lipid metabolic gene expression in the gilthead seabream. The chapter makes some relationships between compositional and gene expression data using regression and correlation analyses. The results show that dietary effects are subtle and detected as trends against dietary VO. This manuscript was published: Houston et al. (2017) British Journal of Nutrition 118, 1010-1022.

VI) **Dietary n-3 LC-PUFA gradients in the European seabass (*Dicentrarchus labrax*); effects on composition and metabolism**

This chapter addresses the dietary effect on composition, tissue fatty acids and lipid metabolic gene expression in the European seabass. The chapter makes some relationships between compositional and gene expression data using of regression and correlation analyses. The response to the dietary gradient of LC-PUFA is compared to seabream in the discussion, drawing attention to the apparent differences between the species. Compositional data regarding the fish is presented and the effects of the diets on lipid metabolic gene expression is also considered.

The hypotheses addressed in chapters V and VI are: What are the effect of a dietary gradient of n-3 PUFA on the proximate composition of juvenile seabream/seabass? What

impact does this gradient have on the fatty acid composition and lipid metabolic gene expression in key tissues?

II. General Methodology

II.1 Introduction

The four result chapters (III – VI) present analysis from two feeding trials conducted in parallel in Hirtshals, Denmark, during April – August 2014. The two species were gilthead seabream (*S. aurata*) and European seabass (*D. labrax*). The design of these trials draws on theory outlined in the Introduction (Section I.3: *Methods for defining EFA requirements*). The two hypotheses driving the experimental design were:

What are the EPA+DHA requirements of gilthead seabream?

What are the EPA+DHA requirements of European seabass?

The wording of these hypotheses, with regards to "EPA+DHA", reveals an important caveat that, although previously introduced (Section I.3), is worthy of mention here. EPA and DHA are treated as though they represent a single nutrient, i.e. the sum of EPA+DHA. This is a consequence of using commercially relevant FO and FM as the sources of LC-PUFA to formulate the experimental feeds. The present chapter gives detailed description of the fish feeding trials and associated biochemical, molecular and statistical analyses. More chapter-specific materials and methods are further provided in the corresponding result chapter where appropriate.

II.2 The feeding trials

The feeding experiments were designed and conducted by BioMar, who were responsible for formulating the diets and executing the experiments. This placed some constraints on the author.

Six experimental diets (D1-D6) were formulated, for each species, to contain precise levels of EPA+DHA. They were formulated to be representative of modern aquafeeds currently fed to the two species, gilthead seabream and European seabass. To achieve these EPA+DHA levels, three commercially available oils were used: A South American FO, rich in EPA+DHA; rapeseed oil (RO), rich in oleic acid (18:1n-9) and linoleic acid (18:2n-6) and palm oil (PO), rich in palmitic acid (16:0) and oleic acid (18:1n-9). Six mixtures of these oils were used as the added oil component of the seabream and seabass diets, with D1 containing only a blend of RO and PO whereas D6 contained only FO as the sole added oil (Table II.1).

Table II.1. The oil mixtures used to formulate the experimental diets expressed as percentage of the mixture and the targeted level of EPA+DHA as a percentage of total fatty acids.

Diet:	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Fish oil	0.0	11.9	17.9	30.3	55.4	100.0
Rapeseed oil	66.8	59.0	55.1	46.5	29.8	0.0
Palm oil	33.2	29.1	27.0	23.2	14.8	0.0
EPA+DHA[1]	1.4	3.5	4.6	6.8	12.3	23.4

[1]% total fatty acids

Other ingredients were selected to meet the known nutrient requirements of gilthead seabream (Oliva-Teles, Lupatsch et al. 2011, National Research Council 2011) and European seabass (National Research Council 2011). Small differences in formulation, between the gilthead seabream and European seabass basal diets reflect the current understanding of the nutrient requirements of these species. Due to imbalances in certain nutrients present in FO but not VO, some nutrients, namely cholesterol and phospholipids (Emulthin G35), required to be balanced so that they did not compromise the growth of the fish and therefore the experiments. As a large part of the dietary protein was supplied as soya protein concentrate and rapeseed meals, the formulations were supplemented with

crystalline lysine and methionine (a common commercial practice). The gilthead seabream and European seabass dietary formulations and proximate analysis are provided in Table II.2 and Table II.3, respectively. An inert marker, yttrium was added to the formulation, however due to time constraints during the experiment faecal samples were not collected and consequentially no digestibility work was carried out.

The diets were produced by extrusion at the BioMar TechCentre, Brande, Denmark. They were produced as two pellet sizes, 3 mm and 4.5 mm to cover the anticipated growth of the fish during the experimental periods. The feeds were delivered to the BioMar Feed Trial Unit, Hirtshals, Denmark, where they were stored at room temperature. Samples of diets were also shipped to the Institute of Aquaculture (IoA), University of Stirling (UoS), where they were stored at -20°C until further analysis of proximate and fatty acid composition.

All experimental fish were maintained under the current European legislation on handling experimental animals (2010/63/EU). Furthermore, this project was submitted to the UoS Animal Welfare and Ethical Review Board (AWERB) for detailed ethical review and was authorised by the board. The gilthead seabream and European seabass used in this study were purchased from a commercial hatchery (Les Poissons du Soleil, Balaruc-les-Bains, France) and transported to Hirtshals. Before commencing the trial, they were fed a commercial diet rich in FM and FO and grown to a weight of approximately 25 g. Approximately 2,700 seabream and seabass individuals were used in the trials.

Table II.2. Diet formulations and proximate analyses of the six experimental diets for gilthead seabream (Chapter III).

DIET **Ingredients (%)**	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Fishmeal	12.5	12.5	12.5	12.5	12.5	12.5
Soya Protein Concentrate	21.9	21.9	21.9	21.9	21.9	21.9
Rape seed meal	10.0	10.0	10.0	10.0	10.0	10.0
Wheat Gluten	4.0	4.0	4.0	4.0	4.0	4.0
Corn Gluten	25.0	25.0	25.0	25.0	25.0	25.0
Wheat	7.1	7.1	7.1	7.1	7.1	7.1
Aminoacids[1]	0.8	0.8	0.8	0.8	0.8	0.8
Micro-ingredients[2]	3.1	3.2	3.2	3.3	3.5	3.9
Yttrium	0.03	0.03	0.03	0.03	0.03	0.03
OILS (%)						
Fish Oil (SA)	0.0	1.8	2.6	4.4	8.0	14.9
Rapeseed Oil	10.4	9.2	8.6	7.3	4.8	0.0
Palm Oil	5.2	4. 6	4.2	3.6	2.4	0.0
PROXIMATE COMPOSITION (% of diet as fed)						
Protein	41.8	43.0	42.9	41.7	42.4	42.5
Lipid	22.2	21.7	21.7	21.5	21.8	20.8
Ash	6.1	6.1	5.9	5.9	6.1	6.2
Moisture	10.3	9.7	9.4	10.7	8.9	8.9
Energy crude (MJ/kg)[3]	22.0	22.1	22.2	21.8	22.2	22.0

[1] Lysine and methionine

[2] Vitamin and mineral premix, monocalcium-phosphate (MCP), cholesterol, Emulthin G35, antioxidants

[3] Calculated by using the mean values of gross energy for proteins, lipids and carbohydrates 23.6, 39.5 and 17.2 kJ/g, respectively

Table II.3. Diet formulations and proximate analyses of the six experimental diets for European seabass (Chapter IV).

DIET **Ingredients (%)**	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Fishmeal	12.5	12.5	12.5	12.5	12.5	12.5
Soya Protein Concentrate	18.3	18.3	18.3	18.3	18.3	18.3
Rape seed meal	10.0	10.0	10.0	10.0	10.0	10.0
Wheat Gluten	5.0	5.0	5.0	5.0	5.0	5.0
Corn Gluten	25.0	25.0	25.0	25.0	25.0	25.0
Wheat	9.6	9.6	9.6	9.6	9.6	9.6
Aminoacids[1]	0.9	0.9	0.9	0.9	0.9	0.9
Micro-ingredients[2]	3.8	3.9	4.0	4.1	4.2	4.5
Yttrium	0.03	0.03	0.03	0.03	0.03	0.03
OILS (%)						
Fish Oil (SA)	0.0	1.8	2.6	4.4	8.0	14.1
Rapeseed Oil	9.9	8.7	8.1	6.8	4.3	0.0
Palm Oil	4.9	4.3	4.0	3.4	2.1	0.0
PROXIMATE COMPOSITION (% of diet as fed)						
Protein	39.8	42.5	41.0	41.4	41.8	41.6
Lipid	21.4	20.7	21.8	21.0	20.6	20.9
Ash	5.8	5.8	5.9	6.0	6.0	5.9
Moisture	10.8	9.3	10.5	10.5	9.8	11.0
Energy crude (MJ/kg)[3]	21.7	21.9	21.9	21.7	21.8	21.6

[1] Lysine and methionine

[2] Vitamin and mineral premix, monocalcium-phosphate (MCP), cholesterol, Emulthin G35, antioxidants

[3] Calculated using the mean values of gross energy for proteins, lipids and carbohydrates 23.6, 39.5 and 17.2 kJ/g, respectively

At the start of the trials 150 fish of each species were randomly distributed to triplicate 1 m^3 tanks (n = 3) in a recirculation saltwater system and initial bulk weights recorded in each tank. The mean initial fish weights (± SD) were 24.3 g (± 0.0) and 23.2 (± 0.9) for seabream and seabass, respectively. Feeding of the experimental diets commenced on the 7th April 2014 and lasted until the 13th August for the seabass (127 days) and 14th August for the seabream (128 days). The 3 mm pellet was fed till the 2nd June 2014 (56 days, P1) and the 4.5 mm was fed till termination (72 and 71 days, P2). The fish were fed to apparent satiation twice per day. Excess feed was delivered using belt feeders and uneaten pellets collected. Delivered and uneaten feed were recorded to accurately determine biological feed conversion ratio (FCR). The densities reached at the end of each trial were 32.5 Kg m^{-3} and 21.3 Kg m^{-3} for seabream and seabass, respectively. During the trials, mortalities were 1.6 % and 0.3 % for the seabream and seabass, respectively. Mortalities were weighed and deducted from biomass calculations. Mortalities were unrelated to dietary treatment. The fish were reared at 24°C and 32 g L^{-1} salinity and under 12:12 L:D photoperiod.

The fish in each tank were bulk weighed at the change of pellet size and on termination of the trial. Prior to bulk weighing feed was withdrawn for 24 h. The fish were anaesthetised with benzocaine (Centrovet, Kalagin, Santiago, Brazil), removed to a table to allow excess water to drain and weighed in manageable batches. After weighing the fish were placed in clean seawater to recover and returned to their tanks. Feeding recommenced the following day.

The following fish and samples were taken from fish that were euthanised with a lethal dose of benzocaine. At the termination sampling, five whole fish from each tank

were taken for proximate composition analysis. Three further fish from each tank were taken and separated to eviscerated carcasses, livers and viscera (minus liver). The fish and body compartments were pooled by tank. These samples were stored at -20°C. Tissue samples (liver and mid-intestine) were collected from three fish from each replicate tank. A small piece of liver and a short piece of mid-intestine (~1 cm) was dissected about 1 cm behind the pyloric caeca. The mid-intestine sample was cleaned of any contents and perivisceral fat removed. A 1 - 2 mm^3 piece of each tissue was cut and placed in RNAlater® (Sigma-Aldrich, UK) and a ~0.5 cm^3 piece was taken for fatty acid analysis. Tissue samples for RNA extraction were perfused with RNAlater® for 24 h at 4°C and subsequently stored at -20°C. Samples for fatty acid analysis were stored at -20°C.

All the samples mentioned above were stored for 1-2 months in freezers at Hirtshals at -20°C. Then they were shipped to the IoA using a 24 h courier service. All samples were packed into polystyrene boxes containing dry ice. On arrival samples were stored at -20°C, whereas samples for RNA extraction were stored at -70°C.

II.3 Laboratory analyses

Most reagents were purchased from Fisher Scientific UK (Loughborough, UK) or Sigma-Aldrich (recently acquired by Merck, Darmstadt, Germany). Solvents (chloroform, methanol, isohexane, diethyl ether, toluene, isopropanol, ethanol) were invariably HPLC grade from Fisher Scientific. Gases were obtained from the British Oxygen Company (Glasgow, UK). Reagents or materials purchased elsewhere will be indicated accordingly in the text.

II.3.1 Proximate composition

The proximate chemical composition of whole fish, carcasses, viscera and livers were determined by standard methods described the Association of Analytical Chemists (2000). Samples, pooled by tank, were homogenised and sub-sampled, all further analyses were carried out with untreated homogenate. Analyses were carried out in duplicate and repeated if technical replicates were not in agreement, coefficient of variation (CV) > 5 %.

Moisture content of samples was determined by drying at 90°C for 24 h. 1.0 - 5.0 g of homogenate (0.5 g for liver) was placed in a pre-weighed dish and then placed in a drying oven until a constant mass was achieved (usually 24 h). The dish was then re-weighed after cooling in a desiccator and the moisture content determined using the equation:

$$Moisture\ (\%) = \frac{Sample\ weight\ (g) - Dry\ sample\ weight\ (g)}{Sample\ weight\ (g)} \times 100$$

Ash content was determined by combustion of samples at 600°C in a muffler furnace. A total of 1.0-2.0 g of sample homogenate (0.5 g for liver) was placed in a pre-weighed porcelain dish. Samples were combusted overnight and then re-weighed after cooling in a desiccator and ash content determined using the equation:

$$Ash\ (\%) = \frac{Ash\ weight\ (g)}{Sample\ weight\ (g)} \times 100$$

Crude protein content of samples was determined using the Kjeldahl method (Nitrogen * 6.25), which assumes that the nitrogen content of animal protein is 16%

(Persson, Wennerholm et al. 2008). Nitrogen content was analysed by titration using a Kjeltec analyser (Foss 2300, Gemini, Apeldoorn, Netherlands) after sample hydrolysis. Briefly, 250 mg of homogenate was weighed into a test tube with two copper kjeltabs (Fisher Scientific) and 5 ml of concentrated sulphuric acid (Fisher Scientific). Two blanks and two standards (pure lysine, Sigma-Aldrich) were routinely run with each batch of samples. Acid hydrolysis was carried out on a hot block at 420°C for 1 h. Samples were cooled to room temperature before adding 20 ml of distilled water and vortexing. All tubes were then titrated on the Kjeltec analyser and protein content determined using the equation:

$$Crude\ Protein\ (\%) = \frac{(Sample\ titre - Blank\ titre) \times 1750.875}{Sample\ wieght\ (mg)}$$

where 1750.875 is a conversion factor to convert sample titre volume to percent protein based on the standardised protein factor.

The total lipid content of samples was determined using the Folch (1957) method and is described in Section II.3.2.1 on fatty acid analysis. Whole body lipid samples were stored at 10 mg ml^{-1} in chloroform/methanol (2:1, v/v) under oxygen free nitrogen (OFN) at -20°C for whole body fatty acid analysis.

II.3.2 Fatty acid analysis

II.3.2.1 Lipid extraction

The total lipids from feeds, whole fish, carcass, viscera and tissue samples were extracted according to Folch et al. (1957) and quantitated to calculate the tissue concentrations of fatty acids. Samples were weighed and homogenised (Ultra-turrax,

Sigma-Aldrich) in 20 ml chloroform/methanol (2:1) and allowed to stand on ice for 1 h in 50 mL glass test tubes. Feeds or feed materials were extracted using 36 ml of solvent and left in a freezer overnight. The samples were washed with 5 ml 0.88 % (w/v) KCl (Sigma-Aldrich) and centrifuged at 400 g (Jouan, C312, Fisher Scientific). The aqueous fraction was removed by aspiration and the solids by filtration of the organic phase. The solvent was evaporated under OFN and then by desiccation *in vacuo* overnight. The total lipids were weighed and then dissolved in chloroform/methanol (2:1) + 0.01 % (w/v) butylated hydroxytoluene (BHT, Sigma-Aldrich) at 10 mg mL^{-1} and stored under OFN at -20°C for fatty acid analysis.

II.3.2.2 Separation and purification of fatty acid methyl esters

Fatty acid methyl esters (FAME) were prepared from total lipids samples by acid catalysed transesterification and purification by thin layer chromatography (TLC) (Christie 2003). An internal standard, 0.1 mg of heptadecanoic acid (17:0, Sigma-Aldrich) was added to 1 mg of total lipid in glass test tubes and the solvent evaporated under OFN. Transesterification was started by adding 1 ml of toluene and 2 ml of 1% (v/v) sulphuric acid (Fisher Scientific) in methanol and heating to 50°C for 16 h. The reaction was stopped by addition of 2 ml of 2% (w/v) $KHCO_3$ (Sigma-Aldrich). FAME were recovered by the addition of 5 ml isohexane/diethyl ether (1:1) with 0.01% (w/v) BHT. Tubes were mixed and centrifuged for 2 min at 400 g (Jouan, C312) and the organic fraction removed by pipette. A further 5 ml isohexane/diethyl ether (1:1) was added to the original tubes and the process repeated to recover any remaining methyl esters. Solvent from the collected fractions was evaporated under OFN and FAME were purified on 20*20 cm TLC silica gel plates (Merck) developed with isohexane/diethyl ether/acetic

acid (90:10:1, v/v/v). FAME bands were visualised by spraying with 1% (w/v) iodine in chloroform, the silica was scrapped off the plate to a fresh tube and purified FAME were eluted by addition of 10 ml isohexane/diethyl ether (1:1) with 0.01% (w/v) BHT followed by centrifugation. The supernatant was pipetted into a new tube and the solvent evaporated under OFN. FAME were dissolved in 0.8 ml of isohexane and stored under OFN at -20°C until analysis by gas chromatography.

II.3.2.3 Gas chromatography

The gas chromatograph used for FAME analysis was a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m x 0.32 mm i.d. x 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK). Samples were injected on-column by an auto sampler and detection was by flame ionisation. The carrier gas was hydrogen with an initial oven thermal gradient from 50 °C to 150 °C at 40 °C min^{-1} to a final temperature of 225 °C at 2 °C min^{-1}. Chromcard for Windows (Version 1.19; Thermoquest Italia S.p.A., Milan, Italy) was the software used to export the data. FAME were identified by comparison to known commercial standards (Supelco 37-FAME mix, Sigma-Aldrich) and a marine oil prepared in the laboratory. Fatty acid contents were expressed as percentage (%) of total fatty acids and absolute values (mg g^{-1}).

Corrected percentage calculation:

Percentage of total fatty acids = (Raw peak area * 100 / ∑ Raw peak area) * (100 / (100 – 17.0 Peak area))

Calculation of fatty acid per unit tissue mass (mg g^{-1}):

Fatty acid sample concentration (mg g^{-1}) = (Raw peak area * 100 / ∑ Raw peak area) * (100 / 17.0 Peak area) * (Sample lipid (%) / 100)

where the Raw peak area is the fraction of total area of the chromatogram of an identified fatty acid and 17.0 is the corrected peak area of the internal standard.

II.3.3 Relative gene expression

II.3.3.1 Extraction of RNA

The method to extract total RNA from fish tissues was based on Tri Reagent (Ambion, Sigma-Aldrich) and use of 1-bromo-3-chloropropane (BCP, Sigma-Aldrich). Approximately 100 mg of tissue was homogenised in 1.0 mL of Tri Reagent using a Mini-Beadbeater 24 (Biospec, Bartlesville, USA). Homogenised samples were stored at -40 °C overnight before continuing with the protocol. The samples were thawed and then centrifuged for 10 min, 4 °C at 12,000 g (SciQuip 4K15, Sigma-Aldrich) and the Tri Reagent transferred to a fresh tube. One hundred µl of BCP was added to separate the aqueous and organic phases. After shaking vigorously, the tubes were incubated for 15 min at room temperature before centrifugation for 15 min at 20,000 g and 4 °C. Then 400 µl of the aqueous phase was pipetted and transferred to a fresh tube. Precipitation of the RNA was achieved by adding 200 µl isopropanol and 200 µl RNA precipitation solution (see below), mixing the tubes gently and incubating at -20 °C for at least 10 min and followed by centrifugation for 10 min at 20,000 g and 4 °C. The supernatant was removed, and the RNA pellet washed twice with ice-cold 75% ethanol in milliQ H_2O (v/v). Ethanol was pipetted from the pellet and the excess allowed to dry at room temperature. The RNA was resuspended in milliQ H_2O and the concentration measured spectrophotometrically (NanoDrop ND-1000, Spectrophotometer, Sussex, UK). Integrity was checked by the

absorbance ratio (260:280 nm) and by agarose gel electrophoresis (Section II.3.3.4). Bands of 18S and 28S ribosomal subunits were visualised under ultra violet light to check for RNA degradation. Total RNA extracts were stored at -80 °C until further analysis.

RNA precipitation solution (1 L)

1.2 M sodium chloride (Sigma-Aldrich), NaCl – 70 g
0.8 M Sodium Citrate Sesquihydrate (Sigma-Aldrich), $C_6H_6Na_2O_7 \cdot 1.5\ H_2O$ – 210.5 g
Made up to 1 L with milliQ water

II.3.3.2 Preparation of cDNA by reverse transcription

Reverse transcription (RT) of the RNA was performed using a High-Capacity cDNA Reverse Transcription kit without RNAase inhibitor (Applied Biosystems, Warrington, UK). A 2.0 µg aliquot of RNA in 10 µl of milliQ H_2O was used as reaction substrate. The total volume was 20 µl and the master mix made up according to manufacturer's protocol, except the addition of oligo(dT) primers (Eurofins Genomics, Ebersberg, Germany) as follows: reverse transcription buffer, 2.0 µl; deoxynucleotide (dNTP) mix (100 mM), 0.8 µl; random primers, 2.0 µl; oligo T primers, 0.5 µl; nuclease-free H_2O, 4.2 µl and reverse transcriptase (RTase), 1.0 µl. Initially, samples were denatured at 75 °C and then the master mix was added. The reaction was performed on a Biometra Thermocycler (Analytik Jena, Goettingen, Germany) using the following program: 25 °C for 10 min; 37 °C for 120 min; 85 °C for 5 min and terminated at 4 °C. Five samples from each tissue were prepared as negative controls (RT^-) in the same way but without addition of reverse transcriptase (RTase) and a non-template control (NTC) was prepared without RNA. These were run on the first plate for RT-qPCR, as described in Section II.3.3.3. RT was performed by tissue, so there were six diets, triplicated with three fish sampled from each

tank (N = 54). The reaction products were used to prepare cDNA samples at 1:20 dilution with milliQ H_2O. A pooled cDNA sample was also prepared using 4.0 µl from each reaction. cDNA samples were stored at -20 °C for further analysis.

II.3.3.3 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with Luminaris Color HiGreen qPCR Master Mix (SYBRgreen, Thermo Scientific, Hemel Hempstead, UK). Each seabream cDNA samples were analysed in duplicate on a Biometra TOptical Thermocycler instrument (Analytik Jena, Goettingen, Germany) in 96 well plates (StarLab, Milton Keynes, UK). As there were 54 samples per tissue, each analysed in duplicate, two plates were used for each gene and a calibration sample (1:20 of the pool) run to check and control for inter assay variation. Plate corrections were rarely required as the two plates were run sequentially using the same master mix. Total reaction volume was 20 µl and each well contained: SYBRgreen, 10.0 µl; milliQ H_2O, 3.0 µl; forward primer 10.0 pM, 1.0 µl; reverse primer 10.0 pM, 1.0 µl and 5.0 µl of cDNA (for reference genes this was reduced to 2.0 µl and 3.0 µl additional milliQ H_2O added). The seabass samples were performed in triplicate on a Lightcycler-480 instrument (Roche Diagnostics Corp, Indianapolis, USA) using 384-well plates (Roch Diagnostic Corp). Total reaction volume was 10.0 µl and each well contained: SYBRgreen, 5.0 µl; milliQ H_2O, 1.5 µl; forward primer 10.0 pM, 0.5 µl; reverse primer 10.0 pM, 0.5 µl and 2.5 µl of cDNA (for reference genes this was reduced to 1.0 µl and 1.5 µl additional milliQ H_2O added). Plates were prepared with a standard curve of pooled cDNA diluted with milliQ H_2O (dilutions: 1/5; 1/10; 1/20; 1/50; 1/100; 1/200 and 1/500), a calibrator (1/20 pool, same dilution as samples) and a NTC. After preparation, the plate was covered, briefly

vortexed and centrifuged. Both seabream and seabass samples were analysed using identical thermocycling protocols as follows: 50 °C for 2 min, 95 °C for 10 min, then 35 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s, and a final melting curve to check for non-specific products.

II.3.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to: 1) determine the specificity of primer pairs to their target genes, 2) visualise the integrity of RNA extractions and 3) purify PCR products. The 1% (w/v) agarose (Sigma-Aldrich) gels were prepared in Tris-Acetate buffer (1x, TAE, see below) with 0.5 µg ml^{-1} ethidium bromide. Electrophoresis tanks were filled with the same 1x TAE buffer. Gels were placed in the tanks and PCR products loaded with an appropriate "ready-to-use" GeneRuler (Fisher Scientific) (100 bp for PCR products and 1 Kb for RNA extractions). Gels were run at 50 – 100 V for the necessary time to visualise the products of interest.

Tris-Acetate buffer (TAE, *50)

242 g Tris base (Sigma-Aldrich)
57.1 mL glacial acetic acid (Fisher Scientific)
100 mL 0.5M Ethylenediaminetetraacetic acid (EDTA pH8, Sigma-Aldrich)
Made up to 1 L with milliQ water.

II.3.3.5 Sequencing

Partial coding DNA sequences (CDS) were sequenced for some of the European seabass candidate genes reported in Chapter VI. Primers were designed on conserved regions identified by alignment of orthologues from several fish species using Bio Edit

(Ibis Therapeutics, Carlsbad, California, USA). PCR were carried out on pooled cDNA as template using MyTaq HS DNA polymerase master mix (Bioline, London, UK) in 20.0 µl reactions containing: 10.0 µl of mastermix; 2.0 µl of 1:20 pooled cDNA and 1.0 µl of forward and reverse primers (10 pM). The reactions were run on a Biometra thermocycler with the following cycling conditions: 95°C for 1 min then 35 cycles of 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. Products were visualised by gel electrophoresis (Section II.3.3.4) and when necessary, products were purified on gel to obtain the fragment of the correct length. Purification of the products was carried out using an Illustra GFX PCR DNA and gel band purification kit (GE Healthcare Lifesciences, Little Chalfont, UK), according to the manufacturer's protocol.

Purified PCR products were sent for DNA sequencing at GATC Biotech ltd. (Cologne, Germany). Sequence reads were checked and assembled in Bioedit and translated to peptide sequences using ExPASy (Swiss Institute for Bioinformatics, Lausanne, Switzerland) (Artimo, Jonnalagedda et al. 2012). Once a clean fragment was obtained it was submitted to GenBank via National Centre for Biotechnology Information (NCBI) online submission tool BankIt (NCBI, Bethesda, USA). Partial sequences for the seabass *srebp2*, *fas* and *pparα1* were submitted to NCBI (Chapter VI).

II.3.3.6 Primer design and verification

For sequencing purposes, primers were designed manually in alignments performed in Bioedit and verified by gel electrophoresis (Section II.3.3.4) and then by use of the NCBI's basic local alignment search tool (BLAST) on the returned sequence. For RT-qPCR primers designed using Primer3 (Untergasser, Cutcutache et al. 2012, Koressaar, Remm 2007), through the NCBI website and purchased from Eurofins Genomics. Primers

were designed to have 60°C annealing temperatures. Primer specificity and annealing temperature was verified with MyTaq HS DNA polymerase master mix in 20.0 µl reactions containing: 10.0 µl of master mix, 2.0 µl of 1:20 pooled cDNA and 1.0 µl of forward and reverse primers (10 pM). The reactions were run on a Biometra thermocycler with the following cycling conditions: 95°C for 1 min then 35 cycles of 95°C for 15 s, temperature gradient 58 - 62°C for 15 s and 72°C for 10 s. A single reaction was carried out at each annealing temperature (58, 60, 62°C). The amplification of a single band of the appropriate length was visualised after gel electrophoresis. The efficiency of the primer was determined using RT-qPCR with a dilution curve of pooled cDNA (1/20 dilution), as described in Section II.3.3.3. Primers with efficiencies < 1.8 were rejected. The melting curves for each well were inspected to check for secondary products.

II.3.3.7 Data handling

Threshold cycle (C_t) values were exported for all wells from the relevant instrument's software and further calculations carried out in Excel. Coefficients of variation (CV, %) were calculated at the level of C_t. The sample was deleted if the CV was over 1.5%. Coefficients for the standard curve were calculated so that reaction efficiency could be calculated using the following equation:

Efficiency (E) = $10^{(-1 \,/\, \text{Slope of standard curve})}$

Expression ratios (Pfaffl 2001) were determined with the following equation:

$$Expression\ ratio = \frac{E(ref)^{Ct(Sample)}}{E(goi)^{Ct(Sample)}} \div \frac{E(ref)^{Ct(Calibrator)}}{E(goi)^{Ct(Calibrator)}}$$

where *E* is the determined efficiency, *ref* is the geometric mean of four reference genes (*ef1α*, *β-actin, tuba1α* and *rplp0*), *goi* is the gene of interest (candidate gene), Ct is the threshold cycle and Calibrator is a 1/20 dilution of the pool. These ratios were $\log_2$ transformed (Hellemans, Vandesompelle 2011). The above calculation was selected because the data are normalised to their centre (the calibrator sample), this means that variance is shared evenly across the data set rather than being loaded unequally to the treatments due to the choice of a control treatment. Taking the $\log_2$ of the expression ratio controls the error distribution for the down-stream statistical analysis (Hellemans, Vandesompelle 2011).

II.4 Statistics

All statistics and figures were carried out in the statistical programming language R (Venables, Smith 2015). There are two major sections under this heading: section II.4.1 deals with the nonlinear regression analysis of biometric data derived from the two experiments that form Chapters III and IV; section II.4.2 deals with statistical treatment of the data presented in Chapters V and VI. The first part of each section will outline the methods of statistical treatment and the second part will give details about implementation of these techniques in the R programming environment, which is necessary for repeatability.

II.4.1 Nonlinear regression models for requirement determination

II.4.1.1 Statistical methods

This section refers to the statistical techniques applied to the biometric data presented in Chapters III and IV. The biometrics were weight gain (WG), daily growth index (DGI) and feed conversion ratio (FCR). These biometrics are defined as follows:

WG = Final mean weight (g) – Initial mean weight (g)

DGI = Final mean weight $(g)^{0.333}$ – Initial mean weight $(g)^{0.333}$ / Days

FCR = Ingested feed (Kg) / (Final biomass (Kg) – Initial biomass (Kg))

These metrics were calculated at the level of tank and for each pellet size (P1 = 3 mm pellet, P2 = 4.5 mm pellet) and over both pellet sizes (OV) to produce the raw data for analysis (N = 18). All the relevant regression models applied to these data are presented in Chapters III and IV. Invariably, the predictor variable (x-axis) was dietary EPA+DHA (percentage of diet as fed) and the response (y-axis) was one of the metrics described above. All model assumptions were checked according to techniques described by Ritz and Streibig (2008) and where violations occurred, these were declared in the text. In Chapter IV, robust fitting algorithms were used, thus relaxing the normality of error assumption. The diagnostic tests of the models included: 1) Levene's test for homogeneity of variances, 2) Shapiro-Wilkinson test for normality of error, 3) the Run's test to check for non-independent errors, 4) the lack-of-fit test, which tests the nonlinear model against an analysis of variance (ANOVA) model, 5) the null test, which tests the significance of the model against the mean of the data (tests for the existence of a relationship in the response), and 6) diagnostic plots were produced as part of the model

assessment work flow. To assess the fit of a function two metrics are given. Firstly, the residual error after fitting was presented as the residual sum of the squares (RSS, in the case of Chapter III) and iteratively reweighted least squares (IRLS, in the case of chapter IV). Secondly, the Akaike information criterion (AIC) is presented, this value compares nonlinear models and penalises models for over parameterisation. Calculation of the uncertainty of a nonlinear function is not trivial, and usually omitted by authors. The model output gives standard errors of the parameter estimates, but these are not useful because it is the error around the model's ability to predict that is of interest. The methods used to calculate the uncertainty (95% prediction interval) were second order propagation and Monte-Carlo simulation (Spiess 2013). Propagation was suitable for asymptotic curved functions (Gompertz and four parameter logistic functions) and Monte-Carlo simulation for the split linear or quadratic functions (see Chapter III). These allowed the calculation of a 95% prediction interval around a model's fitted values, these being shaded in the relevant figures. Plots of the models are given in Chapters III and IV. The components were: the response (y-axis) as a function of the predictor (x-axis), the raw biometric data (coloured points), the fitted values (plotted as a black line), the 95% prediction intervals of the fitted values (shaded) and, in the case of asymptotic functions, the derived requirement estimates. With the asymptotic models an explicit requirement estimate is not given by the function, and this value is derived by using the model backwards (i.e. a value for the predictor is derived from a chosen level of the response). This is somewhat statistically flawed as one of the assumptions of regression is that the predictor has been measured with certainty, yet this value is derived from the response that is not known with certainty. Therefore, it is critical to present the level of uncertainty in the response, and sometimes it is wide. Once a series of models had been produced for

OV, P1 and P2 a technique for objective comparison was required. Therefore, in the model tables presented in Chapters III and IV, the model RSS/IRLS and the AIC values are included. The lower the RSS/IRLS the more variation explained by the model. The lower the AIC value the more parsimonious the model (Sakamoto, Ishiguro et al. 1986). The use of R^2 values for nonlinear functions is debated, but R^2 is calculated from RSS, therefore this indicator of the error was chosen for presentation.

II.4.1.2 Implementation in R

This section introduces the function calls and their "package" of origin that were used to call statistical tests or calculations to the data. It is not a repeat of all the necessary code to reproduce the analyses. The R environment is extremely diverse and powerful containing thousands of "base" functions for statistics and data manipulation, additional functions are available in the form of "packages" downloaded from the Comprehensive R Archive Network (CRAN, https://cran.r-project.org). Furthermore, R is open source and free to anyone.

The environment in R holds data "objects" in the computer's random-access memory (RAM). These are created by functions and manipulated by other functions to produce new objects. Nonlinear regression models are stored in the environment as objects of class: "nls". All nonlinear models were called with R's base function nls(), in the case of Chapter III or nlrob(), in the case of Chapter IV, from the package Robustbase (Rousseeuw, Croux et al. 2015).

The model diagnostics, listed in Section II.4.1.1, were carried out to verify the suitability of the models using the packages Car (Fox, Weisberg et al. 2016) and nlstools

(Baty, Ritz et al. 2015). For 2), 3) and 6) the input is the nonlinear error (residuals), which are extracted from the "nls" object with nlsResiduals() (nlstools) and this object passed to the relevant functions listed below.

1) Levene's test: leveneTest() – Car package
2) Shapiro-Wilkinson: test.nlsResiduals() – nlstools package
3) Run's test: test.nlsResiduals() – nlstools package
4) Lack-of-fit test: lm() to create ANOVA model followed by anova() to compare the nonlinear model with its nested ANOVA model – base R
5) Null test: lm() to create null model (a single intercept model, the mean of the data) followed by anova() to compare the nonlinear model with its null model – base R
6) Diagnostic plots: plot() – base R

As mentioned above, calculation of the uncertainty surrounding nonlinear functions is not trivial. The package propagate (Spiess 2013) contained the function necessary to achieve this task, predictNLS(). This function simultaneously calculates Monte-Carlo simulations and carries out second-order Taylor expansion of a series of model derived fitted values obtained using predict() (base R). The propagation method could not be applied to split models (models that ascend to a static plateau) and so the Monte-Carlo values were used in these cases.

AIC values for a model were called with AIC() (base R). To plot the various models and uncertainty the graphics package ggplot2 was used to bring the relevant information together (Wickham 2016), as described in Section II.4.1.1. The model parameters, RSS/IRLS and AIC and requirements were presented in tables (see Chapters III and IV).

II.4.2 Statistical techniques applied to compositional and gene expression data

The techniques applied to data in Chapters V and VI were: principal components analysis (PCA), linear and quadratic regression and ANOVA. This section is divided under those headings with the appropriate functions summarised in the last sub-section.

II.4.2.1 Principal components analysis

PCA is a dimension reduction technique and is particularly useful to explore multivariate data, such as fatty acid compositions. PCA was applied to fatty acid data as means of identifying the main correlations between fatty acids and, fatty acids that may be physiologically interesting. The data were cleaned of fatty acids (variables) that contained many zeros and a few remaining zeros were imputed with a constant (0.005, chosen because it is approximately one order of magnitude below the detection limit of the gas chromatograph). The C_{20} and C_{22} MUFA were summed to single variables as the peaks do not always separate on the GC instrument. The variables were scaled so that each fatty acid had equal weight in the analysis. The results of PCA are presented graphically in the relevant chapters, this includes a biplot of the first two principal component scores and a loadings plot indicating the contribution of the variables (fatty acids) to the construction of the principal components. Dietary fatty acid profiles were included as "supplementary individuals" in the biplots, allowing the diet fatty acid profiles to be compared to the tissue profiles. The loadings plot contains arrows for each variable, the length of this arrow indicates the quality of projection in the two-dimensional plotting plane.

II.4.2.2 Regression

Regression (linear or quadratic) was applied to composition, fatty acid and gene expression data. The predictor variable was either dietary VO (% diet) or in the case of fatty acids, the dietary level of that fatty acid. The assumptions of linear or quadratic regression were tested, mostly in the same way as the nonlinear regression models described in Section II.4.1.1. In some cases, transformations were required and usually applied as logarithms or power transforms.

II.4.2.3 Analysis of variance

ANOVA was also applied to fatty acid data. To test for the effect of tank it was included as a variable nested in diet and tested against a model without tank. The Levene's test and Shapiro-Wilkinson test checked for heteroscedasticity and normality, respectively. When required, transformations were applied to the data, usually a power transformation to control heteroscedasticity. When differences were identified, Tukey's, honest significant differences (HSD) tests were applied to establish which means differed.

II.4.2.4 Implementation in R

PCA was performed using the function PCA(). From the FactoMineR package (Husson, Josse et al. 2016). This package allows supplementary individuals (rows in a data table) and supplementary variables (columns in a data table) to be added to the analysis. These are not used to generate the analyses or scores but given scores after analysis. The dietary fatty acid profiles were used as supplementary individuals in Chapter VI. To present this data, data from the PCA object was extracted and passed to ggplot2 for plotting.

Regression analyses were performed using the function lm() and models were verified using the tools described previously (Section II.4.1.2) but with R base functions or tools from the Car package. Where necessary power transforms were applied (usually to control heteroscedasticity).

ANOVA models were called using R's base function aov() and models assessed as above. Where necessary power transforms were applied (usually to control heteroscedasticity). The lambda value was obtained using the function powerTransform() from the Car package. When differences were identified (P(>F) > 0.05) Tukey's HSD were identified using the function glht(), set to "Tukey".

III. A comparison of regression models for defining EPA+DHA requirements using the gilthead seabream (*Sparus aurata*) as a model species

Abstract

Carnivorous marine fish species, such as gilthead seabream (*Sparus aurata*) require dietary EPA and DHA for optimal growth and wellbeing. Due to the rapid growth of global aquaculture the quantity of marine oils used in aquafeeds has been limited, yet the overall quantity of oil in an aquafeed has increased to supply energy to rapidly growing farmed finfish. This has been satisfied by vegetable oils (VO) and, therefore, essential fatty acid (EFA) requirements in juvenile marine fish requires reassessment. A dietary trial was carried out with six experimental groups of gilthead seabream (~25 g) fed diets with specific EPA+DHA levels (0.2-3.2 % diet, as fed). For each pellet size, the biometric data (weight gain, daily growth index and feed conversion ratio) were analysed by four different regression strategies, namely split linear, split quadratric, the Gompertz function, and the four-parameter logistic function. Over the whole experimental period (two pellet sizes) the current published requirement (1% of diet) appeared too low and should be increased to at least 1.2%. However, when the first pellet size, for fish of 25 – 80 g was considered the requirement was at least 1.4% of diet. Thus, we demonstrate for the first time in a single trial that EFA requirement is a function of fish mass, decreasing as the fish grows. If FCR is considered, the requirement may be as high as 2 %. A range of regression models were required, for instance the first pellet growth data was best fit by curves but, over both pellet sizes, the split linear fit the data best. For asymptotic models, (Gompertz and four-parameter logistic functions) a novel way of defining the requirement is presented, the "elbow" calculation to identify an optimal point of diminishing returns on the nutrient response curve. Furthermore, it was illustrated how a range of regression approaches should be explored when determining nutrient requirement estimates as no single model is an ideal fit for all response curves.

III.1 Introduction

EPA and DHA are primarily found in marine oils. For marine carnivorous fish species, EPA and DHA are regarded as EFA due to the limited ability that these species possess for their biosynthesis from the C_{18} precursor, ALA (18:3n-3) (Castro et al. 2016). Consequently, marine carnivorous finfish have a dietary requirement for EPA+DHA to guarantee survival, health and normal growth (Oliva-Teles 2012, Tocher, Glencross 2015) and, currently FO is the primary source of these fatty acids in aquafeeds. ARA is also an important EFA and, although usually found at lower levels compared to EPA and DHA, is a key component of diets for larvae and broodstock (Bell, Sargent 2003).

An essential nutrient must be present in the animal's diet because it cannot be synthesised endogenously to fulfil physiological demands (Sargent et al. 2002). The response of an animal to the supply of an essential nutrient can be described by four levels of supply (Figure III.1). At lower levels, there is deficiency and the response (e.g. body weight) may take on negative values (e.g. weight loss). At some level, supply is balanced, and deficiency symptoms are not apparent (Lassiter, Edwards 1982). This can be referred to as the "maintenance requirement". Further increase in the dietary level of the nutrient takes the animal to optimum supply, at this point the response is close to maximum and characteristics such as growth and feed conversion are optimal (Bailleul, Bernier et al. 2000, Hauschild et al. 2010). This is the level that is usually considered a minimum requirement in terms of animal production, particularly for nutrients, such as EPA+DHA, derived from high value materials such as FO (Bacon 2017). Further increases in the nutrient supply only elicit very small gains in the response variable until maximum performance (plateau or asymptote) is reached. It should be noted that an empirical

determination of a nutrient requirement defines a minimum level of nutrient supply to attain an optimum/maximum response, and differs from nutrient specification, a term commonly used in animal feed formulation that refers to the quantity delivered in the feed (Glencross 2009). The exact characteristics of the nutrient response curve depend on the precise nutrient, the species, its life stage, and the selected response (Rodehutscord, Pack 1999). The most commonly used response is growth but not all nutrients will arrest growth and consequently the deficient, and maintenance part of the response may not be realised. This situation applies to EFA for juvenile marine fish (Kalogeropoulos et al. 1992, Skalli, Robin 2004), where low supply leads to slower growth but does not stop growth completely. However other symptoms of deficiency may become apparent (Bou, Berge et al. 2017). The NRC, Nutrient Requirements of Fish and Shrimp (2011) reports that the EPA+DHA requirements for gilthead seabream (*Sparus aurata*) for EPA+DHA is 0.9% of diet (dry weight) based on studies by Kalogeropoulos *et al.* (1992) and Ibeas et al. (1994, 1996, 1997). Moreover, Ibeas et al. (1997) suggested that, in addition to the EPA+DHA dietary level, the DHA:EPA ratio is also an important factor, with a value of 0.5 to be regarded as appropriate at a 0.9% EPA+DHA dietary supply (Ibeas et al. 1997). In fact, many species of fish such as Atlantic salmon (*Salmo salar*), Asian seabass (*Lates calcarifer*), turbot (*Psetta maxima*), sea bass (*Dicentrarchus labrax*), starry flounder (*Platichthys stellatus*), Korean rockfish (*Sebastes schlegeli*) and red drum (*Sciaenops ocellatus*) have been reported to have EPA+DHA (often referred to as n-3 ($\geq C_{20}$), LC-PUFA) requirements of ~1 % of diet (Coutteau et al. 1996, Lee, Lee et al. 2003, Lochmann, Gatlin 1993, Gatesoupe, Leger et al. 1977, Glencross, Rutherford 2011, Glencross, Tocher et al. 2014). However, other species such as yellowtail flounder (*Pleuronectes ferrugineus*), Japanese flounder (*Paralicthys olivaceus*), silver bream

(*Rhabdosargus sarba*) and striped jack (*Pseudocaranx dentex*) have been shown to require EPA+DHA at a higher level (1.3-2.5 %) (T. Takeuchi 1997, T. Takeuchi, Shiina et al. 1992, Whalen, Brown et al. 1998, Leu, Yang et al. 1994). Thus, EFA requirements vary among fish species, but also among developmental stages, with larvae known to require relatively higher levels of LC-PUFA, particularly DHA, to satisfy the high demands required in rapidly growing neural tissues where DHA is required (T. Takeuchi 1997, Tocher 2010).

It is appropriate to revisit the question of EPA+DHA requirements for two key reasons. First, in the last two decades there has been a substantial reduction in the use of FM and FO (sources of EPA and DHA) in aquafeeds. Second, the oil content of modern aquafeeds has increased to supply high energy for growth while sparing protein (Glencross 2009, Sargent et al. 2002), and it has been suggested that the EFA requirement may be dependent on the lipid content of the diet (Watanabe 1982, Glencross, Smith 2001). Therefore, the feed ingredients that contain EPA and DHA are being reduced while dietary energy is increased by the addition of vegetable oils (VO) that lack LC-PUFA, which may affect the requirement. The present study investigated the EFA requirements of a commercially important marine species, the gilthead seabream, fed a dietary gradient of EPA+DHA achieved by blending FO and VO. We herein discuss the benefits and drawbacks of a range of regression models used for nutrient requirement studies in fish (NRC, 2011) and provide estimations of EFA requirements in gilthead seabream juveniles calculated under the different regression strategies involving different responses including WG, DGI and FCR. A cost-benefit analysis associated with the dietary EPA+DHA inclusion levels used in this study was also carried out to explore the economic implications of the experimental diets.

III.2 Methods

III.2.1 Diets, fish husbandry and sampling

As described in Section II.2 and briefly described here, six experimental diets (D1 – D6) were formulated to deliver graded levels of EPA+DHA to juvenile gilthead seabream. The diet formulations and measured proximate compositions are detailed in Table II.2 and diet fatty acid compositions in Table III.1. Commercially available oils including FO, RO and PO were blended to achieve specific levels of EPA+DHA. Other ingredients were selected to meet the known nutrient requirements of this species (National Research Council 2011, Oliva-Teles et al. 2011).

The nutritional trial was carried out at the BioMar Feed Trial Unit (Hirtshals, Denmark) between April and August 2014 as described previously in Section II.2. Briefly, seabream juveniles of approximately 3 g were purchased from a commercial hatchery (Les Poissons du Soleil, Balaruc-les-Bains, France) and initially were fed a commercial diet rich in FM and FO from first feeding until they reached ~24 g. At the start of the trial, 150 fish were randomly distributed in each of 18 x 1 m^3 tanks (n = 3 per diet). The fish were fed two pellet sizes, firstly a 3 mm pellet (P1) for 56 days and, secondly a 4.5 mm pellet (P2) for 72 days. Therefore, three experimental periods were considered, namely P1, P2 and over the whole trial (OV), with the tank biomass being measured at 0, 56 and 128 days. At the end of P1 and P2, the fish biomass from each of the tanks was recorded and the fish counted. The fish were fed twice daily *ad libitum* and the delivered and wasted feed recorded for an accurate determination of biological FCR (see Section II.4.1.1).

Table III.1. Fatty acid composition and total fatty acids ($g.Kg^{-1}$) of the experimental diets given as percentage of total fatty acids. Note fatty acids < 0.05 across all rows removed.

	D1	**D2**	**D3**	**D4**	**D5**	**D6**
Total lipid (%)	22.16	21.71	21.71	21.47	21.82	20.82
% of total fatty acids						
14:0	0.55	1.07	1.47	2.10	3.34	5.73
16:0	15.44	15.62	15.71	16.05	16.69	17.52
18:0	2.53	2.62	2.67	2.80	3.04	3.43
20:0	0.46	0.38	0.43	0.41	0.36	0.26
22:0	0.23	0.23	0.22	0.21	0.19	0.16
ΣSFA	19.35	20.04	20.64	21.70	23.75	27.22
16:1n-7	0.51	1.12	1.48	2.20	3.65	6.71
18:1n-9	47.13	43.91	41.89	37.57	29.37	12.38
18:1n-7	2.47	2.55	2.58	2.69	2.77	2.99
20:1n	0.93	0.97	0.98	1.04	1.11	1.29
22:1n-11	0.08	0.14	0.19	0.28	0.43	0.75
24:1n-9	0.14	0.17	0.19	0.23	0.29	0.47
ΣMUFA	51.32	48.92	47.37	44.10	37.77	24.69
18:2n-6	21.42	20.42	19.69	18.56	16.16	11.22
18:3n-6	0.00	0.03	0.04	0.07	0.12	0.25
20:2n-6	0.06	0.07	0.07	0.08	0.10	0.14
20:4n-6	0.05	0.11	0.15	0.23	0.40	0.78
22:5n-6	0.00	0.04	0.05	0.09	0.16	0.31
Σ n-6 PUFA	21.52	20.67	20.01	19.03	17.01	12.90
18:3n-3	6.05	5.69	5.52	5.03	3.99	1.76
18:4n-3	0.08	0.30	0.44	0.70	1.23	2.40
20:4n-3	0.03	0.08	0.11	0.18	0.32	0.62
20:5n-3	0.70	1.96	2.73	4.32	7.48	14.34
22:5n-3	0.11	0.26	0.35	0.53	0.91	1.71
22:6n-3	0.63	1.47	1.98	3.06	5.23	9.89
Σ n-3 PUFA	7.61	9.76	11.13	13.83	19.16	30.77
EPA+DHA (%)[1]	0.22	0.57	0.78	1.10	1.91	3.22
Total fatty acids ($g.Kg^{-1}$)	168.49	165.14	164.70	148.28	150.12	133.34

[1]These values are the predictor variable for all analyses in this publication, expressed in this table as a percentage of diet.

III.2.2 Growth performance

Before undertaking the requirement analysis, the growth of *S. aurata* was benchmarked against the model of Lupatsch (2003b):

$$Wt = [Wi^{0.486} + 0.01166 \cdot e^{0.060 \cdot T} \cdot days]^{2.058}$$

where Wt is the final weight (g) after days of growth/feeding, Wi is the initial weight (g) and T is the water temperature. This is an exponential model closely related to daily growth index but parameterised for a stock of gilthead seabream in the Red Sea (Lupatsch, Kissil et al. 1998, Lupatsch et al. 2003b). The fish weights attained in this trial were compared to those forecast by the model and expressed as a percentage of mass relative to the model (± %). Based on this growth model (at a single temperature), mass gain over time is exponential, but, assuming a constant temperature, the absolute growth rate (AGR, g $fish^{-1}$) increases by the same amount every day (linear), and therefore new absolute growth rate data from two samplings can be used to provide an approximate estimate of when the fish would reach a market size (~400 g for gilthead seabream).

III.2.3 Requirement analysis

The predictor variable in the following analysis was EPA+DHA% of diet (as fed). To evaluate the requirements of gilthead seabream juveniles for EPA+DHA% of diet, a series of regression analyses were performed on WG (g), DGI and FCR, for the P1 (3 mm), P2 (4.5 mm) and OV (both pellets) response data. All regression analyses were undertaken using the statistical package R (Venables, Smith 2015) (version 3.4.0, Vienna, Austria). The model parameters were obtained using the function nls(). Graphics were produced in ggplot2. There are many types of nonlinear functions that can model these

experimental data and the approach presented is empirical, as opposed to mechanistic. Preference was given to models with low error (residual sum of the squares, RSS), and that were a strong fit to the requirement region of the response. Suitable models were assessed diagnostically according to techniques described by Ritz and Streibig (2008). The four regression models used were: split linear model, split quadratic model, Gompertz model and four-parameter logistic (FPL) model, which are defined below. For the latter two models (asymptotic models) two requirement estimates are presented: 1) 95% of the asymptote (National Research Council 2011), which is referred to as the "NRC criterion" and plotted as ▲ in the relevant figures and 2) the "elbow" calculation (Eickhardt 2017), defined in Figure III.1. Requirements and "Elbow" calculation, and plotted as ● in the relevant figures. Unless otherwise stated N = 18, which in regression is sufficient to detect very large ($f^2 = 0.5$) effects in the response.

III.2.3.1 Split linear model

The first model applied to the responses was the so-called "broken line" or split regression model, in which the first segment is a linear model usually ascending (descending for FCR) to a maximum response, i.e. a horizontal segment (a mean). The requirement is defined as the intersection of these two segments. Split linear regression can be expressed as follows:

$$f(x) = m \cdot x + c \qquad \text{for } x < req$$

$$f(x) = m \cdot req + c \qquad \text{for } x > req$$

where x is the percentage of EPA+DHA in the experimental diet, c is the model's intercept and m is the gradient (of the first segment) and req is x at the breakpoint (the requirement).

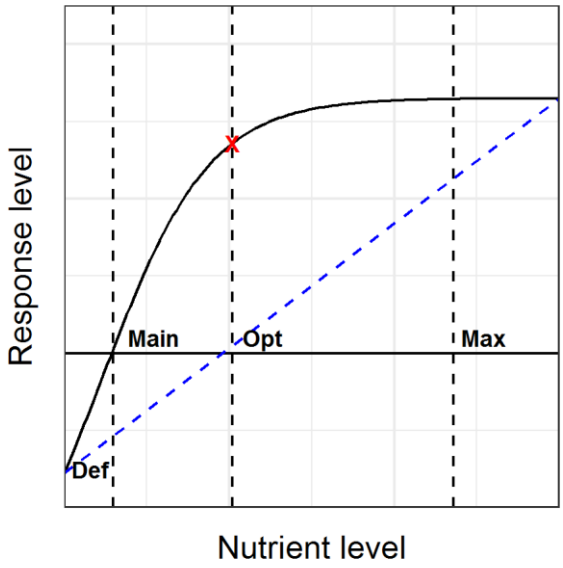

Figure III.1. The effect of nutrient level upon an animal response (a dose response curve). Low nutrient supply indicates deficiency (**Def**), prevention of deficiency indicates maintenance (**Main**), at the "elbow" or corner of the curve the nutrient level can be described as optimal (**Opt**) where additional supply does not elicit significant gains in the response and maximum (**Max**) performance, whereby further increases in nutrient level cannot increase the response level any further (further increases may become detrimental to the response level, toxicity). This publication proposes that the optimum requirement (**X**) can be determined using an "elbow" calculation. 1) A linear model is taken from the y minima to y maxima (**- - -**). 2) The maximum perpendicular distance between this line and the fitted values of the relevant nonlinear function is found. 3) The requirement (**X**) is taken to be the nutrient level at this point. This represents a "point of diminished returns", whereby further gain in the response is small relative to additional nutrient level. However, the "elbow" calculation is still sensitive to experimental design and may not be appropriate for all response curves.

III.2.3.2 Split quadratic model

The second model applied to the data was the split quadratic model, in which the first segment is a quadratic function ascending to a maximum level of response, whereas the second segment is horizontal. The requirement is defined as the intersection of these two segments. Split quadratic regression can be expressed as follows:

$f(x) = m \cdot x + (-0.5 \cdot m / req) \cdot x^2 + c$ for $x < req$

$f(x) = m \cdot req + (-0.5 \cdot m / req) \cdot req^2 + c$ for $x > req$

where x is the percentage of EPA+DHA in the experimental diet, c is the model's intercept, m is the rate constant and req is x at the breakpoint (the requirement).

III.2.3.3 Gompertz model

The first asymptotic function fitted to the response data was the Gompertz which can be defined as:

$f(x) = a \cdot \exp(b \cdot c^x)$

where x is the percentage of EPA+DHA in the experimental diet, a is the asymptote or Y maxima, b sets the displacement on the x-axis and c is the scaling parameter, setting the rate of growth towards the asymptote.

III.2.3.4 Four parameter logistic model

The second asymptotic function fitted to the response was the FPL model which is defined as:

$f(x) = b + (a-b) / (1 + \exp((xmid-x)/c))$

where x is the percentage of EPA+DHA in the experimental diet, a is the upper asymptote, b is the lower asymptote, $xmid$ is the value of x at the infection point and c is the scaling parameter.

III.2.4 Cost-benefit analysis

To assess the economic implications of the experimental diets a simple cost benefit-analysis was conducted. The economic analysis was based on a production plan of 10,000 seabream juveniles starting at 24.34 g (mean weight of fish in this trial), at a constant temperature of 24°C, and growth forecast for 300 days of feeding. To estimate the future mean weight of the fish, the two sampling values for absolute daily gain (g $fish^{-1}$ day^{-1}) were used to predict the linear increase in daily gain, the latter being used to calculate daily mean weight. This assumption is reasonable at a constant water temperature as considered in this analysis (Lupatsch et al. 1998). Daily feed rates were calculated according to Lupatsch and Kissil (2003b) as:

Feed (g $fish^{-1}$ day^{-1}) = $0.029 * Wt^{0.598} * e(0.057*T)$

where Wt is the average weight and T is the water temperature. This function marginally underestimated the actual feeding rates in this study, but extrapolation was required so an established equation for *ad libitum* feeding was chosen. Mortality was assumed to be 6 % over 300 d and feed waste assumed to be 5 % across the six different diets. Prices for the key commodities were found online and taken at their "Free-on-board" prices (Anonymous 2017a, Anonymous 2017b, Bacon 2017) and are reported in Euro (€). Feed prices were calculated based on a typical gilthead seabream grower feed with 15 % added

oil (Crude lipid = 21%) and adjusted for the different oil prices at the ratios used in this study.

III.3 Results

III.3.1 Growth performance

Table III.2 shows the mean initial weights, P1 weights, P2 weights, feed intake, DGI and FCR. During the feeding trial, weight of fish in all dietary groups increased. However, the increase in dietary EPA+DHA (particularly diets D5-D6) resulted in higher weight gain in gilthead seabream juveniles. All the experimental fish grew faster than predicted by the Lupatsch (2003b) model.

III.3.2 Requirement analysis

A series of regression analyses were conducted to explore different ways to determine the EPA+DHA requirement of juvenile gilthead seabream. All functions and their respective parameters are given in Table III.3. The requirement estimates derived from these models are given in Table III.4, along with measures of the model fit, the Akaike's AIC and RSS. Below we present the EFA requirement estimations resulting from applying the four models to growth parameters (WG and DGI) and FCR.

Table III.2. Mean (± SD) values for the dietary treatments for the biometric data analysed in this study (N=3) over the different feeding periods, P1, P2 and OV.

	D1		**D2**		**D3**		**D4**		**D5**		**D6**	
Initial Weight (g)	24.39	± 0.27	24.28	± 0.10	23.98	± 0.12	24.63	± 0.33	24.30	± 0.10	24.53	± 0.33
Weight P1 (g)	83.75	± 1.97	89.08	± 0.69	89.58	± 2.06	93.78	± 1.67	98.00	± 1.25	98.50	± 0.80
Weight P2 (g)	198.63	± 1.30	219.41	± 6.59	223.73	± 5.03	241.30	± 3.09	244.96	± 3.98	248.31	± 1.52
Feed intake P1 (Kg)	10.52	± 0.39	11.50	± 0.03	11.64	± 0.40	11.97	± 0.13	12.14	± 0.07	11.99	± 0.36
Feed intake P2 (Kg)	22.16	± 0.24	24.47	± 0.73	25.07	± 0.43	26.68	± 0.35	26.04	± 0.53	26.35	± 0.76
Feed intake OV (Kg)	32.67	± 0.54	35.98	± 0.74	36.71	± 0.77	38.65	± 0.47	38.19	± 0.58	38.34	± 0.67
FCR[1] P1	1.19	± 0.02	1.21	± 0.01	1.20	± 0.01	1.17	± 0.01	1.11	± 0.01	1.09	± 0.00
FCR P2	1.38	± 0.03	1.37	± 0.03	1.35	± 0.04	1.29	± 0.01	1.25	± 0.02	1.24	± 0.02
FCR OV	1.33	± 0.02	1.33	± 0.02	1.31	± 0.02	1.27	± 0.01	1.22	± 0.01	1.21	± 0.01
DGI[1] P1	2.63	± 0.04	2.80	± 0.02	2.83	± 0.05	2.91	± 0.02	3.05	± 0.02	3.06	± 0.04
DGI P2	2.02	± 0.04	2.17	± 0.07	2.21	± 0.02	2.33	± 0.00	2.28	± 0.02	2.31	± 0.01
DGI OV	2.29	± 0.01	2.44	± 0.05	2.48	± 0.04	2.58	± 0.01	2.62	± 0.02	2.63	± 0.01
Lupatsch (+%)[3]	42.30	± 1.20	57.50	± 4.80	62.20	± 3.30	72.10	± 1.20	75.70	± 2.60	77.4	± 1.10

[1]Feed conversion ratio = (Feed fed (kg) – feed waste (kg)) / Δ Biomass (kg)

[2] Daily Growth Index = Final weight (g) $^{0.333}$ – Initial weight (g) $^{0.333}$ / Days feeding

[3] In terms of weight during the experimental period. The model is defined: $Wt = [Wi^{0.486} + 0.01166 \cdot e^{0.060 \cdot T} \cdot days]^{2.058}$ where Wt is the final weight (g) after days of growth/feeding, Wi is the initial weight (g) and T is the water temperature.

Table III.3. Model parameters fitted to the biometric data of *Sparus aurata* juveniles in this trial to derive estimates for the EPA+DHA requirement of this species. The four models presented are the split linear, split quadratic, Gompertz and the four parameter logistic (FPL) function. The models were applied to the overall data (OV, both pellet sizes), the 3 mm pellet size (P1, in the text) and the 4.5 mm pellet size (P2 in the text). The name of the parameter and the notation used in the text is indicated in the column headers. N = 18 except for the Gompertz model applied to FCR data, where N=15.

Model type	**Metric**	**Period**	**Parameters**			
			Plateau	Gradient	Intercept	Requirement
				m	*c*	*req*
Split regression	WG (g)	OV	222.22	47.13	165.25	1.21
		3mm	78.81	11.02	57.61	1.47
		4.5mm	148.38	36.03	107.62	1.13
	DGI	OV	2.63	0.33	2.23	1.20
		3mm	3.05	0.32	2.59	1.46
		4.5mm	2.30	0.32	1.96	1.09
	FCR	OV	1.21	-0.07	1.36	2.03
		3mm	1.09	-0.06	1.23	2.50
		4.5mm	1.25	-0.10	1.41	1.62
			Plateau	Rate constant	Intercept	Requirement
				m	*c*	*req*
Ascending polynomial	WG (g)	OV	222.43	75.26	158.52	1.70
		3mm	74.04	15.62	56.43	2.25
		4.5mm	148.76	59.18	102.31	1.57
	DGI	OV	2.63	0.55	2.17	1.64
		3mm	3.06	0.48	2.54	2.15
	FCR	OV	1.21	-0.11	1.37	3.03
		3mm	1.02	-0.06	1.23	6.94
		4.5mm	1.24	-0.13	1.42	2.71
			Plateau	Displacement	Scale parameter	
			a	*b*	*c*	
Gompertz	WG (g)	OV	224.42	-0.38	0.17	
		3mm	72.84	-0.31	0.28	
		4.5mm	149.99	-0.42	0.14	
	DGI	OV	2.64	-0.22	0.16	
		3mm	3.08	-0.21	0.26	
		4.5mm	2.31	-0.23	0.10	
	FCR	OV	1.20	0.23	0.26	
		3mm	1.06	0.21	0.47	
		4.5mm	1.24	0.26	0.20	
			Plateau	Bottom (b)	Inflection	Scale parameter
			a	*b*	*xmid*	*c*
FPL	WG (g)	OV	223.00	150.65	0.45	0.32
		3mm	74.62	24.75	-0.32	0.66
		4.5mm	149.00	106.37	0.55	0.25
	DGI	OV	2.63	2.00	0.29	0.36
		3mm	3.08	-6.10	-2.03	0.76
		4.5mm	2.31	1.98	0.55	0.19
	FCR	OV	1.21	1.34	1.04	0.10
		3mm	1.09	1.21	1.41	0.29
		4.5mm	1.25	1.38	0.95	0.19

Table III.4. Requirement estimates for EPA+DHA (% of diet as fed) of juvenile gilthead seabream, *Sparus aurata*, from the models listed in Table III.3. The models were applied to the overall data (OV, both pellet sizes), the 3 mm pellet size (P1, in the text) and the 4.5 mm pellet size (P2 in the text). In the Gompertz and FPL models, the EPA+DHA estimate derived from the "elbow" calculation is given. The residual sum of the squares (RSS) and the Akaike information criterion (AIC) are also given as an indication of how well the models fit the experimental data.

Metric	**Pellet**	**Split linear**	**RSS**	**AIC**	**Split quadratic**	**RSS**	**AIC**	**Gom[1]**	**RSS**	**AIC**	**FPL**	**RSS**	**AIC**
WG (g)	OV	1.21	336.00	109.75	1.70	341.00	112.02	1.22	368.00	113.40	1.25	343.00	114.14
	3mm	1.47	38.20	70.61	2.25	33.50	70.24	1.34	33.10	70.00	1.37	32.90	71.92
	4.5mm	1.13	210.00	101.33	1.57	241.00	105.76	1.18	274.00	108.07	1.19	244.00	107.98
DGI	OV	1.20	0.016	-67.27	1.64	0.014	-69.86	1.18	0.015	-68.75	1.21	0.014	-67.60
	3mm	1.46	0.033	-56.22	2.15	0.027	-57.86	1.30	0.026	-58.72	1.30	0.026	-56.72
	4.5mm	1.09	0.027	-57.90	No fit-			1.08	0.037	-52.20	1.09	0.033	-52.53
FCR	OV	1.97	0.0079	-80.19	3.03	0.0088	-78.25	1.53	0.0048	-70.09	1.55	0.0064	-81.76
	3mm	2.50	0.0062	-85.29	6.94	0.0071	-82.00	1.67	0.0021	-82.84	2.03	0.0037	-91.85
	4.5mm	1.62	0.015	-70.53	2.71	0.016	-67.28	1.47	0.011	-57.70	1.45	0.013	-68.64

[1] **Gom -** Gompertz

III.3.2.1 Weight gain and daily growth index

Using the split linear model to fit the WG data, the requirements were estimated as 1.21, 1.47 and 1.13 EPA+DHA% of diet for OV, P1 and P2, respectively (Figure III.2A). Using the split linear model to fit the DGI data, the requirements were 1.20, 1.46 and 1.09 EPA+DHA% for OV, P1 and P2, respectively (Figure III.2B). The requirement values agree between the two metrics of growth, WG and DGI. The distribution of error for P1, WG and DGI exhibited a curve. When using the split quadratic model for the WG data, the EFA requirements were estimated as 1.70, 2.25 and 1.57 EPA+DHA% for OV, P1 and P2, respectively (Figure III.2C). The error distribution in the P1 data was better using the quadratic model. Using the split quadratic model to fit the DGI data, the requirements were 1.64 and 2.15 EPA+DHA% for OV and P1, respectively. This model failed to fit the DGI data of P2 due to the high DGI values attained with fish fed diet D4 (Figure III.2D). The OV and P2 data was best modelled by the split linear method, but the P1 data was best described by the split quadratic model (Table III.4). The asymptotic nonlinear models, namely the Gompertz and the four-parameter logistic (FPL) models, applied to the WG and DGI data are presented in Figure III.3. Using the Gompertz model and elbow calculation (Figure III.1) to fit the WG data, the EFA requirements were estimated as 1.22, 1.34 and 1.18 EPA+DHA% for OV, P1 and P2, respectively. Considering the NRC recommendation of 95% of the maximum response, the EFA requirements were calculated as 1.13, 1.40 and 1.07 EPA+DHA% for OV, P1 and P2, respectively (Figure III.3A). With regards to the DGI data, the Gompertz function and the elbow calculation estimated EFA requirements of 1.18, 1.30 and 1.08 EPA+DHA % for OV, P1 and P2, respectively.

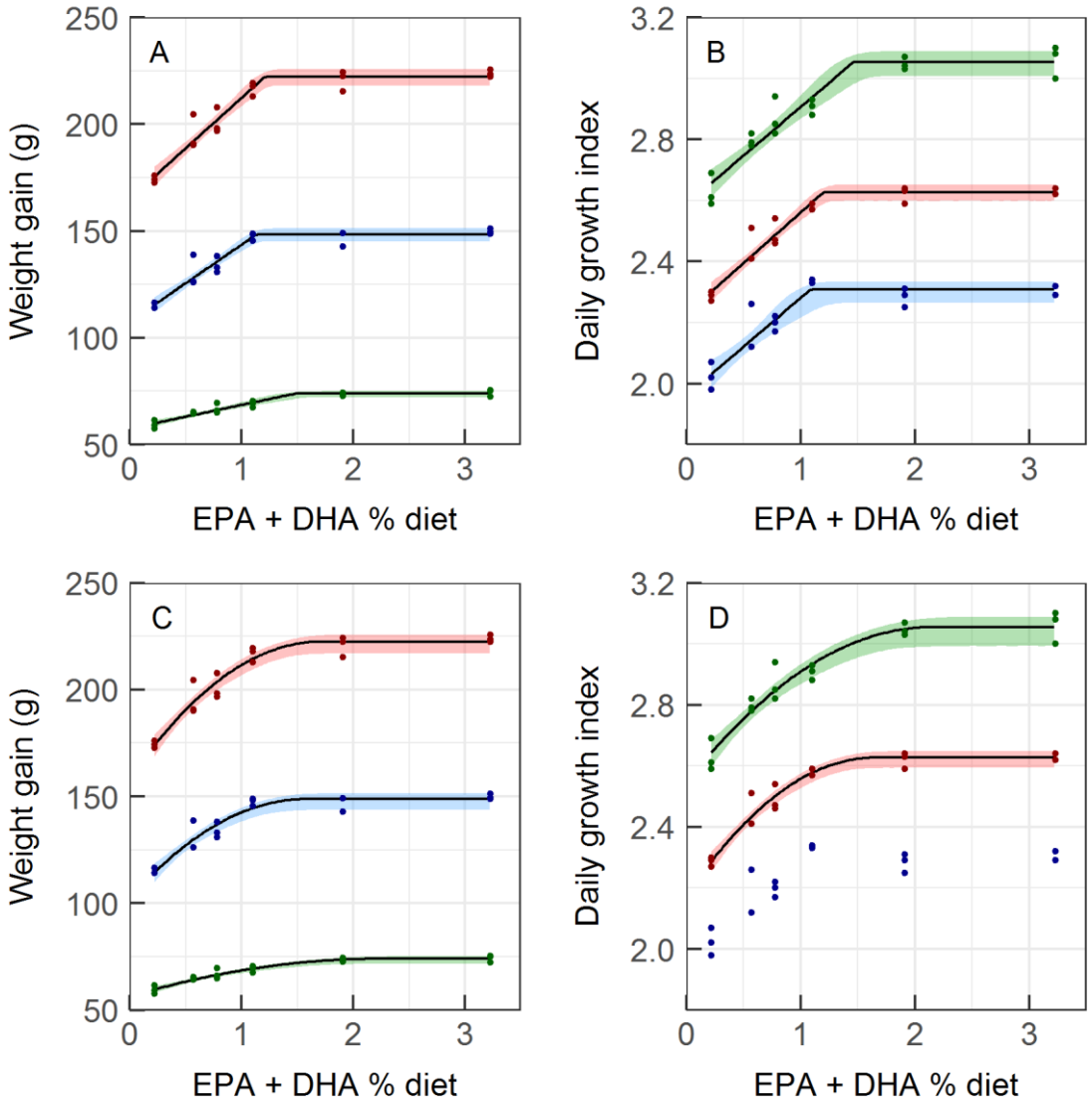

Figure III.2. The split linear (A and B) and split quadratic (C and D) models applied to the weight gain (A and C) and daily growth index (B and D) data against EPA+DHA % of diet (as fed, x-axis). The raw data are marked with coloured spots, the relevant model fit with an unbroken line, 95 % prediction intervals (calculated by Monte-Carlo simulation) are shaded in colour. Colours for the different periods are as follows: the whole trial, OV = ; the 3 mm pellet size, P1 = and the 4.5 mm pellet size, P2 = . Note, in panel D the quadratic model would not fit the P2 data due to the high performance of D4. Estimated requirement values are given in Table III.4.

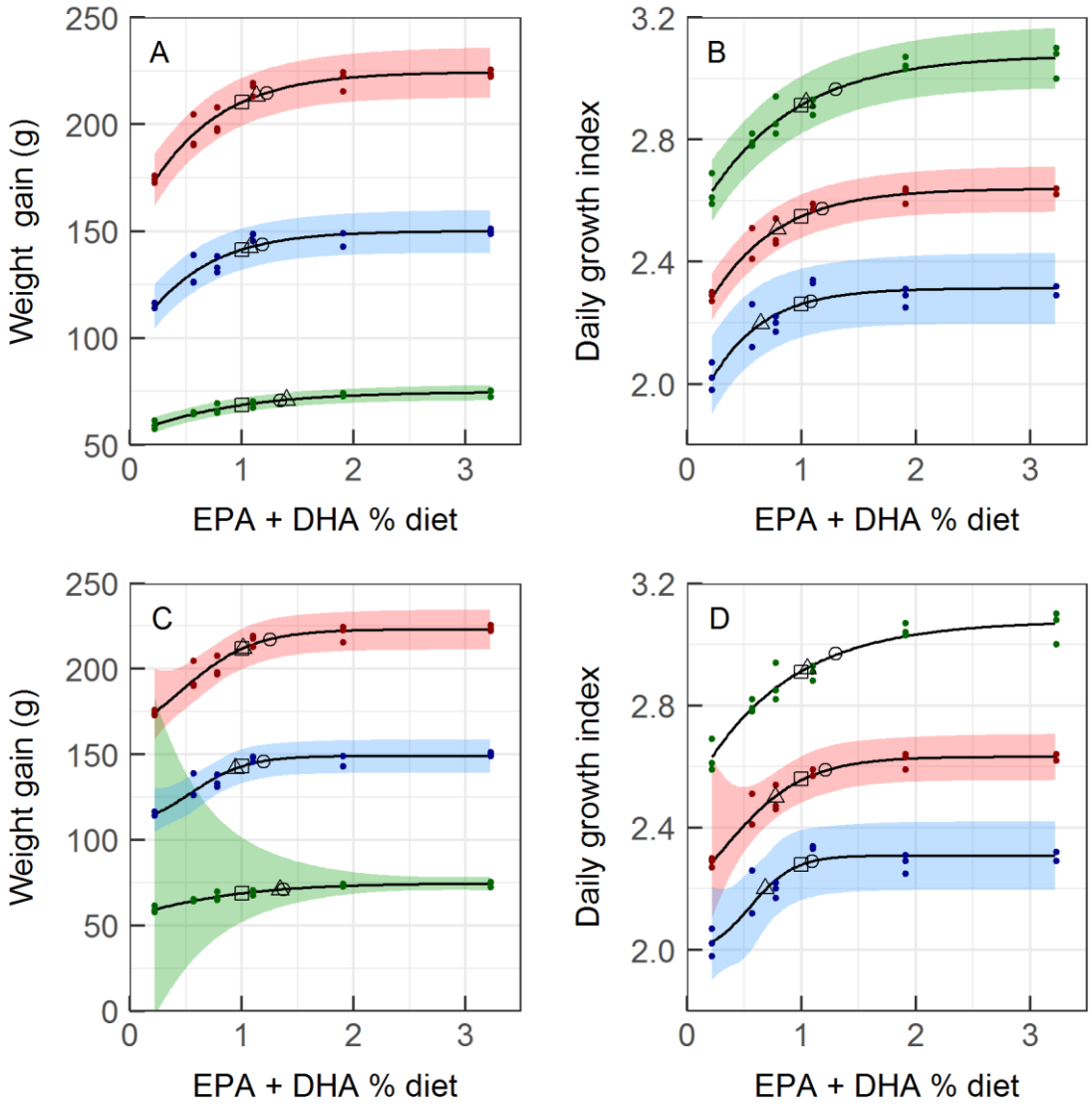

Figure III.3. The nonlinear Gompertz model (A and B) and four-parameter logistic model (FPL in the text, C and D) applied to the weight gain (A and C) and daily growth index (B and D) data against EPA+DHA % of diet (as fed, x-axis). The raw data are marked with coloured spots, the relevant model fit with an unbroken line, requirements with black symbols and the 95 % prediction intervals (calculated with 2nd order Taylor expansion) are shaded in colour. Symbols for the requirement estimates are as follows: Published = □, National Research Council = △ (NRC criterion in text) and Elbow = ○. Colours for the different periods are as follows: the whole trial, OV = ; the 3 mm pellet size, P1 = and the 4.5 mm pellet size, P2 = . Note in panel D in P1, the error was not plotted for clarity because it was very wide, an indication that the curve for this data was over-parameterised. Estimated requirement values are given in Table III.4.

When the NRC criterion (95% of the maximum response) was applied to estimate EFA requirements from DGI data, much lower values were obtained, namely 0.79, 1.04 and 0.64 EPA+DHA % for OV, P1 and P2, respectively (Figure III.3B). Using the FPL model to fit the WG data, EFA requirements were estimated as 1.25, 1.37 and 1.19 EPA+DHA% (elbow calculation) and 1.01, 1.34 and 0.94 % of dietary EPA+DHA (NRC criterion) for OV, P1 and P2, respectively (Figure III.3C). For the DGI data, the EFA requirements were estimated as 1.21, 1.3 and 1.09 % (elbow calculation) and 0.77, 1.05 and 0.68 % of dietary EPA+DHA (NRC criterion) for OV, P1 and P2, respectively (Figure III.3D). The fourth parameter allowed the model to fit the growth data well in OV and P1. However, parameter estimates for *b* and, or *xmid* had large standard errors, due to the absence of data for the lower part of the curve. It should be noted that for P1 the model parameters were not significant leading to wide prediction intervals (Figure III.3D).

III.3.2.2 Feed conversion ratio

The split linear and quadratic models provided requirement estimations that were high. This was due to the inadequacy of these models to fit the unusual distribution of the real-world FCR data. Therefore, the values are not discussed in detail here but are reported in Table III.4 and Figure III.4 A and B for completeness and to demonstrate the lack of fit. To fit the Gompertz models to the FCR data it was necessary to remove data of diet D1 (N = 15). Using the Gompertz curve to fit the FCR data, the EFA requirements were estimated as 1.53, 1.67 and 1.47 % (elbow calculation) and 1.15, 1.92 and 1.03 EPA+DHA % (NRC criterion), for OV, P1 and P2, respectively (Figure III.4C). The removal of diet D1 data removed error and therefore the model is not comparable to the FPL model. Despite the unusual shape of the FCR data, the FPL model was flexible

enough to fit these data without the removal of diet D1 data. With FCR as response, the EFA requirements were 1.55, 2.03 and 1.45 EPA+DHA % (elbow calculation) and 1.05, 1.45 and 0.99 EPA+DHA % (NRC criterion) for OV, P1 and P2, respectively (Figure III.4D). The values attained for FCR were higher than for growth, and the FPL model was the only model to offer a satisfactory fit to the real-world data.

III.3.3 Cost-benefit analysis

The six diets were economically analysed in terms of a standardised production objective and recent costs and prices (June 2017) for the key dietary components and farm-gate value of gilthead seabream. The best performing diet in this analysis was D4 (EPA+DHA = 1.1 %). The time to reach a harvest weight of 400 g was 182, 181 and 183 days, respectively for diets D4-D6. These theoretical growth trajectories were plotted for 300 days (Figure III.5). Diet D4 attained the highest value for margin over feeds, which is the price per Kg sea bream minus the value of its feed. The results of this analysis are summarised in Table III.5.

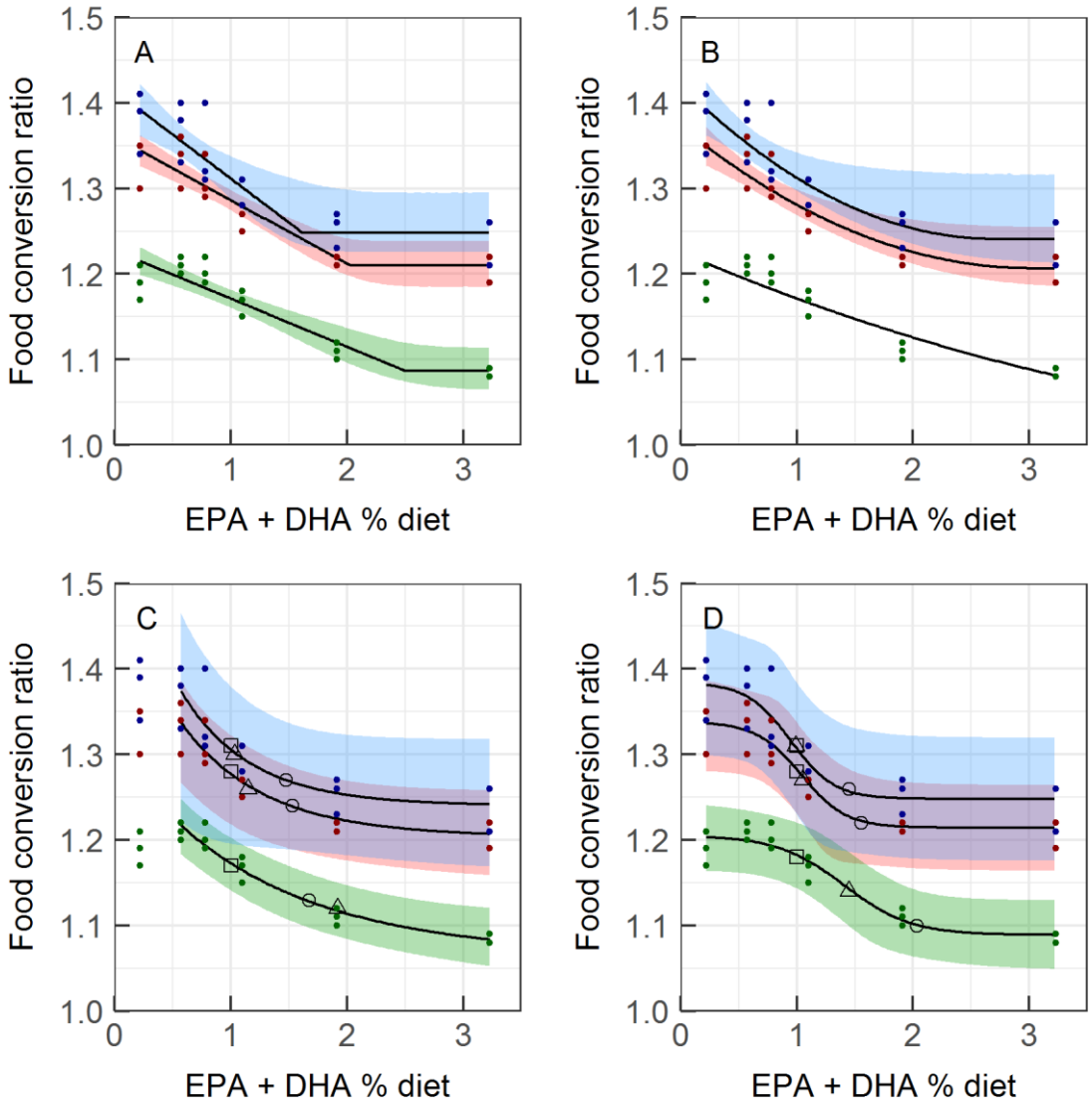

Figure III.4. The linear (A) and quadratic (B) split regression models applied to the FCR data against EPA+DHA % of diet (as fed, x-axis), the 95% prediction intervals (shaded colour) were calculated by Monte-Carlo simulation. The nonlinear Gompertz (C) and four parameter logistic (FPL in the text, D) applied the FCR data against EPA+DHA % of diet (as fed, x-axis), the 95% prediction intervals (shaded colour) were calculated by second-order Taylor expansion. The raw data are marked with coloured spots, the relevant model fit with an unbroken line and requirements with black symbols. Symbols for the requirement estimates are as follows: Published = □, National Research Council =△ (NRC criterion in text) and Elbow = ○. Colours for the different periods are as follows: the whole trial, OV = ; the 3 mm pellet size, P1 = and the 4.5 mm pellet size, P2 = . Note in panel B, the error for P1 was not plotted, but clearly it is a poor fit to the data.

Table III.5. Summary of production economic analysis of *Sparus aurata* production in terms of feeding to a market size of 400 g. Mortality was assumed to be 6 % and production and feed waste 5%. Economically, there is no benefit in terms of time or margin in raising EPA+DHA beyond 1.1%, the level in D4.

Item	**D1**	**D2**	**D3**	**D4**	**D5**	**D6**
Fish oil (% inclusion)	0.0	1.8	2.6	4.4	8.0	14.8
Rapeseed oil (% inclusion)	10.4	9.2	8.6	7.3	4.8	0.0
Palm oil (% inclusion)	5.2	4.6	4.2	3.6	2.4	0.0
Cost of oil (€ t^{-1})	689.05	782.26	830.17	926.51	1123.06	1513.00
Feed cost (€ t^{-1})	936.84	950.72	957.80	971.84	1000.00	1054.16
Results						
Days to reach 400g‡	216	198	195	182	183	181
Economic FCR	1.49	1.36	1.35	1.25	1.27	1.26
Feed cost (€ Kg seabream^{-1})	1.31	1.21	1.2	1.13	1.18	1.24
Margin over feed (€ Kg^{-1})	3.15	3.25	3.26	3.33	3.28	3.22

Commodity	**Price**	**Source**
EURO/Dollar (€/$)	0.89	
Fish oil (€)	1513.00	†https://hammersmithltd.blogspot.co.uk/
Rapeseed oil (€)	741.79	†http://www.indexmundi.com/commodities/
Palm oil (€)	583.36	†http://www.indexmundi.com/commodities/
Farm gate (€)	4.46	†https://www.eumofa.eu/ad-hoc-queries
Market weight (g)	400.00	†https://www.eumofa.eu/ad-hoc-queries

† All sources accessed 21/06/17

‡ Calculated by a linear projection of absolute growth rate (g day^{-1}) to generate a series of fitted weights by days.

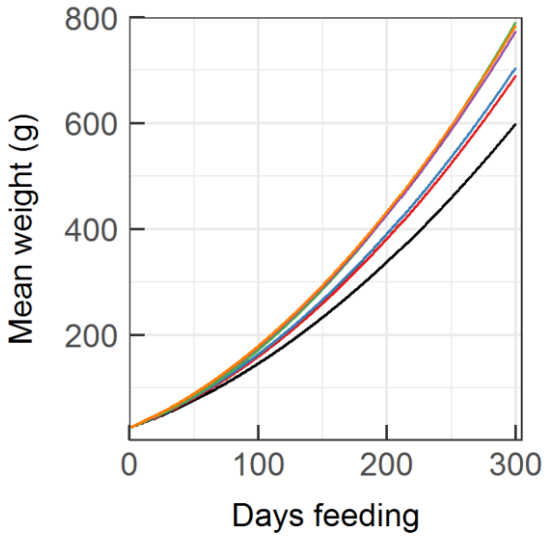

Figure III.5. Average weight of a fish plotted over days feeding on diets D1-D6. The values of weights are those predicted by the growth forecasts in the cost-benefit analysis, note the similarity in performance between the D4, 5 and 6 mean weights. D1 = ; D2 = ; D3 = ; D4 = ; D5 = ; D6 = .

III.4 Discussion

The present study explored four different models and three performance responses that can be used to advance EPA+DHA requirement estimates. The experimental design involved diets formulated with commercially relevant oils and energy densities, and strong performance was observed from all dietary groups. There were two key conclusions from this study. Firstly, we showed for the first time in a single study, that the requirement for EPA+DHA is a function of fish mass (decreasing with increasing weight) and could therefore be modelled with data from more time points. Secondly, the current published requirement for EPA+DHA of 0.9 % (dry diet) appeared too low for juvenile gilthead seabream (Kalogeropoulos et al. 1992, National Research Council 2011). Recommendations for the two pellet sizes (P1 and P2) can be made. For juveniles

of 24-80 g, the diet (3 mm pellet) should contain at least 1.4 % for an optimum requirement but to achieve maximum performance (highest growth and feed conversion) the level is 2 %. This can be reduced in larger fish (80 – 250 g; 4.5 mm pellet) to 1.2 % for optimal requirement but 1.5 % to achieve maximum performance. The data suggested that the current published guidance (~1%) would become adequate at some body weight during this growth phase (P2). The previous requirement estimate for juvenile gilthead seabream employed smaller fish than this study (1.2 g) and used split linear regression to analyse weight gain (g). The reason for the previously estimated lower EFA requirement is related to the lower level of growth attained by the juvenile seabream in the earlier study (DGI: 0.74-0.87). In a further study in European seabass also employed split linear regression with DGI as the response and reported a requirement of 0.7 % LC-PUFA (of diet dry diet) for fish of 14 g initial weight (DGI: 0.84 – 1.0) (Skalli, Robin 2004). The different approaches presented in the present study also showed that there is no one model that suits every parameter or each of the experimental periods, as suggested previously (Baker 1986, Rodehutscord, Pack 1999).

The higher EFA requirements, especially for DHA, of larval fish is relatively well understood (Izquierdo 1996, Sargent et al. 1997). However, the present study has shown a change in EFA requirement between two fish (pellet) sizes and, therefore demonstrated the existence of a relationship between fish mass and EFA requirement. It is likely that this relationship takes on a similar form to digestible protein (requirement = $-a \cdot$ fish mass $(Kg)^{b}$) (Lupatsch et al. 2003b), to define this relationship the precise requirement for EPA+DHA would need to be known over a range of fish masses, ideally in a single experiment achieving optimal growth. Here, we only provide requirement estimates for fish of around 25 – 80 g and 100 – 250 g, and so only the negative slope between these

fish weights can be estimated, with the value being approximately -1.8. Alternatively, if the requirement is viewed per unit energy (Glencross 2009) or lipid (Watanabe 1982) the decreasing requirement (% of diet) may be interpreted as a consequence of the rising energy (DE) demand for maintenance as the fish grows (Lupatsch 2005). Regardless of how the requirement is numerically expressed, or even understood, its fraction in the diet (% or g kg^{-1}) still needs to be known to inform diet specification. The discussion below focuses on the strengths and weaknesses of the models.

The first two models applied to the data were linear and quadratic split regression. Conceptually, these models derive the maximum requirement because the equation describes a model approaching a horizontal plateau, at this plateau the response is equivalent to the mean of the highest performing dietary groups. For this reason, it is critical for these techniques that the maximum response is known with at least two dietary treatments. The exception to this is where an effect of toxicity is observed at higher levels of nutrient supply. An example of this can be found in blackhead seabream (*Acanthopagrus schlegelii*) where high levels of n-3 LC-PUFA led to lower growth, and in this situation the authors used quadratic regression, without the horizontal segment (Jin, Lu et al. 2017). In the present study, we did not observe a detrimental effect of higher EPA+DHA levels, so quadratic regression was not appropriate. As split linear and quadratic define the requirement at the intersection of the model with the plateau, an unequivocal requirement is provided by application of the model (Glencross 2009). Linear split regression is the most frequently applied model in fish nutrient requirement studies (Kalogeropoulos et al. 1992, Skalli, Robin et al. 2006, Murillo-Gurrea, Coloso et al. 2001, Luzzana, Hardy et al. 1998, Mai, Lu Zhang et al. 2006). Quadratic split regression, to our knowledge, has only been applied in a meta-analyses of fish data

(Hernandez-Llamas 2009) but there are more examples in terrestrial animals (Hauschild et al. 2010). Interestingly, the split linear model had the best fit for WG response in OV and P2 data, and it produced EPA+DHA requirement estimates marginally higher than the two asymptotic models (Gompertz and FPL), this being somewhat unexpected as this method has been found to underestimate nutrient requirements (Shearer 2000, Hernandez-Llamas 2009). In the present study, the quadratic split regression gave higher estimates than the linear split regression. Neither of these methods seemed applicable to the FCR data due to the unusual shape of this response. As a result, the EPA+DHA requirements estimated with FCR as response variable were considerably higher than published requirement estimates for *S. aurata* (Kalogeropoulos et al. 1992) when any of the split regression models were used. Feed conversion/feed efficiency ratio is usually given in requirement papers, but rarely modelled as a parameter. An example with lysine requirements of the Japanese seabass (*Lateolabrax japonicus*) used split regression with feed efficiency (Mai et al. 2006).

Asymptotic functions (such as the Gompertz and FPL) can often be a strong fit to real-world data (Mercer, May et al. 1989) since they adequately address the concept of the increase in the response slowing as the plateau is reached, which in economics, is referred to as the concept of diminishing returns (Spillman, Lang 1924). A limitation of nonlinear models, however, stems from the fact that the nutrient requirement is defined arbitrarily at 0.95 · asymptote (Cowey 1992, National Research Council 2011). Our present results suggested that this method was highly sensitive to parameter selection and might lead to erroneous requirement estimates, at least in the context of EFA. Thus, the requirement estimates during the OV period for both growth responses (DGI and WG) disagree when either the FPL or Gompertz models are applied. An alternative to the 0.95

· asymptote criterion, the proposed "elbow" calculation was developed as a means of defining a "point of diminishing returns" (Spillman, Lang 1924) or optimal requirement on a response curve. In nutrient requirement studies, it is important that both the deficient and excess part of the nutrient response curve are well-defined (Shearer 2000, Glencross 2009). It was anticipated that the elbow calculation would be less sensitive to the dietary levels of nutrient supply. Unfortunately, this is not the case and the elbow calculation still relies on the nutrient levels determined at the experiment's conception. However, this calculation did seem useful in analysing the data obtained in this study as it gave similar requirement estimates for both metrics of growth and both models (albeit, marginally higher for the DGI parameter). This is due to its insensitivity to the scale of the response. However, it is advocated that the elbow calculation is only applied to asymptotic functions.

The two asymptotic models used in the present study (Gompertz and FPL) have been used previously to fit nutrient responses in terrestrial animals (Gahl, Finke et al. 1991, Pesti, Vedenov et al. 2009) but, to our knowledge, have not been applied to fish. Other asymptotic functions (four and five-parameter saturation kinetics models) were used in a study investigating the tryptophan requirements of hybrid striped bass (*Morone chrysops* · *M. saxatilis*) finding requirements just over 2 g kg^{-1} when the asymptote was multiplied by 0.95 (Gaylord, Rawles et al. 2005). Two meta-analyses in fish have employed a variety of nonlinear models to previously published data and found that often the curves fit responses better, but that no one model was appropriate for all data sets (Rodehutscord, Pack 1999, Shearer 2000). Therefore, we explored a range of nonlinear models and the two attaining the best fit were reported here. In the present study, the Gompertz model was a good fit for most of the growth data, especially for P1. The FPL model was also

able to fit these data, but this model carried high uncertainty because it has parameters that are not covered by the data (*b* and *xmid*). To apply the Gompertz model to the FCR data it was necessary to disregard data for diet D1, and therefore it does not capture reality well, which created problems comparing fits as error associated with diet D1 was discarded. The only model that could describe the FCR response to the diets was the FPL model, as such requirements for FCR differed between the two asymptotic models and it is difficult to advance an estimate, but it can be said that the requirement to achieve the best FCR was higher than for the growth metrics.

The economic analysis showed that there was no obvious economic benefit of increasing EPA+DHA beyond the level of that in diet D4 (1.1 % EPA+DHA) both in terms of margin over feed cost and time taken to grow to a market weight of 400 g. This type of analysis is highly sensitive to several factors. The first is the market price of the three oils used, fish, palm and rapeseed oils. The second is the method of growth projection. Over the first period of the experiment, diet D4 had absolute growth rate (AGR) values (mean = 1.23 g day^{-1}) intermediate to diets D3 and D5, however, over P2 the AGR values (mean = 2.05 g day^{-1}) were equivalent to diets D5 and D6, and this meant that the linear projection of daily AGR was the steepest out of the six diets (this would not occur). This is a weakness in this method, but it does reveal that, at some point during P2, diet D4 had sufficient EPA+DHA and growth rates were on par with the fish consuming diets D5 and D6. This forecast, therefore lends support to the conclusion that the requirement reduces as fish increase in mass and at some bodyweight the current recommendations would be sufficient. This is in line with larvae and small juveniles having much higher relative requirement for LC-PUFA (Sargent et al. 2002, T. Takeuchi et al. 1992, Watanabe 1993). It could be speculated that the best performance could be

achieved by feeding 1.5 % EPA+DHA up to a size of 100 g and then reducing the level of FO equivalent to the values reported for the OV requirement, ~1.2 % EPA+DHA of diet.

It is evident from these empirical data that the current requirement of 0.9 (dry weight) or ~1 % EPA+DHA is probably too low for juveniles of this species, especially when considering fish of 25 – 80 g. The present study has demonstrated that no model is a fit for each situation and the NRC definition of a requirement at 95 % of a response maximum does not seem to be appropriate for EPA+DHA requirements. One reason for this is that the models suggest that n-3 LC-PUFA (i.e. EPA and DHA) are not completely essential to growth (all models have positive intercepts, implying that there is no maintenance demand and no weight loss), at least over the timescale of this study. Interestingly, results modelling the turnover of individual fatty acids in Asian seabass (*Lates calcarifer*) suggested zero maintenance requirement for EPA and DHA (Salini, Poppi et al. 2016). Therefore, multiplying the optimum response by 0.95 can give a result that is too low on the response curve. The elbow-calculation was applied to determine a point on an asymptotic response curve that was less sensitive to the design of the experiment although, unfortunately, it is not completely insensitive to experimental design. The calculation, however, does bisect the curve well and the data demonstrated it returned values that agreed with each other across models and metrics. However, its application was not appropriate unless the maximum response and deficient response was well defined, and we only advocate its use for asymptotic models. The elbow calculation defines a point on the curve that obeys the concept of diminishing returns and, further increases in dietary EPA+DHA levels lead to little gain in the response and, therefore, denotes an optimum requirement.

In summary, the key conclusions of this work are that 1) *S. aurata* juveniles of 25 g – 80 g (3 mm pellet) have a higher requirement for EPA+DHA of at least 1.4 % of diet but perhaps as high as 2% if FCR is considered, 2) the requirements for EPA+DHA are a function of fish mass, the implication of this being that requirements could be modelled but the trial would need a larger number of sampling points than the two reported here and, 3) the exact definition of EPA+DHA requirement is a question of context; maintenance, optimum production, maximum performance, health, well-being or product quality could all ultimately reflect different end-points and therefore give different requirement estimates.

IV. Application of robust nonlinear regression to determine the requirements of juvenile European seabass (*Dicentrarchus labrax*) for eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids

Abstract

The long-chain polyunsaturated fatty acids (LC-PUFA), EPA and DHA are essential to marine fish species, such as European seabass (*Dicentrarchus labrax*), for optimal growth and health. Vegetable oils (VO) are added to fish feed formulations to increase the diet's energy density while "sparing" protein for growth. This higher dietary lipid level may influence the requirement for essential fatty acids (EFA). This formulation practise has been introduced since the EPA and DHA requirements were defined in most cultured fish species and, therefore, EFA requirements need to be reassessed in many farmed species. This study was carried out to reassess the requirement of EPA+DHA in juvenile European seabass. Six diets were formulated with graded levels of EPA+DHA (0.2 – 3.1 % of diet as fed) and fed to triplicate groups of European seabass as two pellet sizes (3 mm and 4.5 mm) for 127 days. For each pellet size, the biometric data (weight gain and daily growth index) were analysed by three different robust nonlinear regression strategies, namely split linear, split quadratric and the Gompertz function. The FCR data was also considered in the analysis. Over the whole experimental period (two pellet sizes) the current published requirement (1% of diet) appeared too low and should be increased to at least 1.1 - 1.2% diet (as fed). However, when the first pellet size for fish of 25 – 80 g was considered, the requirement was 1.3 – 1.5 % diet (as fed). The experimental diets did not have large effects on FCR, particularly during the feeding of the 4.5 mm pellet. The growth data was best fit using the Gompertz function. In accordance to the earlier study in gilthead seabream (Chapter III) it was demonstrated that the requirement for EFA falls as the fish increases in weight. However, in European seabass the effects of size on growth and FCR were smaller than in the seabream.

IV.1 Introduction

Fish oils are rich in LC-PUFA of the n-3 series. The two principal n-3 LC-PUFA are EPA and DHA. For many fish, especially marine species, these two fatty acids are regarded as EFA due to the absence of functional pathways to synthesise n-3 LC-PUFA from ALA (18:3n-3) (Castro et al. 2016). Therefore, EPA and DHA need to be supplied preformed to most marine fish species to ensure wellbeing and normal growth (Oliva-Teles 2012, Tocher, Glencross 2015). Arachidonic acid (20:4n-6) is also an important EFA and, although usually found at lower levels in FO compared to EPA and DHA, is a key component of diets for larvae and broodstock (Bell, Sargent 2003). Currently, FO is the main source of n-3 LC-PUFA in marine fish diets but in the future, wild caught fish will be unable to supply demands of aquaculture and nutraceutical industries (Quaas, Reusch et al. 2016, Cashion, Le Manach et al. 2017, Fisheries 2016). Therefore, efforts are currently underway to develop new sources of these crucial EFA. Possible future sources of EFA are algal derived oils (Barclay, Weaver et al. 2010, Sprague, Walton et al. 2015) and genetically modified crops (Qi, Fraser et al. 2004, Ruiz-Lopez, Haslam et al. 2014, Betancor, Sprague et al. 2015). However, the finite nature of FO and the technical sophistication of novel EFA sources means that a firm understanding of EFA requirements for all cultured fish are required to utilise all sources of EFA as efficiently as possible (Glencross 2009).

Current aquafeeds are rich in dietary energy to support rapidly growing finfish, this is supplied as dietary oil to “spare” higher value protein for growth (Sargent et al. 2002). Typically, this is a combination of VO with FO added to cover primarily the requirements for EFA. It has been suggested that EFA requirements are dependent on the energy (or

lipid content) of the diet (Glencross, Smith 2001, T. Takeuchi, Watanabe 1982). Furthermore, the FM content (which contains some n-3 LC-PUFA) of aquafeeds is also under pressure due to the same reasons outlined above for FO, which will further reduce the EFA content of a feed. Therefore, the feed ingredients that contain EPA and DHA are being reduced while dietary energy is increased by the addition of VO that lack LC-PUFA, which may affect the requirement. Due to these developments, it is necessary to redefine the EFA requirements of European seabass and other species of fish (National Research Council 2011).

The European seabass is an important cultured species, farmed primarily in Mediterranean countries, such as Greece and Turkey. Previous research concluded that the n-3 LC-PUFA requirement of European seabass was 0.7 % of diet (dry wt.) (Skalli, Robin 2004). The fish in this trial achieved DGI values of 0.9 - 1.1, using fish in the 15-35 g range (Skalli, Robin 2004). Today, with improved nutritional knowledge, higher energy diets, and improved genetic stock, it is possible to attain higher DGI, especially in small fish (Torrecillas et al. 2017) and, therefore, the EFA requirement for European seabass should be revisited. Many studied species have been shown to require n-3 LC-PUFA at ~1 % of diet, for example Atlantic salmon (*Salmo salar*), Asian seabass (*Lates calcarifer*), turbot (*Psetta maxima*) and starry flounder (*Platichthys stellatus*) (Lee et al. 2003, Gatesoupe et al. 1977, Glencross, Rutherford 2011, Glencross et al. 2014). However, some species, for example yellowtail flounder (*Pleuronectes ferrugineus*) a higher requirement of 2.5% of diet has been reported (Whalen et al. 1998).

In Chapter III, four regression models were compared to analyse response data (weight gain, daily growth index and FCR) using the gilthead seabream as a model. There

were two key conclusions: firstly, that the requirement for EPA+DHA declines as the seabream grows, which means a requirement for EFA is dynamic and a function of fish mass; secondly, that the precise number determined is sensitive to both model selection and the parameter used as a response variable. This study will employ similar methodological approaches as described in Chapter III, applied in this occasion to data collected from the European seabass growth trial run as described in Section II.2. Due to the differences between the gilthead seabream (Chapter III) and European seabass (present chapter) biometric data, the FPL function was not useful for the European seabass data and so the results are not presented. The first aim of the present study was to establish requirement estimates for EPA+DHA for juvenile European seabass. The second aim was to establish if the dynamic nature of the EPA+DHA requirement found in gilthead seabream is applicable to European seabass juveniles, which would imply that this extends to more species of fish.

IV.2 Methods

IV.2.1 Diets, fish husbandry and sampling

The diets and trial design has been described in Section II.2. Briefly, to empirically determine the EFA requirements for EPA+DHA, six experimental diets were formulated to deliver a gradient of EPA+DHA (D1-D6; 0.21-3.31% of diet), to juvenile European seabass. The diet formulations and measured proximate compositions are detailed in Table II.3 and fatty acid compositions in Table IV.1. The graded levels of EFA were achieved by blending commercially available oils (FO, rapeseed oil and palm oil). Other ingredients were selected to meet the known nutrient requirements of this species (National Research Council 2011). The diets (D1 – D6) were fed to triplicate (n = 3) tanks

of 150 juvenile European seabass (~ 23 g) for a period of 127 days. The diets were delivered as two pellet sizes, firstly, P1 a 3.0 mm pellet for 56 days and secondly, P2 a 4.5 mm for 71 days. Therefore, three experimental periods were considered, namely P1, P2 and over the whole trial (OV), with the tank biomass being measured at 0, 56 and 127 days. The fish were fed twice daily *ad libitum* and delivered and wasted feed weighed for an accurate determination of biological FCR.

IV.2.2 Growth performance and feed conversion

Before undertaking the requirement analysis, the growth of *D. labrax* was benchmarked against the model of Lupatsch (2005):

$$Wt = [Wi^{0.483} + 0.009418 \cdot e^{0.065 \cdot T} \cdot days]^{2.070}$$

where Wt is the final weight (g) after days of growth/feeding, Wi is the initial weight (g) and T is the water temperature. This is an exponential model closely related to daily growth index, but parameterised for a stock of European seabass in the Red Sea (Lupatsch 2005). The fish weights attained in this trial were compared to those forecast by the model and expressed as a percentage of mass relative to the model (± %). The FCR data were modelled with OLS linear regression and the existence of a relationship supported by F-test of the model's gradient term.

IV.2.3 Requirement analysis

To estimate the EFA requirements of juvenile European seabass, three nonlinear regression models were applied to WG and DGI data, for the P1 (3 mm), P2 (4.5 mm) and OV (both pellets) response data. The predictor variable used was the dietary

percentage of EPA+DHA. The data contained some outliers, so a robust approach was used in this study for all models, so they were comparable. Robust regression automatically detects and weights outliers; thus, the assumptions for normality of error are relaxed. When using robust regression, the usual ordinary least squares (OLS) are replaced with iterated reweighted least squares (IRLS), if the algorithm detects no outliers these are equivalent to OLS and parameter estimates are identical to an OLS fit. An M-estimator algorithm was used to obtain all parameter estimates. Figure IV.1 illustrates the rational for this approach. The implication of this approach will be dealt with in the discussion. All regression analyses were undertaken using the statistical package R (Venables, Smith 2015) (version 3.4.0, Vienna, Austria). The model parameters were obtained using the function nlrob(), from the package robustbase (Rousseeuw et al. 2015). The package robustbase contains the commands to call robust nonlinear fits to derive parameters. Model diagnostics were carried out according to techniques described by Ritz and Streibig (2008).

The three regression models used were: split linear model, split quadratic model and Gompertz model. The Gompertz curve is an asymptotic equation and therefore two requirement estimates are given based on the model. The first is calculated by multiplying the asymptote by 0.95 (National Research Council 2011), which in the text is referred to as the "NRC criterion" and the second is the "elbow" calculation described in Chapter III. Unless otherwise stated N = 18, which in regression is sufficient to detect very large (f^2 = 0.5) effects in the response. The existence of a response was further supported by testing each model against its null model (the mean of the data), no models failed this test.

Table IV.1. Total lipid, total fatty acids (g.Kg^{-1}) and fatty acid composition of the six experimental diets, D1 – D6, given as percentage of total fatty acids. Note fatty acids < 0.05 % across all rows removed.

	D1	**D2**	**D3**	**D4**	**D5**	**D6**
Dietary Lipid (%)	21.44	20.73	21.79	21.04	20.60	20.88
% of total fatty acids						
14:0	0.57	0.85	1.42	2.15	3.31	5.87
16:0	15.60	15.70	15.77	16.50	16.89	17.74
18:0	2.55	2.66	2.70	2.86	3.04	3.43
20:0	0.46	0.44	0.43	0.40	0.37	0.27
22:0	0.23	0.22	0.23	0.21	0.31	0.16
24:0	0.15	0.15	0.15	0.14	0.14	0.27
ΣSFA	19.56	20.01	20.69	22.26	24.06	27.74
16:1n-9	0.06	0.06	0.06	0.07	0.08	0.11
16:1n-7	0.53	1.21	1.55	2.24	3.59	6.53
18:1n-9	46.57	42.49	41.08	36.63	28.92	12.36
18:1n-7	2.45	2.53	2.53	2.64	2.73	2.93
20:1n	0.94	0.96	1.00	1.02	1.11	1.28
22:1n	0.09	0.16	0.19	0.25	0.42	0.74
24:1n-9	0.13	0.17	0.17	0.20	0.27	0.40
ΣMUFA	50.77	47.58	46.58	43.04	37.11	24.35
18:2n-6	21.89	21.30	20.25	19.31	17.25	12.37
18:3n-6	0.00	0.02	0.05	0.07	0.12	0.24
20:2n-6	0.06	0.06	0.07	0.08	0.10	0.13
20:3n-6	0.00	0.00	0.00	0.00	0.08	0.14
20:4n-6	0.05	0.12	0.16	0.24	0.39	0.76
22:5n-6	0.00	0.04	0.06	0.09	0.15	0.30
Σ n-6 PUFA	21.99	21.55	20.59	19.78	18.09	14.00
18:3n-3	5.99	5.63	5.39	4.87	3.88	1.76
18:4n-3	0.05	0.34	0.46	0.70	1.19	2.32
20:3n-3	0.00	0.00	0.00	0.00	0.02	0.06
20:4n-3	0.00	0.09	0.12	0.18	0.31	0.60
20:5n-3	0.71	2.21	2.86	4.27	7.21	13.77
22:5n-3	0.11	0.28	0.36	0.53	0.87	1.65
22:6n-3	0.62	1.64	2.07	3.04	5.03	9.51
Σ n-3 PUFA	7.48	10.19	11.26	13.59	18.51	29.67
EPA+DHA (%)[1]	0.21	0.58	0.82	1.13	2.04	3.10
Total fatty acids (g.Kg^{-1})	155.93	150.36	167.13	155.01	166.70	133.0

[1]These values are the predictor variable for all analyses in this publication, expressed in this table as a percentage of diet.

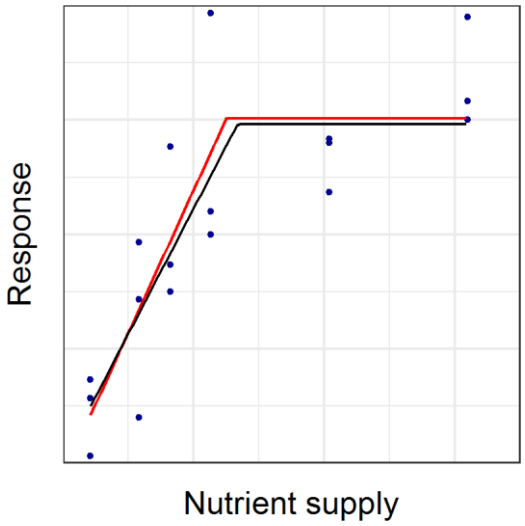

Figure IV.1. A comparison of two fitting methods for the same data (●). The red line is an ordinary least squares (OLS) fit (called using the function nls()), and the black line an iterated reweighted least squares (IRLS) fit (called using the function nlrob()). The latter function is a robust method that derives a more conservative estimate of the requirement by limiting the influence of unusual observations. In this example the two highest observations in the response have too great an effect on the parameters, the influence of this approach was to provide a moderately more conservative (higher) requirement estimate.

IV.2.3.1 Split linear model

The first model applied to the responses was the so-called "broken line" or split linear model, in which the first segment is a linear model ascending to a maximum response, i.e. a horizontal segment (a mean). The requirement is defined as the intersection of these two segments. Split linear regression can be expressed as follows:

$f(x) = m \cdot x + c$ for $x < req$

$f(x) = m \cdot req + c$ for $x > req$

where *x* is the percentage of EPA+DHA in the experimental diet, *c* is the model's intercept and *m* is the gradient (of the first segment) and *req* is *x* at the breakpoint (the requirement).

IV.2.3.2 Split quadratic model

The second model applied to the data was the split quadratic model, in which the first segment is a quadratic function ascending to a maximum level of response, whereas the second segment is horizontal. The requirement is defined as the intersection of these two segments. Split quadratic regression can be expressed as follows:

$f(x) = m \cdot x + (-0.5 \cdot m / req) \cdot x^2 + c$ for $x < req$

$f(x) = m \cdot req + (-0.5 \cdot m / req) \cdot req^2 + c$ for $x > req$

where *x* is the percentage of EPA+DHA in the experimental diet, *c* is the model's intercept, *m* is the rate constant and *req* is *x* at the breakpoint (the requirement).

IV.2.3.3 Gompertz model

The asymptotic Gompertz function was fitted to the response data, which can be defined as:

$f(x) = a \cdot \exp(b \cdot c^x)$

where *x* is the percentage of EPA+DHA in the experimental diet, *a* is the asymptote or Y maxima, *b* sets the displacement on the x-axis and *c* is the scaling parameter, setting the rate of growth towards the asymptote.

IV.3 Results

IV.3.1 Growth performance

The biometric data analysed in this paper are summarised as means (±SD) in Table IV.2. Regardless of diet, all experimental groups increased in weight, from an initial weight of ~23 g to final weights ranging between 133.5 – 156.7 g, with fish consuming D1 (the VO diet) gaining less weight than fish consuming D4 - D6. Accordingly, DGI values ranged between 1.78 – 1.99. The effect size (difference between D1 – D6) is not very large, an important point that will form part of the discussion. The range of FCR values were 1.26 – 1.22, the higher values attained for the VO diets, D1 - D4. When compared to the Lupatsch model, all experimental groups outperformed the model, D1 by 7.57 % and D6 by 26.20 %. Thus, all groups attained good performance in this trial.

IV.3.2 Requirement analysis

A series of regression analyses were performed on the biometric data from this trial. The split linear and quadratic and Gompertz model were applied to the three experimental periods, P1, P2 and OV for the WG and DGI data. It should be noted that the presentation of the P2 data is only to demonstrate the time effects, each dietary treatment has already diverged when they begin feeding on this pellet and therefore requirement derived from P2 are not valid estimates. All model parameters, requirement estimates, IRLS and AIC values are given in Table IV.3.

Table IV.2. Mean (± SD) values for the performance of European seabass (*Dicentrarchus labrax*) juveniles analysed in this study (n = 3) over the three experiment periods, P1 P2 and OV.

	D1		**D2**		**D3**		**D4**		**D5**		**D6**	
Initial Weight (g)	22.96	± 0.92	23.26	± 0.78	22.59	± 0.93	23.93	± 1.05	22.96	± 0.51	23.18	± 0.68
Weight P1 (g)	72.34	± 1.27	75.02	± 2.59	76.78	± 1.23	80.07	± 1.8	78.93	± 1.02	81.24	± 0.67
Weight P2 (g)	133.53	± 2.55	145.69	± 2.94	146.07	± 3.46	154.15	± 5.1	153.28	± 2.46	156.66	± 2.93
Feed intake P1 (Kg)	8.75	± 0.29	9.06	± 0.07	9.25	± 0.03	9.71	± 0.3	9.58	± 0.12	9.77	± 0.15
Feed intake P2 (Kg)	11.56	± 0.48	13.60	± 0.98	13.72	± 0.24	14.25	± 0.64	14.24	± 0.37	14.13	± 0.4
Feed intake OV (Kg)	20.31	± 0.23	22.66	± 0.92	22.97	± 0.26	23.96	± 0.94	23.82	± 0.49	23.90	± 0.55
DGI[1] P1	2.36	± 0.05	2.43	± 0.04	2.54	± 0.09	2.55	± 0.12	2.58	± 0.02	2.64	± 0.06
DGI P2	1.33	± 0.07	1.48	± 0.11	1.46	± 0.06	1.49	± 0.04	1.50	± 0.02	1.48	± 0.05
DGI OV	1.78	± 0.04	1.89	± 0.05	1.92	± 0.05	1.95	± 0.08	1.97	± 0	1.99	± 0.03
FCR[2] P1	1.19	± 0.02	1.19	± 0.03	1.15	± 0.03	1.17	± 0.03	1.14	± 0.02	1.12	± 0.03
FCR P2	1.31	± 0.02	1.34	± 0.05	1.35	± 0.05	1.34	± 0.02	1.32	± 0.01	1.30	± 0.02
FCR OV	1.26	± 0.02	1.27	± 0.01	1.26	± 0.04	1.26	± 0.02	1.24	± 0.01	1.22	± 0.01
Lupatsch (+%)[3]	7.57	± 2.50	17.37	± 2.94	17.77	± 3.41	24.19	± 5.01	23.46	± 2.41	26.20	± 2.87

[1] Daily Growth Index = Final weight (g) $^{0.333}$ – Initial weight (g) $^{0.333}$ / Days feeding

[2] Feed conversion ratio = (Feed fed (kg) – feed waste (kg)) / Δ Biomass (kg)

[3] In terms of weight during the experimental period. The model is defined: $Wt = [Wi^{0.486} + 0.01166 \cdot e^{0.060 \cdot T} \cdot days]^{2.058}$ where Wt is the final weight (g) after days of growth/feeding, Wi is the initial weight (g) and T is the water temperature (Lupatsch et al. 2003a).

IV.3.2.1 Weight gain and daily growth index

Using the split linear model to fit the WG data, the requirement estimates were 1.21, 1.34 and 1.00 for the OV, P1 and P2 data, respectively. Using the split linear model to fit the DGI data the requirement estimates were 1.04, 1.56 and 0.87 for the OV, P1 and P2 data, respectively. These data are shown in Figure IV.2 (A and B). Using the split quadratic model to fit the WG data, the requirement estimates were 1.64, 1.93 and 1.33 for the OV, P1 and P2 data, respectively. Using the split quadratic model to fit the DGI data, the requirement estimates were 1.84, 3.14 and 0.82 for the OV, P1 and P2 data, respectively.

The Gompertz function was also fitted to WG and DGI data. As mentioned in the methods, two calculations of the requirement are given, namely the NRC criterion and the elbow calculation. Using the Gompertz model to fit the WG data, the NRC criterion requirement estimates were 0.89, 1.19 and 0.73 and the elbow calculation were 1.15, 1.31 and 1.01 for the OV, P1 and P2 data, respectively. Interestingly, the latter elbow values agree with the values derived from split linear regression. Using the Gompertz model to fit the DGI data, the NRC criterion requirement estimates were 0.68, 1.23 and 0.41 and the elbow calculation were 1.21, 1.41 and 0.85 for the OV, P1 and P2 data, respectively. For the NRC criterion, only in P1 do the derived requirement estimates agree across the WG and DGI data because this calculation is sensitive to the scale of the response variable (y-axis). The elbow calculation achieves reasonable agreement across the WG and DGI data. In terms of model fit, the Gompertz function fit the data best (attained the lowest values for IRLS and AIC) in all cases except the P1 DGI data, where the split quadratic attained the lowest values.

Table IV.3. Model parameters fitted to the biometric data of European seabass (*Dicentrarchus labrax*) juveniles in this trial to derive estimates for the EPA+DHA requirement of this species. The three models presented are the split linear, split quadratic and Gompertz function. The models were applied to the overall data (OV, both pellet sizes), the 3 mm pellet size (P1, in the text) and the 4.5 mm pellet size (P2 in the text). The name of the parameter and the notation used in the text is indicated in the column headers. The iterated reweighted least squares (IRWS) and Akaike information criteria (AIC) as a means of comparing fit quality (for like Metrics and Periods) (N = 18).

Model type	**Metric**	**Period**	**Parameters**				**Fit**	
			Plateau	**Gradient** *m*	**Intercept** *c*	**Requirement** *req*	**IRLS**	**AIC**
Split linear	**WG (g)**	OV	131.98	19.88	107.93	1.21	287.46	108.95
		3 mm	56.92	6.57	48.12	1.34	38.58	72.8
		4.5 mm	74.85	16.24	58.61	1.00	213.03	103.56
	DGI	OV	1.98	0.22	1.75	1.04	0.04	-49.34
		3 mm	2.61	0.18	2.33	1.56	0.07	-40.49
		4.5 mm	1.49	0.23	1.29	0.87	0.07	-41.74
			Plateau	Rate constant *m*	Intercept *c*	Requirement *req*	IRLS	AIC
Split quadratic	**WG (g)**	OV	131.76	33.04	104.67	1.64	256.16	106.88
		3 mm	56.90	9.89	47.36	1.93	38.32	72.68
		4.5 mm	74.81	27.93	56.24	1.33	212.91	103.55
	DGI	OV	1.99	0.26	1.75	1.84	0.03	-54.83
		3 mm	2.63	0.19	2.33	3.14	0.06	-42.3
		4.5 mm	1.49	0.68	1.21	0.82	0.07	-41.2
			Plateau *a*	Displacement *b*	Scale parameter *c*	Requirement Elbow	IRLS	AIC
Gompertz	**WG (g)**	OV	132.48	-0.26	0.16	1.15	234.73	105.31
		3 mm	57.77	-0.2	0.32	1.31	27.42	66.66
		4.5 mm	75.07	-0.35	0.07	1.01	208.69	103.19
	DGI	OV	1.99	-0.14	0.22	1.21	0.03	-55.61
		3 mm	2.66	-0.14	0.44	1.41	0.07	-41.98
		4.5 mm	1.49	-0.25	0.02	0.85	0.07	-41.78

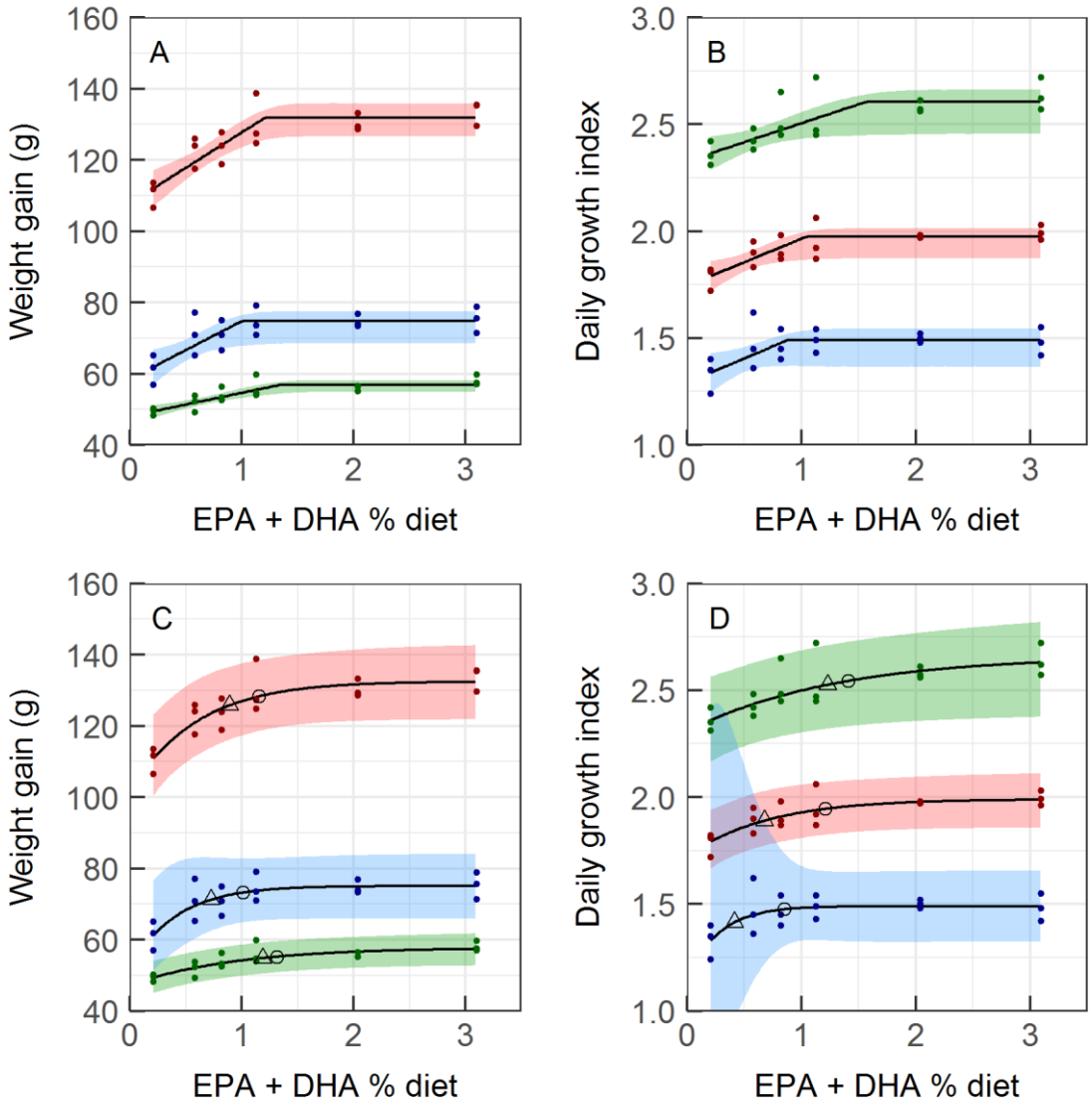

Figure IV.2. The split linear (A and B) and Gompertz (C and D) models applied to the weight gain (A and C) and daily growth index (B and D) data against EPA+DHA % of diet (as fed, x-axis). The raw data are marked with coloured spots, the relevant model fit with an unbroken line, 95 % prediction intervals (calculated by Monte-Carlo simulation in A and B and by second order propagation in C and D) are shaded in colour. Colours for the different periods are as follows: the whole trial, OV = ; the 3 mm pellet size, P1 = and the 4.5 mm pellet size, P2 = . The National Research Council (△, NRC criterion in text) and the Elbow (○) calculation are marked in plots C and D.

IV.3.2.2 Feed conversion ratio

The feed conversion data (estimated as FCR) only exhibited small effect sizes. Furthermore, different regressions had to be applied to each period due to the shape of the data changing as the experiment progressed. It was possible to model the OV data with a split linear model, and the requirement derived from this model was 1.04 % EPA+DHA. However, this estimate had quite large standard error and therefore a high degree of uncertainty. The P1 data was fit with a linear model, which has no breakpoint and therefore a requirement cannot be estimated. However, the gradient term was small. Neither of these models had a significant gradient term for the P2 data and, therefore, in P2 this data set is best described by its mean. These analyses are illustrated in Figure IV.3 and the parameters given in Table IV.4. Therefore, a dietary effect on FCR exists, but it is more important when the fish are smaller (P1).

Table IV.4. Parameters for the analysis of the FCR data: OV by split linear regression, P1 by linear regression, and P2 by a single term model (the mean). These data are illustrated in Figure IV.3.

Model	**Metric**	**Period**	**Plateau**	**Gradient** *m*	**Intercept** *c*	**Requirement** *req*
Split linear	FCR	OV	1.27	-0.02	1.29	1.04
Linear		P1	na[1]	-0.02	1.19	na
Mean		P2	na	na	1.32	na

[1]Not applicable

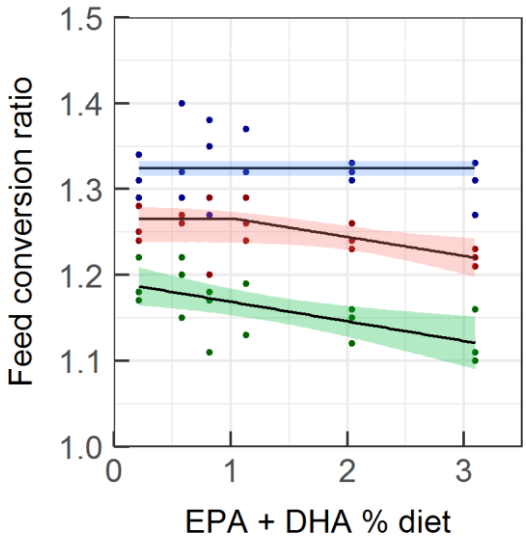

Figure IV.3. The feed conversion ratio (FCR) data from this trial (coloured points) plotted against dietary EPA+DHA. In the case of the OV data it was possible to fit a split regression function, and this is plotted with Monte-Carlo prediction intervals. For P1, a linear fit was possible, and this is plotted with standard error. In P2, neither of these fits described the data better than the global mean ($P > 0.05$) and therefore a single term model (the mean) is plotted with standard error for consistency. Colours for the different periods are as follows: the whole trial, OV = ; the 3 mm pellet size, P1 = ; and the 4.5 mm pellet size, P2 = . Despite the small effect size in FCR there is a significant relationship in P1 and OV, but this is diminished by P2.

IV.4 Discussion

Using a dose-response experiment and a variety of regression strategies, this study set out to provide new EPA+DHA requirement estimates for European seabass juveniles. In accordance with previous work on gilthead seabream (Chapter III), it was demonstrated that the EPA+DHA requirement for the European seabass also fell as the fish increased in weight. Practically, this means that EFA requirements should be viewed as dynamic figures and each pellet size could have an optimum quantity of EFA to achieve optimum performance for the size of the fish. The EFA dietary specification could fall with increasing pellet size, which would preserve the use of marine oils in aquafeeds fed to larger fish.

A study in gilthead seabream with very similar dietary design was carried out in parallel to the present study providing excellent opportunity for direct comparison. The most obvious difference between the two data sets is that the gilthead seabream grew faster achieving DGI values of 2.3 – 2.6, which is considerably higher than the 1.8 – 2.0 reported here for European seabass. In the European seabass, the measured effect size (difference between D1 – D6) available to analyse in the data was lower compared to that of gilthead seabream. This meant that outliers had a stronger impact on these data, which necessitated a robust statistical approach. This approach was usually on the conservative side (i.e. gave an estimate slightly higher than an OLS fit), but usually less than 10% higher. The outliers in the dataset were tanks performing better than expected from the other replicates in D3 and D4. These diets were supplied with EPA+DHA levels, 0.82 % and 1.13 % for D3 and D4, respectively, close to the requirement estimates given for the OV requirements (Table IV.3).

For juvenile European seabass the current published dietary requirement is 0.7 % dietary n-3 LC-PUFA (Skalli, Robin 2004), although the NRC (2011) gives a value of 1% and cites Coutteau *et al.* (1996). From the data reported here two new recommendations for minimum requirements can be made for European seabass of 23 g (3 mm pellet) and 80 g (4.5 mm pellet). European seabass requires approximately 1.3 – 1.5 % EPA+DHA (as fed) as an optimum requirement to achieve good growth from 23 – 80 g. After this size the OV requirement estimate can be considered, with most estimated values being between 1.1 - 1.2 % EPA+DHA. During P2 (4.5 mm) the requirement falls further and the ~1% of diet recommended for most cultured species would become sufficient to support optimal growth (National Research Council 2011). However, the presence of outliers and the smaller effect size in growth cast more uncertainty of the growth performance of European seabass in response to the dietary EPA+DHA gradient. Interestingly, these figures are only marginally lower than those derived from the study in gilthead seabream (Chapter III). This may indicate that, rather than the species itself, body mass and growth rate are more important in determining a requirement for EFA in marine fish, although further studies would be required to validate this statement. Studies regarding the amino acid requirements in pigs also show that lysine requirements per unit dietary energy also fall as the pig increases in weight through different body mass ranges (26 – 106 Kg) (Hauschild et al. 2010). Similar findings have been also made in ducks (Timmler, Rodehutscord 2003). Consequently, the dynamic EFA requirement could be viewed as an indirect result of the fishes rising demand for energy (which causes an increase in absolute feed intake). While further studies are required to test this hypothesis, if confirmed, it would then be reasonable to express EFA requirements per unit protein (or even perhaps per unit protein deposited), rather than per unit energy as has been

previously suggested (Glencross, Smith 2001). In this study EPA and DHA are considered as a single nutrient. In these diets, the DHA:EPA ratio ranged between 0.87 – 0.69, from D1 – D6, therefore the diet containing the lowest level of LC-PUFA (D1) had the highest ratio. This study cannot offer advice on the most suitable ratio of DHA:EPA for juvenile European seabass and, to my knowledge, no studies have been conducted in juveniles of this species. However, a study in the Japanese seabass (*Lateolabrax japonicus*) from 10 – 65 g showed that the best ratio was 2. This is considerably higher than the ratios of the diets used in the present study and, so it must be accepted that an additional gain in performance may have been realised with higher dietary levels of DHA relative to EPA, which would lead to lower requirement estimates for EPA+DHA. A further study in juvenile blackhead seabream (*Acanthopagrus schlegelii*) did not find any significant effects of the DHA:EPA ratio, but SGR was marginally higher than other treatments (0.65 – 2.67) with a dietary DHA:EPA ratio of 1.6 (Jin, Monroig et al. 2017).

Previous studies defining the EFA requirements of fish have shown mixed results. Many species such as Atlantic salmon (*Salmo salar*), Asian seabass (*Lates calcarifer*), turbot (*Psetta maxima*), starry flounder (*Platichthys stellatus*), Korean rockfish (*Sebastes schlegeli*) silvery black porgy (*Sparidentix hasta*) and red drum (*Sciaenops ocellatus*) have been reported to have n-3 LC-PUFA requirements of ~1 % of diet (Lee et al. 2003, Lochmann, Gatlin 1993, Gatesoupe et al. 1977, Glencross, Rutherford 2011, Glencross et al. 2014, Mozanzadeh, Marammazi et al. 2015). However, like reported for gilthead seabream (Chapter III), yellowtail flounder (*Pleuronectes ferrugineus*), Japanese flounder (*Paralicthys olivaceus*), silver bream (*Rhabdosargus sarba*), and striped jack (*Pseudocaranx dentex*) have been shown to require n-3 LC-PUFA at a higher dietary level (1.3-2.5 %) (T. Takeuchi 1997, T. Takeuchi et al. 1992, Whalen et al. 1998, Leu et al.

1994). Some of the former and latter species are phylogenetically close (e.g. silvery black porgy and silver bream) and have similar trophic habits. As the n-3 LC-PUFA requirement appears to be a function of fish mass, this may explain the different n-3 LC-PUFA requirements reported in species that share close phylogeny and feeding habit (e.g. black porgy and silver bream). However, smaller fish were used in former trials that investigated the n-3 LC-PUFA requirements in European seabass (Skalli, Robin 2004) and gilthead seabream (Kalogeropoulos et al. 1992), in these cases the lower requirement estimates can be related to the lower growth rates attained in these experiments. Studies in the Asian seabass (*L. calcarifer*) have examined the effect of dietary EPA (Salini, Wade et al. 2016) and DHA (Glencross, Rutherford 2011) gradients in isolation from one another. Moreover, these studies employed a pair feeding regime (all treatments supplied with an equal ration). In both studies, neither EPA or DHA had a stimulatory effect on growth of juvenile Asian seabass in isolation. A positive control treatment in the DHA study (Glencross, Rutherford 2011) supplied additional EPA and this did have a positive influence on fish growth rates. This raises the question that in the EPA (and ARA) experiment, dietary DHA may have been limiting and in the DHA experiment dietary EPA may have been limiting. However, in the context of paired feeding regimes LC-PUFA effects on growth may be negligible, leading to the conclusion that LC-PUFA enhance physiological processes but only lead to performance increases when feed is supplied to satiation.

Further discrepancies in EFA requirement estimates may be caused by selection of models that do not suit the shape of the response variable (Shearer 2000). As such, a requirement estimate derived from an empirical analysis of biometric data is affected by 1) the size of the fish, 2) the performance attained by the fish and 3) the analytical methods

selected by the authors, as well as the feeding ecology and phylogeny of the species in question. As aquaculture is a rapidly developing food production sector, technical knowledge is also rapidly developing. Any advancement in fish nutrition, husbandry or breed development that leads to improved performance will elevate the demands for key nutrients to support that gain. Therefore, no requirement should be viewed as a static dietary percentage, but rather as a figure that supports a certain level of animal performance at a certain animal mass.

In all the models reported here, except the P1 DGI data, the Gompertz function fit the data the best as indicated by the IRLS and AIC values in Table IV.3. In the case of the P1 DGI data, the split quadratic model achieved a marginally better fit to the data. However, the EPA+DHA requirement estimate was much higher due to the ascending curve fitting all the data points. This emphasises two points: firstly, the data are best modelled by curves; secondly, in P1 some improvement in performance was achieved by the EFA level of D5 and D6. In gilthead seabream for comparison (Chapter III), the OV and P2 data were often best fit using the split linear model, whence several approaches should be explored to find a function that best suits the data (Shearer 2000, Hernandez-Llamas 2009). When an asymptotic function is used there is no obvious breakpoint and therefore some criterion is required to determine where upon the response curve to place the requirement. In Chapter III, the proposed "elbow" calculation (Eickhardt 2017) for establishing a "point of diminishing returns" (Spillman, Lang 1924) was found to be less sensitive to the selected response parameter/variable and to the scale (y-axis) of the parameter/variable. However, the "elbow" method is a geometric calculation, based on the shape of the relationship, and the precise value given still depends on the experimental demonstration of deficiency and surplus (the asymptote). A point of criticism of the

"elbow" calculations used in the present trial could be that the demonstration of deficiency in this experiment is not strong due to the small effect size in growth and FCR responses. Therefore, in this case, with the European seabass dataset it could be argued that this method may have marginally overestimated the requirement for EPA+DHA. However, the estimates derived from the "elbow" calculation gave very similar estimates to the split linear model.

In the gilthead seabream (Chapter III), the FCR data could be modelled effectively using the four-parameter-logistic function. However, in the present study there were no large effect sizes for the FCR data. Therefore, it must be concluded that EPA+DHA has less effect on FCR in European seabass than in gilthead seabream. There was a FCR response in the P1 data, but this took the shape of a straight line. Therefore, the conclusion for the P1 data is that the highest level of EPA+DHA gave the lowest FCR values. The OV FCR data could be fit with a split linear model and a requirement was derived at just over 1 % EPA+DHA, however, this value had wide uncertainty. The P2 data showed no relationship between dietary EPA+DHA and FCR. Despite the smaller effect of EPA+DHA on FCR in European seabass it was certainly present at the first sampling (P1), whence further supporting the notion that the requirement was falling as fish increased in weight. The parameter FCR has rarely been modelled in the context of n-3 LC-PUFA requirements, one example is use of split linear model in the silvery black porgy. In this study the authors found that the requirement, in terms of FCR (0.6 % diet) was lower than for growth (0.8 % diet) (Mozanzadeh et al. 2015). Feed efficiency ratio (FER, inverse of FCR) has also been modelled with split regression in Japanese seabass (*Lateolabrax japonicus*) to establish lysine requirements. The authors found that the lysine requirement (2.61% dry diet) was marginally higher when FER was used as a

response than when SGR was considered (2.49% dry diet) (Mai et al. 2006). Whence, whether growth or FCR give a higher requirement can vary depending on the dataset, so both should be considered.

EPA and DHA are well known to be absolutely critical in larval and juvenile nutrition while fish tissues are developing rapidly (Sargent et al. 1997, Rainuzzo, Reitan et al. 1997, Watanabe 1993). The conclusions drawn from the present study are consistent with this and emphasise that LC-PUFA are best provided earlier in fish development to support rapid growth stages. It is known that LC-PUFA particularly DHA, are retained in the polar fatty acids of crucial tissues such as brain and retina (Mourente, Tocher et al. 1991, Sargent, Bell et al. 1993) However, it has been demonstrated in Asian seabass (*L. calcarifer*) between 10 g – 450 g have no maintenance requirement for EPA or DHA (these fatty acids are not catabolised in response to starvation), this would suggest that the role of EPA and DHA is primarily structural (Salini et al. 2016). Interestingly, when exogenous feeding begins in the marine fish larvae, these crucial tissues (eyes, brain and notochord) form a much greater proportion of the body mass, compared to when the fish has grown to the size of an adult (Rainuzzo et al. 1997). Therefore, the dynamic nature of EFA requirements can be partially explained by the changing allometry that occurs throughout fish ontogeny.

In summary this study and the data in Chapter III present evidence of the dynamic nature of EPA+DHA requirements. It is very likely that these conclusions partially explain the range of LC-PUFA requirements reported for fish species of similar habit (National Research Council 2011). For European seabass from 23 – 80 g an optimum requirement of 1.3 – 1.5 % EPA+DHA (as fed) and from 80 - <150 g an optimum

requirement of 1.1 – 1.2 % EPA+DHA (as fed) can be recommended. At some point in the latter period the requirement would fall to the current recommendations (National Research Council 2011). These data are highly relevant to the formulation of aquafeeds for European seabass (23 g - ~150 g).

V. The compositional and metabolic responses of gilthead seabream (*Sparus aurata*) to a gradient of dietary long-chain (≥ C_{20}) polyunsaturated fatty acids

Abstract

The replacement of fish oil (FO) with vegetable oil (VO) in high energy feed formulations reduces the availability of n-3 LC-PUFA to marine fish such as gilthead seabream, one of the main species cultured in the Mediterranean. The aim of the present study was to examine compositional and physiological responses to a dietary gradient of n-3 LC-PUFA. Six isoenergetic and isonitrogenous diets (D1-D6) were fed to triplicate groups of seabream, with the added oil being a blend of FO and VO to achieve a dietary gradient of n-3 LC-PUFA. Fish were sampled after four months feeding, to determine biochemical composition, liver and mid-intestine fatty acid concentrations and lipid metabolic gene expression. The results indicated a disturbance to lipid metabolism, with fat in the liver increased and fat deposits in the viscera reduced. Liver and mid-intestine fatty acid profiles were altered towards the fatty acid compositions of the diets. There was evidence of endogenous modification of dietary PUFA in the liver which correlated with the expression of fatty acid desaturase 2 (*fads2)*. Expression of sterol regulatory element-binding protein 1 (*srebp1*), *fads2* and fatty acid synthase (*fas*) increased in the liver, while peroxisome proliferator-activated receptor alpha 1 (*ppara1*) pathways appeared to be supressed by dietary VO in a concentration-dependent manner. These findings suggested that suppression of beta-oxidation and stimulation of *srebp1*-mediated lipogenesis may play a role in contributing toward steatosis in fish fed n-3 LC-PUFA deficient diets. These data are relevant to the formulation of modern, sustainable feeds for marine teleosts.

V.1 Introduction

Sustainable expansion of aquaculture requires reduction in the use of FM and FO in aquafeed formulations (Glencross 2009, Pike, Jackson 2010, Merino et al. 2012, Tacon, Metian 2015). Both raw materials, particularly FO, are rich in the two key n-3 LC-PUFA, EPA and DHA recognised as EFA for the majority of marine fish species (Tocher 2015). DHA is an essential component of neural and retinal membranes (Gawrisch et al. 2003) and both EPA and DHA are precursors for an extensive range of autocrine signalling molecules (e.g. eicosanoids, resolvins, protectins, etc.) (Serhan, Chiang et al. 2008). Dietary deficiency of n-3 LC-PUFA has impacts on the health (Oliva-Teles 2012), metabolism (Tocher 2003, Jordal, Torstensen et al. 2005), composition (Izquierdo et al. 2005, Benedito-Palos, Navarro et al. 2008) and growth (Kalogeropoulos et al. 1992) of marine fish.

Typically, an aquafeed for a given marine fish species contains a combination of FO to supply essential n-3 LC-PUFA and VO that, while devoid of LC-PUFA, supply dietary energy (Sargent et al. 2002, National Research Council 2011). While freshwater fish and salmonids are largely able to effectively utilise dietary VO to satisfy their EFA requirements, marine carnivorous fish are not (Tocher 2015). In terms of fatty acid composition, the key effects of high inclusion levels of VO are an increase in C_{18} unsaturated fatty acids (ALA, LOA and oleic acid) in the fish tissues at the expense of LC-PUFA that is reflective of the altered composition of the dietary fatty acids (Turchini et al. 2010). With regards to lipid metabolism, some studies have observed that inclusion of dietary VO leads to reduced fatty acid catabolism and the accumulation of lipid in the liver (Caballero, Izquierdo et al. 2004, Morais, Pratoomyot, Taggart et al. 2011, Jin et al.

2017). Studies examining the influence of VO on lipid biosynthesis have yielded conflicting results with some reporting increased gene expression (Morais et al. 2011, Jin et al. 2017) and others reporting decreased enzyme activity (Menoyo, Izquierdo et al. 2004) although, in mammals, EPA has been shown to supress lipogenesis (Y. Takeuchi, Yahagi et al. 2010).

Lipid homeostasis is maintained in animals through a balance of catabolic and anabolic processes. Fatty acids and cholesterol can be synthesised *de novo* by pathways that are activated by sterol regulatory element binding proteins (Srebp) 1 and 2, respectively. Srebp are transcription factors involved in energy homeostasis and have many target genes with examples of those in lipid metabolism including fatty acid synthase (*fas*) and fatty acid desaturase 2 (*fads2*), the latter being key enzymes in the LC-PUFA biosynthesis pathway (Daemen, Kutmon et al. 2013, C. Castro, Corraze et al. 2016). Fatty acids are catabolised by the β-oxidation pathway in either mitochondria or peroxisomes, and expression of genes encoding proteins involved in these pathways are regulated by, among others, peroxisome proliferator-activated receptors (Ppar) (Mandard, Müller et al. 2004). Upon binding ligands and retinoid X receptor, Ppars bind to peroxisome proliferator response elements in the promoter regions of target genes, many of which are involved in β-oxidation, such as carnitine palmitoyl transferase I (*cpt1a*) and liver-type fatty acid binding protein (*fabp1*), both proteins involved in the intracellular transport of fatty acids destined for catabolism (Mandard et al. 2004, Leaver, Villeneuve et al. 2008).

Despite recent advances in our knowledge regarding the impacts of dietary VO some questions remain, for instance, are dietary differences in gene expression dependent on

precise concentrations of dietary nutrients or are genes activated/deactivated at thresholds of nutrient supply? Furthermore, are lipid metabolic processes related to tissue compositions, which are themselves reflective of diet? Therefore, the present study aimed to examine the impact of modern (high lipid, low FM) aquafeed formulations across a gradient of n-3 LC-PUFA, achieved by blending commercially available oils (FO, rapeseed oil and palm oil), on the biochemical composition of body compartments, fatty acid compositions and gene expression in the liver and mid-intestine of a marine teleost, the gilthead seabream (*Sparus aurata*).

V.2 Methods

V.2.1 Fish husbandry and diets

The diets and trial design has been described in Section II.2. Briefly, 150 juvenile seabream were randomly distributed between 18 x 1 m^3 tanks. Initially, the fish were fed with commercial fry feeds rich in FM and FO until they reached ~24 g. After acclimation, each tank was assigned one of six isoenergetic and isonitrogenous diets and the fish were fed for 18 weeks. The six diets were formulated to deliver specific levels of LC-PUFA by progressively replacing FO with blends of RO and PO, whereas the other dietary ingredients were selected to meet the known nutrient requirements of seabream (Oliva-Teles et al. 2011) (Table II.2). The experimental diets were numbered to reflect the VO/FO inclusion so that diet D1 contained the VO blend as sole exogenously added oil source, diet D6 contained only FO and diets D2 - D5 contained graded levels of VO and FO (Table II.2). The fatty acids that increased with dietary FO were: 16:1n-7, 24:1n-9, 20:3n-6, 20:4n-6, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3, while 20:0, 22:0, 18:1n-9, 18:2n-6 and 18:3n-3 increased with dietary VO (Table III.1).

V.2.2 Sampling

Fish were sampled at the initiation of the trial and at termination after being euthanised with a lethal dose of benzocaine (Centrovet, Kalagin, Santiago, Brazil). Five whole fish and three eviscerated carcasses, liver and viscera were sampled from each tank for compositional analysis. Three fish per tank were also sampled for gene expression and fatty acid composition taking samples of liver and mid-intestine. Samples for RNA analysis were incubated with 1 mL RNA*later*® at 4 °C for 24h (Sigma-Aldrich, UK) before storage at -70°C, while samples for fatty acid analysis were immediately frozen and stored at -20 °C before shipment on dry ice to the Institute of Aquaculture, University of Stirling.

V.2.3 Proximate composition

Feed samples were ground prior to analyses. Whole fish, carcass and viscera samples were homogenised in a blender (Waring Laboratory Science, Winsted, CT, USA) to produce pates. Proximate compositions of feeds and fish were determined according to standard procedures (Horwitz 2000). Moisture contents were obtained after drying in an oven at 110 °C for 24 h and ash content determined after incineration at 600 °C for 16 h. Crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyser, Foss, Warrington, U.K) and total lipid content determined as described below.

V.2.4 Fatty acid analysis

The total lipids were extracted from liver and mid-intestine samples according to Folch (1957) as described in Section II.3.2.1. Fatty acid composition of liver and

mid-intestine total lipid samples were determined as described in Sections II.3.2.2 and II.3.2.3, according to methods described by Christie (2003). Tissue fatty acid levels were expressed as mg g^{-1} of tissue and estimated using the response of the internal standard. The coefficient of variation estimated using mg DHA g^{-1} over a subset of 20 samples was 2.80 ± 2.51 %.

V.2.5 RNA extraction

Total RNA was extracted from ~100 mg of liver and mid-intestine tissue according to the method described in Section II.3.3.1. The concentration and quality were verified spectrophotometrically and by agarose gel electrophoresis to visualise the presence of 18S and 28S ribosomal subunits (Section II.3.3.4). Extracts were stored at -70 °C until cDNA synthesis.

V.2.6 cDNA synthesis

Reverse transcription was performed using a High Capacity Reverse Transcription kit according to the kit manufacturer's protocol (Applied Biosystems) as described in Section II.3.3.2. A no template control (NTC) reaction and reverse transcriptase-free reactions were prepared as blank and negative controls. A pool of cDNA samples was created for serial dilutions, calibrator samples and primer validations. Samples of cDNA were diluted 20-fold with nuclease-free water as template for qPCR, and stored at -20 °C.

V.2.7 Gene expression analysis

Relative gene expression was determined for candidate genes involved in key lipid metabolic pathways by quantitative real time PCR (qPCR, Section II.3.3.3). Primers for

qPCR were designed using Primer3 through the NCBI database's "Primer-BLAST" against known gene sequences including sequences from the *S. aurata* expressed sequence tag (EST) NCBI database that were confirmed to be the gene of interest by BLAST searches, as described in Section II.3.3.6. Primer sequences for genes in the present study are given in Table V.1. Gilthead seabream primer sequences.Primers were tested to confirm that they functioned optimally at annealing temperatures of 60 °C and that a single amplicon of appropriate length was visualised on agarose gel (Section II.3.3.4). Duplicated qPCR reactions were carried out on 96-well plates using a Biometra TOptical Thermocycler (Analytik Jena) instrument according to the conditions described in Section II.3.3.3. As there were 54 samples per tissue (6 treatments, N = 9) two plates were run per gene with treatments equally represented on both plates. A single master mix was used for all the reactions required per gene and both plates were run consecutively. Data were acquired through the software package qPCRsoft 3.1 (Analytik Jena) and calculations for sample expression ratios were carried out according to Pfaffl (2001):

$$Expression\ ratio = \frac{E(ref)^{Ct(Sample)}}{E(goi)^{Ct(Sample)}} \div \frac{E(ref)^{Ct(Calibrator)}}{E(goi)^{Ct(Calibrator)}}$$

where *E* is the determined efficiency, *ref* is the geometric mean of four reference genes, *goi* is the gene of interest and *Ct* is the threshold cycle. Gene expression data are presented as log2 expression ratios (Hellemans, Vandesompelle 2011). The average intra assay coefficient of variation was 0.44 ± 0.12 % at the level of quantification cycle (C_q).

Table V.1. Primer sequences used for gene expression analysis by quantitative reverse-transcriptase PCR. Amplicon sizes (base pair) and GenBank accession numbers also are provided.

Transcript	Sequence (5'-3')	Amplicon(bp)	Accession no
fads2	F:GCAGGCGGAGAGCGACGGTCTGTTCC	72	AY055749
	R:AGCAGGATGTGACCCAGGTGGAGGCAGAAG		
elovl5	F:CCTCCTGGTGCTCTACAAT	112	AY660879
	R:GTGAGTGTCCTGGCAGTA		
cpt1a	F:GTGCCTTCGTTCGTTCCATGATC	82	JQ308822
	R:TGATGCTTATCTGCTGCCTGTTTG		
srebp1	F:AGGGCTGACCACAACGTCTCCTCTCC	77	JQ277709
	R:GCTGTACGTGGGATGTGATGGTTTGGG		
pparα1	F:TCTCTTCAGCCCACCATCCC	116	AY590299
	R:ATCCCAGCGTGTCGTCTCC		
fabp1	F:CATGAAGGCGATTGGTCTCC	165	KF857311
	R:GTCTCCAAGTCTGCCTCCTT		
srebp2	F:GCTCACAAGCAAAATGGCCT	240	AM970922.1
	R:CAAAACTGCTCCCTTCCCCA		
fas	F:TGCCATTGCCATAGCACTCA	172	JQ277708.1
	R:ACCTTTGCCCTTTGTGTGGA		
actb	F:TCCTGCGGAATCCATGAGA	50	X89920
	R:GACGTCGCACTTCATGATGCT		
ef1α	F:ACGTGTCCGTCAAGGAAATC	109	AF184170
	R:GGGTGGTTCAGGATGATGAC		
tuba1α	F:ATCACCAATGCCTGCTTCGA	214	AY326430.1
	R:CTGTGGGAGGCTGGTAGTTG		
rplp0	F:GAACACTGGTCTGGGTCCTG	159	AY550965.1
	R:TTCAGCATGTTGAGGAGCGT		

V.2.8 Statistical analysis

Three individuals from each tank were randomly sampled giving 54 fish in total, this is the minimum number of fish to detect medium effect sizes ($f^2 = 0.15$; power = 0.8) by ordinary least squares regression. Proximate composition, fatty acid and gene expression data were analysed using linear regression to identify the existence of trends across the experimental diets. Trends were reported as significant if the slope was significantly different ($P < 0.05$) to 0. For fatty acid profiles of tissues (N = 54) the first step of analysis was to reduce the dimensions of the data by principal component analysis (PCA), which enabled the identification of fatty acids that were correlated with each other and that should be analysed further. For further details see Section II.4.2.

V.3 Results

V.3.1 Proximate composition of gilthead seabream

Significant effects of diet on proximate compositions of gilthead seabream were observed in liver, mid-intestine and viscera. In liver and mid-intestine, total lipid contents increased from 18.6 – 31.8 % diet ($R^2 = 67.8$, $P < 0.001$) and 8.6 – 13.9 % ($R^2 = 67.8$, $P < 0.001$), respectively, as dietary VO increased from 0 – 15.6 % of diet. In viscera (minus liver), total lipid content decreased from 53.5 – 45.2 % ($R^2 = 24.0$, $P < 0.001$) as dietary VO increased in the diet. The lipid contents of whole fish and carcass were unaffected by dietary treatment ($P > 0.05$). Protein and ash contents were not affected in any body compartment examined in the present study ($P > 0.05$). A summary of these data is presented in the Appendix, Table A1.

V.3.2 Fatty acid composition of liver and mid-intestine

Fatty acid profiles of total lipid of two major lipid metabolic sites, namely liver and mid-intestine, were determined and further analysed by PCA. In both the liver and mid-intestine the first principal component (PC1) was correlated to dietary FO and explained 57.5 % and 60.1 % of the variance in fatty acid compositions, respectively (Figure V.1 and Figure V.2). The fatty acids associated with FO, namely 14:0, 16:1n-7, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3, had positive correlations to PC1, and those associated with VO, specifically 18:1n-9, 18:2n-6 and 18:3n-3, a negative correlation to PC1 in liver and mid-intestine. PC2 accounted for 15.3 % and 15.5 % of the variance in liver and mid-intestine fatty acid profiles, respectively. The contributions of the fatty acids towards PC2 differed between liver and mid-intestine. In liver, 20:2n-6 and 20:1 had a positive influence on PC2, and Iso 18:2n, Iso 20:2n, 18:3n-6, 20:3n-6 a negative influence. In the case of mid-intestine samples, 18:0, 20:0 and 22:0 had a positive influence, and 18:1n-7 a negative influence on PC2 while Iso 18:2n and Iso 20:2n were not detected. It was also apparent from biplots that there was considerable overlap between the fatty acid profiles of liver and mid-intestine of fish consuming diets D1 - D4, although fatty acid profiles from individuals fed on diets containing the highest inclusion levels of FO (D5 and D6) formed clearly separated groups (Figure V.1 and Figure V.2). PCA indicated that the n-3 LC-PUFA (20:3n-3, 20:4n-3, EPA, DPA and DHA) were all correlated with each other in both liver and mid-intestine and therefore these fatty acids were summed as a single variable reflecting their origin from FO. Absolute levels of tissue n-3 LC-PUFA were strongly related to dietary n-3 LC-PUFA in liver ($R^2 = 0.75$, $P < 0.001$) and mid-intestine ($R^2 = 0.87$, $P < 0.001$) (Figure V.3). Absolute levels of monounsaturates (MUFA) responded positively to dietary MUFA in liver ($R^2 = 0.57$, $P < 0.001$) and mid-intestine

(R^2 = 0.76, P < 0.001) (Figure V.4). Contents of MUFA in liver were typically 75 - 175 mg g^{-1} in fish fed diets D1 - D4, and < 50 mg g^{-1} in fish consuming the FO diet (D6).

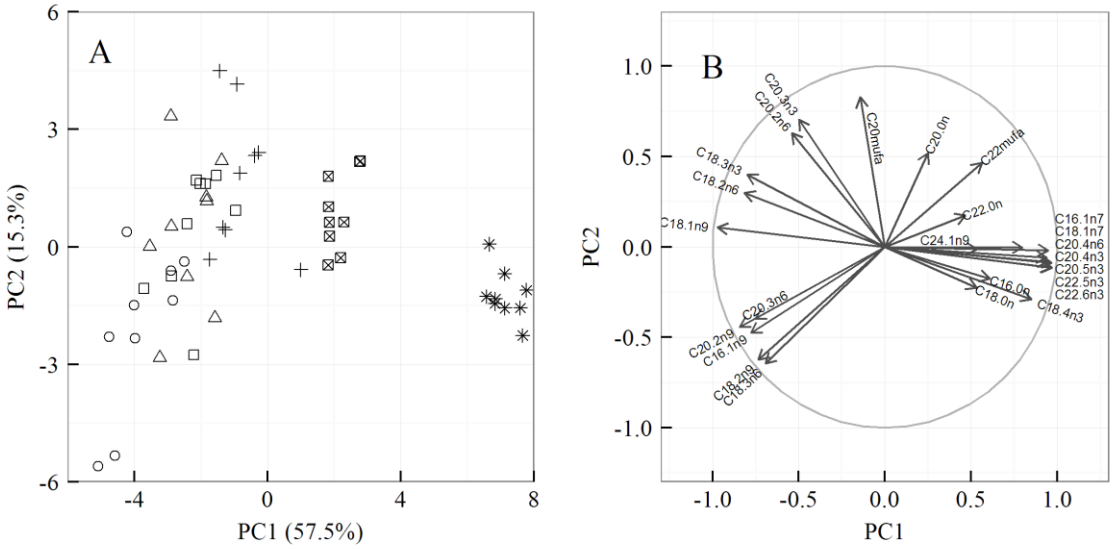

Figure V.1. Graphical representation of principal components analysis (PCA) of fatty acid profiles from liver (n=54) of fish fed experimental diets (D1 - D6). For explanation of figures see section II.4.2.1. The fatty acid profiles of fish consuming diets D5 and D6 formed succinct groups to the right of the plot and they were well defined by PC1. The fatty acid profiles of fish consuming diets D1 - D4 were negatively correlated to PC1 and there was considerable overlap (variability) between the dietary treatments. PC2 accounted for 15.3 % of the total variance and was important to distinguish individual liver fatty acid profiles of fish consuming diets D1 - D3. Several points can be made to describe the data, the fatty acids derived from fish oil (e.g. 22:6n-3) were strongly correlated to PC1, the main fatty acids from vegetable oil (e.g. 18:1n-9) were negatively correlated to PC1. Several fatty acids were best explained by a combination of PC1 and PC2, including Iso 18:2n and Iso 20:2n, and it can be said that these fatty acids are important to distinguish liver fatty acid profiles from fish consuming diets D1 - D4. D1 = ○ ; D2 = ∆ ; D3 = □ ; D4 = + ; D5 = ⊠ ; D6 = ✳

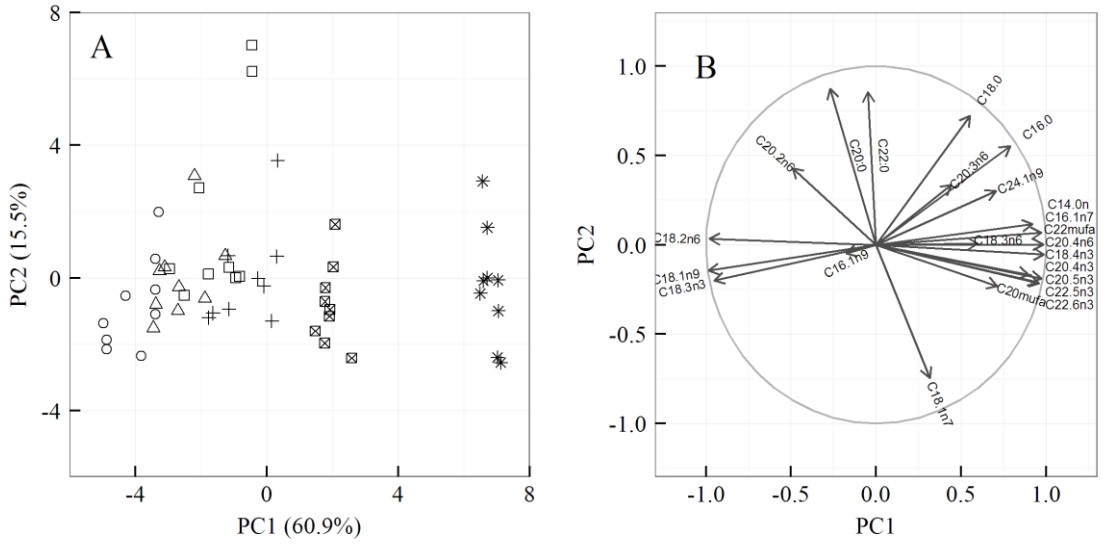

Figure V.2. Graphical representation of principal components analysis (PCA) of fatty acid profiles from mid-intestine (n=54) of fish fed experimental diets (D1 - D6). The fatty acid profiles of fish consuming diets D5 and D6 formed succinct groups to the right of the plot. The fatty acid profiles of fish consuming diets D1 – D3 were negatively correlated to PC1 and there was considerable overlap in the points. PC2 separated the fatty acid profiles vertically and explained 15.5 % of the variance, two outliers appeared in D3 and these samples contained unusually high levels 20:0 and 22:0 and, other than these samples, the variance was quite evenly distributed amongst the diets when compared to liver. The arrows indicate how the fatty acids contributed to the formation of PC1 and PC2 and thus the formation of plot A. D1 = ○ ; D2 = ∆ ; D3 = □ ; D4 = + ; D5 = ⊠ ; D6 = ✳.

Two fatty acids, Iso 18:2n and Iso 20:2n, were identified in liver in spite of their absence in diets and mid-intestine, so they were plotted against dietary VO. Levels of Iso 18:2n increased with the dietary VO ($R^2 = 0.81$, $P < 0.001$) (Figure V.5A). The level of Iso 18:2n was correlated ($R^2 = 0.41$, $r = 0.64$, $P < 0.001$) to the $\log_2$ expression of *fads2* (Figure V.5B). Dietary levels of saturates were relatively stable across the experimental diets (32 - 36 g kg^{-1}). Despite this low range, livers of fish fed diet D1 (VO rich) contained 50 - 70 mg g^{-1} saturates and those of fish fed diet D6 (FO rich) ranged between 10 - 40 mg g^{-1}, and there was a significant relationship with dietary saturates ($R^2 = 0.27$, $P < 0.001$) (data not shown). Examination of percentage data indicated that absolute levels of saturates was mainly associated with the increasing lipid level of the liver, lower levels of saturates were observed in the mid-intestine (15 – 35 mg g^{-1}). Quantitatively, palmitic acid was the dominant saturated fatty acid in both tissues and only 14:0 increased with dietary FO. Liver and mid-intestine fatty acid data are summarised in the Appendix, Tables, A2 and A3, respectively.

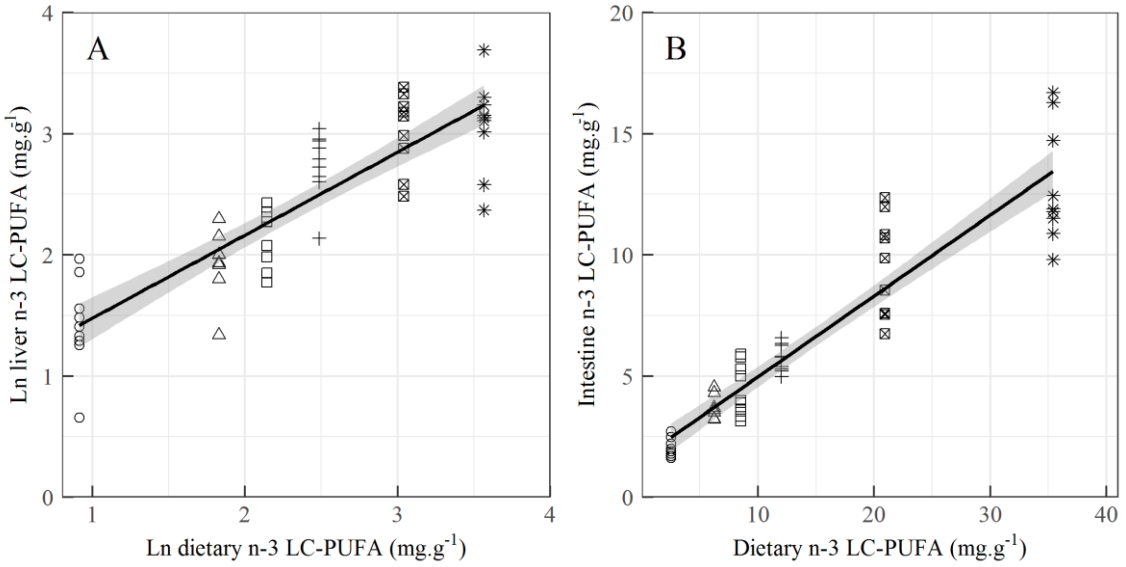

Figure V.3. Levels of n-3 long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA) in gilthead seabream liver (A) and mid-intestine (B) against the dietary levels of n-3 LC-PUFA. Note the natural logarithm (ln) transformation applied to the data in panel A indicating that in liver this relationship was not linear. Both models were linear ordinary least squares fits with the standard error shaded in grey (n=54). Diet 1 = ○ ; Diet 2 = ▵ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 = ✳

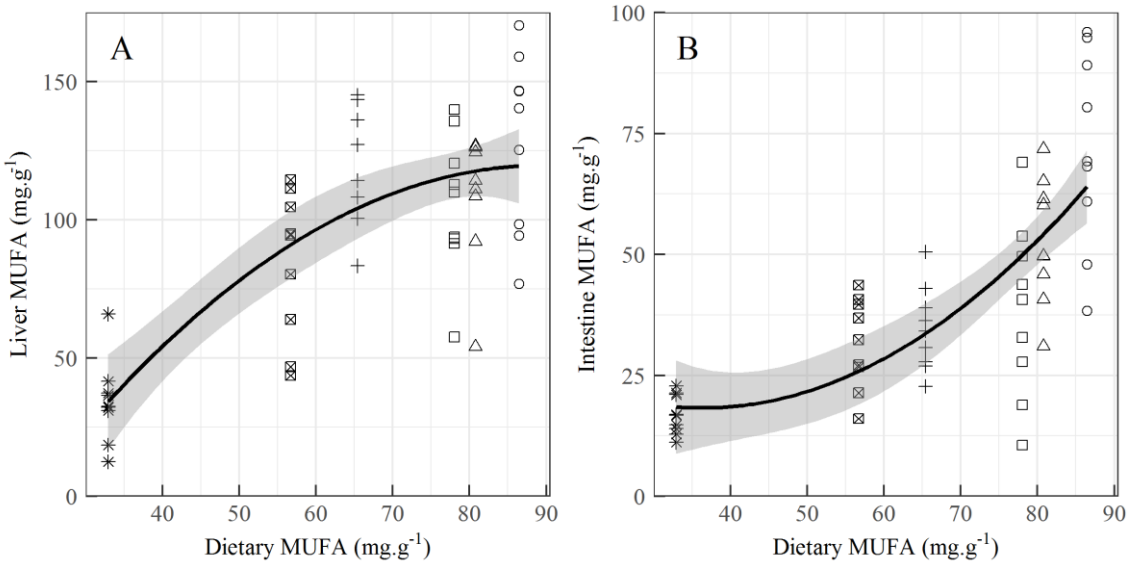

Figure V.4. Levels of monounsaturated fatty acids (MUFA) in gilthead seabream liver (A) and mid-intestine (B) against the dietary levels of MUFA. Both are quadratic ordinary least squares fits with the standard error shaded in grey (n=54). Diet 1 = ○ ; Diet 2 = ▵ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 = ✳

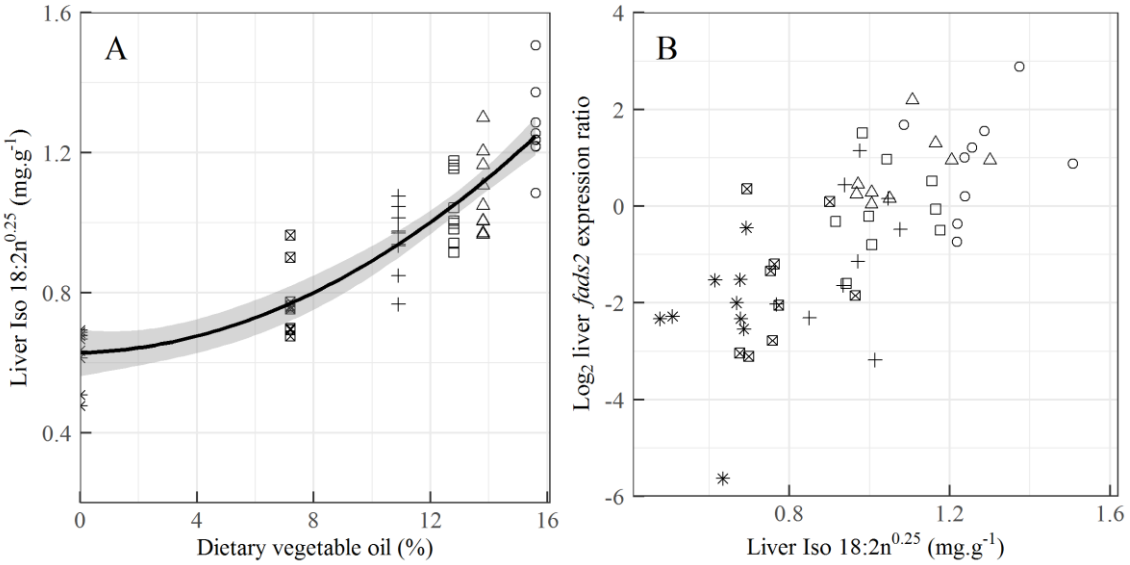

Figure V.5. Levels of Iso 18:2n, which is not present in the diets, in gilthead seabream liver against dietary vegetable oil (A), and the level of *fads2* expression correlated (r = 0.64; P < 0.001) with the levels of Iso 18:2n in liver (B). The model applied in A is a quadratic ordinary least squares fit with the standard error shaded in grey (n=54). Note the transformation ($x^{0.25}$) applied to the Iso 18:2n data. Diet 1 = ○ ; Diet 2 = △ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 = ✳

V.3.3 Gene expression

Genes representing lipid metabolic pathways were assayed by qPCR, and the expression of target genes plotted against dietary VO and, although variability (R^2 = 19 – 52 %), between individuals was high, significant trends showed that the diets had an impact on the regulation of lipid metabolism. Negative trends were found in the liver between dietary VO and the expression of *pparα1* (R^2 = 0.32, P < 0.001) and its target genes *cpt1α* (R^2 = 0.26, P < 0.001) and *fabp1* (R^2 = 0.19, P < 0.001) (Figure V.6). The level of variation in the mid-intestine prevented the application of suitable models to the data. In the liver, positive trends, fitted with quadratic functions were found between VO

and *srebp1* (R^2 = 0.37, P < 0.001) and its target genes *fas* (R^2 = 0.42, P < 0.001) and *fads2* (R^2 = 0.52, P < 0.001) (Figure V.7). Effects of VO on the expression of *srebp2* and *elovl5* in liver were not detected (data not shown). However, in mid-intestine, the effect on *srebp1* expression was not as strong and linear (R^2 = 0.23, P < 0.001), but *srebp2* showed a strong up-regulation in fish fed diets D1 and D2 (R^2 = 0.49, P < 0.001) and *elovl5* was responsive to dietary VO (R^2 = 0.26, P < 0.001) (Figure V.8). Effects of VO on the expression of *fads2* were not detected in mid-intestine (data not shown).

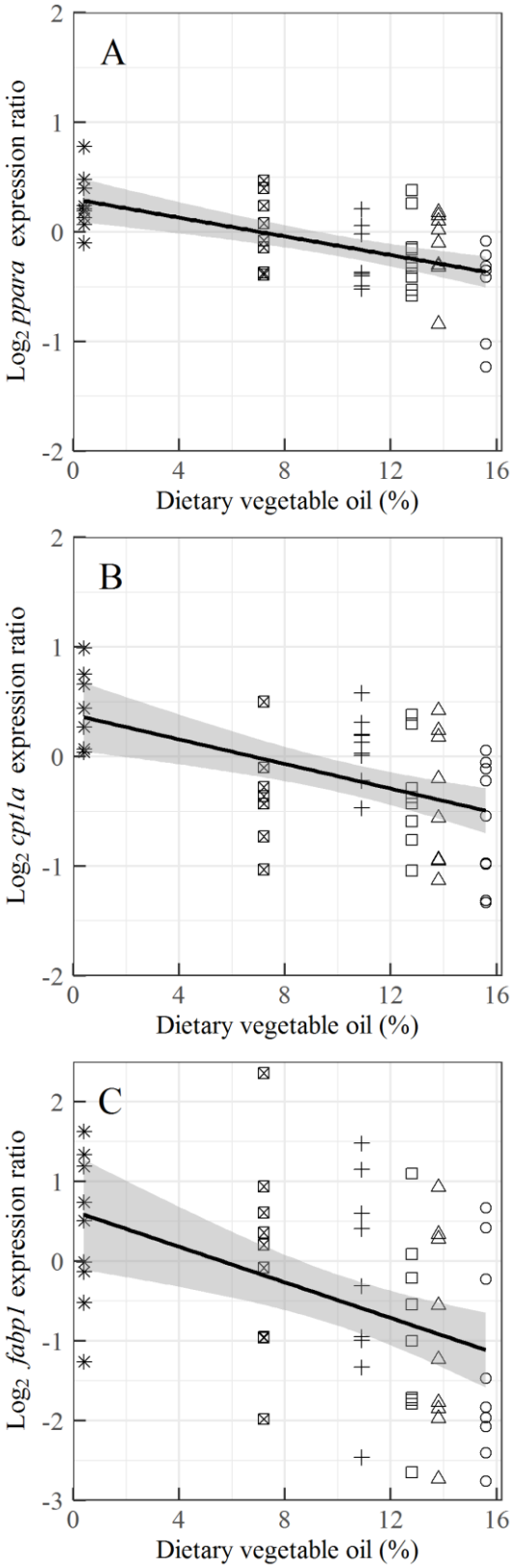

Figure V.6. Hepatic gene expression of *ppara1* (A), *cpt1a* (B) and *fabp1* (C) against dietary vegetable oil. Data are $\log_2$ (expression ratios) normalised to four reference genes and then to the calibrator sample. Fitted lines are linear functions with the standard error highlighted in grey (n=54). Diet 1 = ○ ; Diet 2 = ∆ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 = ✳

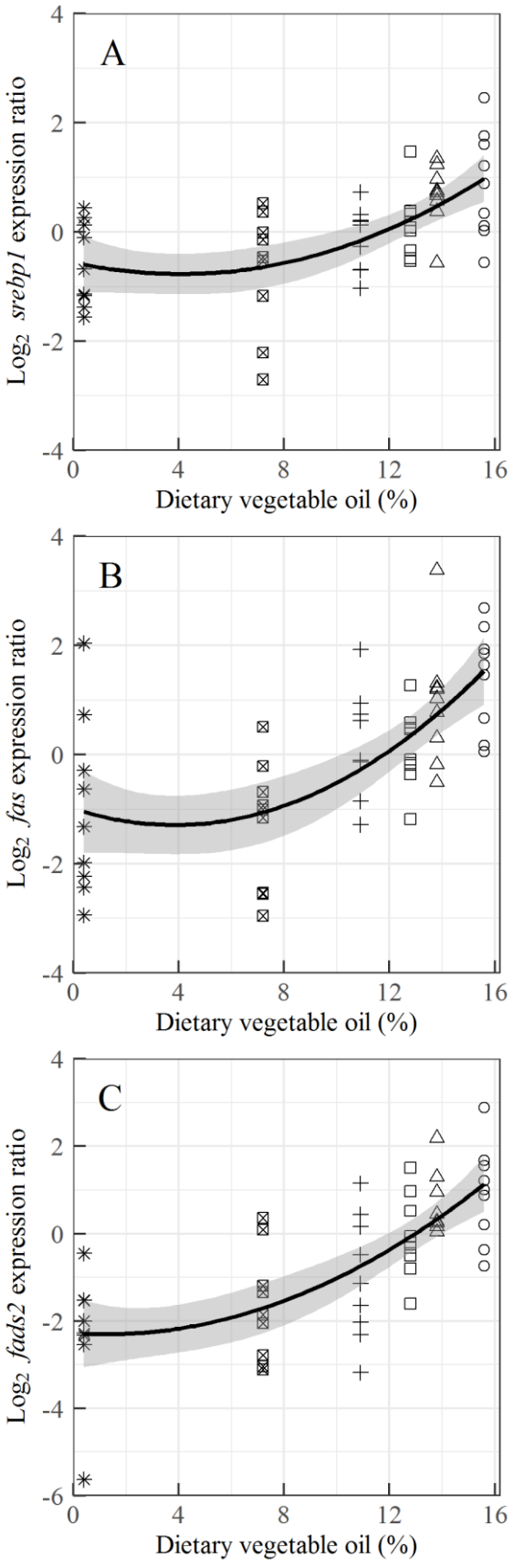

Figure V.7. Hepatic gene expression of *srebp1* (A), *fas* (B) and *fads2* (C) against dietary vegetable oil. Data are $\log_2$ (expression ratio) normalised to four reference genes and then to the calibrator sample. Fitted lines are second order quadratic functions with the standard error highlighted in grey (n=54). The similarity between the responses is striking. Diet 1 = ○ ; Diet 2 = ▵ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 = ✳

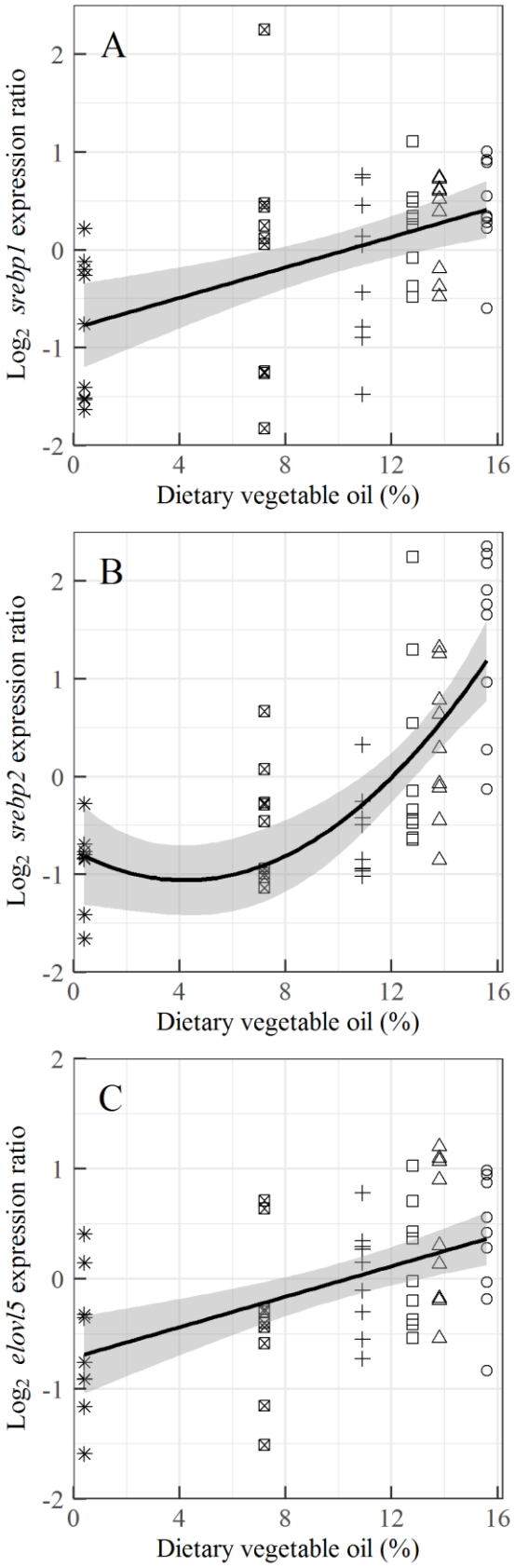

Figure V.8. Mid-intestine gene expression of *srebp1* (A), *srebp2* (B) and *elovl5* (C) against dietary vegetable oil. Data are $\log_2$ (expression ratio) normalised to four reference genes and then to the calibrator sample. Fitted lines are linear fits for *srebp1* and *elovl5* and a second order quadratic function is fitted to *srebp2*. The model standard errors are highlighted in grey (n=54). Diet 1 = ○ ; Diet 2 = △ ; Diet 3 = □ ; Diet 4 = + ; Diet 5 = ⊠ ; Diet 6 = ✳

V.4 Discussion

Replacement of FO by alternative oils in aquafeeds has been an extensively investigated research topic over the last two decades (Turchini et al. 2010). The most common FO alternatives are VO, for example rapeseed oil or soya bean oil, which are devoid of essential LC-PUFA and, consequently, their use has important implications, not only on the nutritional value of the product for consumers (Henriques, Dick et al. 2014, Sprague, Dick et al. 2016, Shepherd, Monroig et al. 2017), but also effects on metabolism and fish health (Glencross 2009). We employed a dietary gradient of LC-PUFA to span the EFA requirements reported for a commercially relevant teleost, the gilthead seabream (Oliva-Teles et al. 2011) and show how this gradient modifies the composition and the expression of lipid metabolic and regulatory genes in gilthead seabream juveniles.

The results of the present study show that dietary provision of n-3 LC-PUFA below the reported requirements for gilthead seabream (NCR, 2011) led to alterations in lipid metabolism as indicated by increased lipid in liver and decreased lipid in viscera. The visceral cavity is the lipid storage site in this species (McClelland, Weber et al. 1995). These results were in agreement with previous studies regarding increased hepatic lipid content as a result of dietary deficiency of EPA and DHA in gilthead seabream (Kalogeropoulos et al. 1992, Ibeas et al. 1996, Caballero et al. 2004) and other fish species (Glencross 2009, Tocher, Bell et al. 2001). Interestingly, increased lipid contents in liver have been also described when dietary lipid was increased to boost the energy content of the diet (Vergara, López-Calero et al. 1999). The range of values for hepatic total lipid was 17 - 32 % (wet wt) and these were higher than those reported previously, 15 - 25 %

(wet wt), in gilthead seabream fed graded levels of soya bean oil (Kalogeropoulos et al. 1992). This may be due to the larger size of the fish in the present trial (24-230g) whereas the previous study used fry (1.2-12.4g), the longer feeding period of four months in the present study, or the higher crude lipid levels in the diets. In the previous study there was a threshold level of soya bean oil (~ 50 % of oil) that increased hepatic lipid content whereas data in the present study suggested VO increased hepatic lipid in a concentration-dependant manner (linear increase).

Quantitatively, the main fatty acids driving the increased liver lipid were oleic acid, LOA and ALA, all major constituents of rapeseed oil used in the experimental diets. Such accumulation of dietary fatty acids observed in liver, occurring as well in mid-intestine, has been commonly reported in FO replacement studies in gilthead seabream (Kalogeropoulos et al. 1992, Montero, Robaina et al. 2001, Benedito-Palos et al. 2008). Interestingly, in fish fed VO, two fatty acids, namely Iso 18:2n and Iso 20:2n, that were not present in the diets, were found in the liver. The presence of Iso 18:2n is likely to be the result of Δ6 desaturation of 18:1n-9, with Iso 20:2n being the elongation product of Iso 18:2n. Fads2 is typically a Δ6 desaturase in marine teleosts (C. Castro et al. 2016) and, although its activity towards 18:1n-9 has not been demonstrated in gilthead seabream (Zheng, Seiliez et al. 2004), it is highly likely that Fads2 activity was responsible for observed production of Iso 18:2n in liver. Indeed, this is consistent with increased expression of *fads2* in liver of fish fed high VO diets, a regulatory mechanism often reported in literature not only on desaturases, as observed herein, but also elongases such as *elovl5* (Jin et al. 2017). Interestingly, an up-regulation of hepatic *elovl5* was not observed, that would support the production of Iso 20:2n mentioned above. Instead, *elovl5* was down regulated in the mid-intestine with dietary VO, in agreement with

previous studies on seabream (Castro et al. 2016). Hepatic *fads2* expression was variable between individual fish fed diets with high VO inclusion (D1 - D4). The PCA analysis showed that this was also the case for the fatty acid profiles of fish consuming these diets and that Iso 18:2n and Iso 20:2n were important fatty acids in driving this variability. Furthermore, the level of Iso 18:2n was strongly correlated to the level of *fads2* transcripts in the liver. It may be possible to exploit this individual variability in response to VO to select seabream that are better adapted to diets that are rich in VO, as has been described previously in Atlantic salmon (*Salmo salar*) (Morais et al. 2011).

In addition to the distinctive patterns of *fads2* and *elovl5* expression described above, the regulatory mechanisms by which dietary fatty acids modulate metabolic responses in liver and mid-intestine appear to differ. In liver, *srebp1*, but not *srebp2*, was increased in gilthead seabream fed diets D1 - D3, with a threshold between diets D3 and D4. In mid-intestine, both *srebp1* and *srebp2* expression were increased with dietary VO. Srebp signalling is responsible for maintaining lipid levels in balance and, although there is some overlap between the functions of Srebp1 and Srebp2, the former is mainly associated with fatty acid/lipid synthesis, whereas the latter is associated with cholesterol synthesis in mammals (Pai, Guryev et al. 1998, Amemiya-Kudo, Shimano et al. 2002, Daemen et al. 2013) and fish (Carmona-Antoñanzas, Tocher et al. 2014). While the up-regulation of *srebp1* has been often associated with increased expression of *fads2* in response to VO-rich diets (Morais et al. 2011, Jin et al. 2017), the up-regulation of *srebp2* in mid-intestine suggested putative activation of cholesterol biosynthesis. Dietary cholesterol was corrected for but VO are known to contain a range of phytosterols that may interfere with cholesterol metabolism (Leaver et al. 2008, Morais, Pratoomyot, Torstensen et al. 2011, Sanden, Liland et al. 2016).

The *fas* gene is also regulated by Srebp1 (Daemen et al. 2013) and its product, Fas, is an enzyme complex responsible for *de novo* synthesis of saturated fatty acids (Smith 1994, Chirala, Wakil 2004). Despite the inclusion of 20 % lipid in the diets, the inclusion of VO resulted in up-regulation of *fas* in liver. There have been reports of VO increasing *fas* expression in Atlantic salmon (Morais et al. 2011) and blackhead seabream (*Acanthopagrus schlegelii*) (Jin et al. 2017). Nevertheless, this is not always supported by measurements of Fas activity in gilthead seabream fed diets with 80 % of dietary FO replaced with linseed oil (Menoyo et al. 2004). However, in turbot (*Scophthalmus maximus*), Fas activity was stimulated by dietary VO although the differences were not significant (Regost, Arzel et al. 2003). It is unclear what exactly is responsible for the apparent discrepancy between these results. However, the increased expression of *srebp1* in response to dietary VO, particularly notable in liver, suggested increased regulatory activity of Srebp1 towards potential target genes including *fas* and thus increasing their transcription. The patterns of dietary regulation of *srebp1* and *fas* share a similar shape in liver (modelled by quadratic functions) suggesting co-regulation. Their up-regulation in response to dietary VO indicated that *de novo* lipogenesis may contribute to increased lipid deposition, as suggested previously by Morais et al. (2011) when studying diet/genotype interactions in Atlantic salmon.

Beyond the anabolic processes described above, the impact of dietary VO was further evidenced in lipid catabolic processes such as β-oxidation. Generally, the expression of catabolic genes (*pparα1* and *cpt1α*) in liver and, to some extent, in mid-intestine, decreased with increasing dietary VO. The reduction in catabolic gene expression is coherent with the observed increased hepatic lipid levels associated with dietary VO. Furthermore, the results were consistent with previous studies in rats (Harris,

Bulchandani 2006, Shearer, Savinova et al. 2012) and Atlantic salmon (Morais et al. 2011) that revealed that dietary FO increased the expression of *pparα* and the activity of β-oxidation enzymes. In contrast, a recent study demonstrated expression of *pparα1* and its target *cpt1α* were increased in liver of gilthead seabream fed diet containing both wild-type *Camelina sativa* oil (a low LC-PUFA diet) and containing genetically-modified camelina oil (containing n-3 LC-PUFA), although both these diets contained sufficient FM to satisfy EFA requirements (Betancor, Sprague et al. 2016). Dong *et al*. (2017) have recently shown that *pparα1* expression in response to VO was different in three species of fish. The authors observed, dietary VO increased expression of *pparα1* and *pparα2* in rainbow trout (*Oncorhynchus mykiss*) and decreased expression of *pparα2* in Japanese seabass (*Lateolabrax japonicus*) but had no effect in livers of yellow croaker (*Larimichthys crocea*). This finding complicates the regulation of lipid homeostasis but means that lipid catabolic processes can be regulated by a broad range of endogenous stimuli.

In conclusion, the present study demonstrated that diets deficient in essential LC-PUFA can cause alterations to lipid metabolism, fatty acid composition of liver and the regulation of genes involved in lipogenesis and catabolism. Despite the high energy (high lipid) diets used in the present study, expression of lipogenic genes such as *fas* involved in *de novo* biosynthesis was increased by dietary VO. In contrast, *pparα1* and its target *cpt1α* were down-regulated and the expression was linear and therefore appeared to be modulated in a concentration-dependant manner. However, the expression of *srebp1* and its gene targets was modelled by a curve, which was indicative of a threshold concentration at which gene expression was activated. Overall, the results reported in the present study were consistent with those reported in rats (Ide 2000), that the LC-PUFA

found in FO have a stimulating effect on β-oxidation and an inhibitory effect on *de novo* lipogenesis. These opposing biochemical activities would be expected to contribute towards the increased hepatic lipid observed in fish fed increasing VO in the present study and others investigating EFA nutrition. These physiological effects of VO have direct relevance to decisions regarding sustainable and modern feed formulations for marine fish species.

VI. Dietary n-3 LC-PUFA gradients in the European seabass (*Dicentrarchus labrax*); effects on composition and metabolism

Abstract

Replacement of fish oil (FO) with vegetable oil (VO) in energy-rich aquafeeds reduces the availability of omega-3 (n-3) long-chain polyunsaturated fatty acids (LC-PUFA) to marine fish, such as European seabass, a major cultured finfish in the Mediterranean. The aim of the present study was to examine the compositional and physiological responses of European seabass to a dietary gradient of n-3 LC-PUFA. Six isoenergetic and isonitrogenous diets (D1-D6) were fed to triplicate groups of juvenile European seabass (23 g), with the added oil being a blend of FO and VO to achieve a dietary gradient of n-3 LC-PUFA (0.23 – 3.40 % of diet as fed). Fish were sampled after four months feeding to determine biochemical composition, fatty acid concentration and lipid metabolic gene expression in liver and mid-intestine. The only effect on proximate composition was an increase in hepatic lipid, while diet modified fatty acid composition of both tissues. Production of LC-PUFA biosynthesis intermediates was observed in both tissues with some bias observed to n-6 fatty acids. Only two genes in liver were moderately influenced by dietary VO (*fads2* and *igf1*) while, in mid-intestine, genes involved with lipogenesis (*srebp1*, *srebp2* and *fas*) and LC-PUFA biosynthesis (*fads2* and *elovl5*) were markedly upregulated in response to dietary VO. No differences were found in the expression of genes related to β-oxidation (*pparα*, *cpt1α* and *fabp1*). Overall, the transcriptional response in mid-intestine was much more pronounced than in liver. These findings provide insights relevant to the formulation of diets rich in VO for this species.

VI.1 Introduction

Expansion in global aquaculture has placed increasing demand on marine raw materials, especially FM and FO (Shepherd, Pike et al. 2005, Tacon, Metian 2015, Shepherd et al. 2017), resulting in their increasing replacement with plant-based ingredients in aquafeeds (Kaushik 1990, Turchini et al. 2010). FO and, to a lesser extent, FM, are rich in LC-PUFA, including EPA and DHA that are regarded as EFA for marine fish as they cannot be biosynthesised endogenously (Tocher, Glencross 2015). Both EPA and DHA play critical roles in vertebrate physiology as structural components of biomembranes and as autocrine signal molecules (Gawrisch et al. 2003, Serhan et al. 2008). Fish consuming EFA-deficient diets present a range of pathologies including steatosis (Watanabe 1982, Caballero et al. 2004), shock syndrome (Lochmann, Gatlin 1993) and reddening of fins (Tacon 1996). Currently, the aquafeed sector relies heavily on FO to supply EFA in diets for carnivorous marine fish (National Research Council 2011), whereas dietary VO supplies energy required for rapidly growing fish (Sargent et al. 1999). However, dietary VO has been shown to have important influences on fish composition and physiology (Glencross 2009), with fatty acid compositions of tissues altered towards that of the diet, showing increased levels of C_{18} MUFA and PUFA that are abundant in VO (Turchini et al. 2010). Although many vertebrates can synthesise LC-PUFA from 18:3n-3 and 18:2n-6, most carnivorous marine fish cannot due to incomplete or insufficient activity of the LC-PUFA biosynthesis pathway (Shepherd et al. 2017). The pathway involves desaturation and elongation of C_{18} PUFA substrates and, while some enzymes are present in marine fish, such as fatty acid desaturase 2 (Fads2) and elongation of very long chain fatty acids 5 (Elovl5), others such as a Δ5-desaturase are not (Castro et al. 2016).

Fish can biosynthesise fatty acids and cholesterol, with the pathways known to be regulated by sterol regulatory element binding proteins (Srebp) 1 and 2, respectively (Carmona-Antoñanzas et al. 2014). Srebp are transcription factors regulating energy homeostasis and have many target genes including those involved in lipogenesis such as fatty acid synthase (*fas*), as well as *fads2* (Daemen et al. 2013, C. Castro et al. 2016, Minghetti, Leaver et al. 2011). Excess dietary lipids must be stored as triacylglycerol or catabolised by β-oxidation, both processes regulated by peroxisome proliferator-activated receptors (Ppar) (Mandard et al. 2004). Target genes of Ppar include, fatty acid binding proteins (Fabp) and carnitine palmitoyl transferase I (Cpt1α), proteins involved in transport of fatty acids destined for breakdown via β-oxidation (Mandard et al. 2004, Leaver et al. 2008). Genes involved in lipid catabolism are also known to be impacted by dietary VO with some studies reporting reduced catabolism (β-oxidation) contributing to excess lipid deposition in liver of many species, including Atlantic salmon (*Salmo salar*) (Morais et al. 2011), gilthead seabream (Houston et al. 2017) and blackhead seabream (*Acanthopagrus schlegelii*) (Jin et al. 2017). However, in European seabass, *pparα* expression was unaffected by VO (Geay et al. 2010). Studies on lipogenic processes have also yielded conflicting results, with lipogenic genes increased by dietary VO in Atlantic salmon (Morais et al. 2011), European seabass (Geay et al. 2010), gilthead seabream (Houston et al. 2017) and blackhead seabream (Jin et al. 2017), but reduced enzyme activity has also been reported in gilthead seabream (Menoyo et al. 2004).

Although many studies have investigated the impact of dietary VO in fish, major questions remain including: 1) are differences in gene expression dependent on precise concentrations of dietary nutrients or are genes activated/deactivated at certain dietary thresholds? 2) Can tissue composition be related to transcriptional response? The aim of

the present study was to determine the effects of a gradient of dietary n-3 LC-PUFA in the context of modern feed formulations (high lipid and low FM) on tissue compositions and expression of lipid metabolic genes in European seabass. The tissues being liver and mid-intestine, both important sites of lipid and fatty acid metabolism.

VI.2 Methods

VI.2.1 Fish husbandry and diets

The experimental diets and trial design are detailed in Section II.2. European seabass juveniles (~3 g) were purchased from a commercial hatchery and transported to the BioMar FTU (Hirtshals, Denmark). Subsequently, 150 fish were distributed randomly between 18 x 1 m^3 tanks, and fed a commercial diet until they reached a size of ~23 g. Six experimental diets were formulated at the BioMar Tech-Centre (Brande, Denmark) to achieve specific levels of LC-PUFA by progressively replacing FO with blends of RO and PO (Table II.3). All other dietary ingredients were selected to meet the known nutrient requirements of European seabass (National Research Council 2011). The experimental diets were numbered to reflect the VO/FO inclusion so that diet D1 contained the VO blend as sole exogenously added oil source, diet D6 contained only FO, and diets D2 - D5 contained graded levels of VO and FO. The dietary fatty acid profiles are given in Table IV.1. The feeding trial started in April 2014 and ran for 18 weeks, initially feeding a 3 mm pellet (8-weeks) and then with a 4.5 mm pellet to the end of the trial. Fish were fed to satiation twice per day using automatic feeders and uneaten feed was collected to accurately measure feed consumption.

VI.2.2 Sampling

The tank biomass was weighed before and after the trial so that mean weight and specific growth weight (SGR) could be calculated. After four months feeding, five whole fish and three eviscerated carcasses, liver and viscera (minus liver) were sampled from each tank for compositional analysis. Livers were also weighed to determine hepato-somatic index (HSI). Liver and mid-intestine samples were sampled from three fish per tank for gene expression and fatty acid analysis. Further details of sampling can be found in Section II.2

VI.2.3 Proximate composition

Proximate composition analyses were carried out according to standard procedures (Horwitz 2000), as described in Section II.3.1. Total lipid content was carried out according to Folch (1957) as described in Section II.3.2.1.

VI.2.4 Lipid extraction and fatty acid analysis

Total lipid for fatty acid analyses was extracted from tissues according to Folch *et al.* (1957). Fatty acid composition of liver and mid-intestine samples were determined as described in Sections II.3.2.2 and II.3.2.3, according to methods described by Christie (2003). Tissue fatty acid levels were expressed as mg g^{-1} of tissue and estimated using the response of the internal standard. The coefficient of variation estimated using mg DHA g^{-1} over a subset of 20 samples was 2.80 ± 2.51 %.

VI.2.5 RNA extraction

Total RNA was extracted from ~100 mg of liver and mid-intestine tissue according to the method described in Section II.3.3.1. The concentration and quality were verified spectrophotometrically and by agarose gel electrophoresis to visualise the presence of 18S and 28S ribosomal subunits (Section II.3.3.4). Extracts were stored at -70 °C until cDNA synthesis.

VI.2.6 cDNA synthesis

Reverse transcription was conducted using a High Capacity Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol and as described in Section II.3.3.2. Each tissue was run in a single run and included a no template control (NTC) and reverse transcriptase-free reactions as blank and negative controls, respectively. Reverse transcription was performed on a Biometra Thermocycler (Analytik Jena). A pool of cDNA samples was created for serial dilutions, calibrator samples and primer validations. Samples of cDNA were diluted 20-fold with nuclease-free water as template for qPCR, and stored at -20 °C.

VI.2.7 Gene expression analysis

Gene expression was determined for candidate genes involved in key selected pathways by quantitative real time PCR (qPCR). Primers for qPCR were designed using Primer3 using the National Centre for Biotechnology Information (NCBI) database's tool "Primer-BLAST" against known gene sequences. Three genes, namely *srebp2*, *fas* and *ppara1*, were sequenced as part of this work and sequences deposited in NCBI with accession numbers as shown in Table VI.1. All qPCR analyses were performed using a

Lightcycler-480 instrument (Roche Diagnostics Corp, USA), as described in Section II.3.3.3. Triplicated qPCR reactions were carried out on 384-well plates. Data were acquired through the instrument's software and calculations for sample expression ratios were carried out according to Pfaffl (2001):

$$Expression\ ratio = \frac{E(ref)^{Ct(Sample)}}{E(goi)^{Ct(Sample)}} \div \frac{E(ref)^{Ct(Calibrator)}}{E(goi)^{Ct(Calibrator)}}$$

where *E* is the determined efficiency, *ref* is the geometric mean of four reference genes, *goi* is the gene of interest and *Ct* is the threshold cycle. The genes of interest were normalised to the geometric mean of four reference genes, elongation factor 1α (*ef1α*), beta-actin (*β-act*), alpha-tubulin (*tuba1α*) and ribosomal protein P0 (*rplp0*), whose expression was not influenced by dietary treatment. Gene expression data are presented as $\log_2$ expression ratios (Hellemans, Vandesompelle 2011). The average intra assay coefficient of variation was 0.31 ± 0.25 % at the level of threshold cycle (C_t).

VI.2.8 Statistical analysis

Three individuals from each tank were randomly sampled giving a total of 54 fish, this being the number of fish to detect medium effect sizes ($f^2 = 0.15$; power = 0.8) by OLS regression. For fatty acid profiles of tissues (n = 54) a PCA was first run to explore the dataset. The fatty acid profiles of the diets were supplied to PCA as supplementary individuals (they were not used to generate the PC scores but allocated scores after analysis). Fatty acids and gene expression data were further analysed using regression. Some variables including final weight, SGR and HSI were analysed by one-way ANOVA after verifying the absence of tank effects (by inclusion of tank as a nested variable in the

model). Where differences were identified, Tukey's HSD tests were used to determine which groups differed. For further details see Section II.4.2.

Table VI.1. Primer sequences used for gene expression analysis by quantitative reverse-transcriptase PCR. Amplicon sizes (base pair) and GenBank accession numbers also are provided, accession numbers beginning with "MF" were sequenced for this study.

Transcript		**Sequence (5'-3')**	**Amplicon (bp)**	**Accession no.**
cpt1α	F:	AGGCTAGGTGAGATTCGGGT	204	KF857302.1
	R:	ACTGGACAATCCTTCGGCTG		
elovl5	F:	GCCGTACCTTTGGTGGAAGA	234	FR717358.1
	R:	GATACGGGAGAGCCGTTCTG		
fabp1	F:	CCGGCTCAAAGGTCCTCATT	223	FL486710.1
	R:	TACATGCGTTTGCTCGTCCT		
fads2	F:	TGTATGGCCTGTTTGGCTCG	152	EU439924.1
	R:	GGTGGCTTGTAACTGCATGG		
fas	F:	CGTCAAGCTCTCCATCCCTG	238	MF566098
	R:	GGTGGTGTCTAGGCAGTGTC		
igf1	F:	ACAAAGTGGACAAGGGCACA	138	AY800248.1
	R:	CCGTGTTGCCTCGACTTGA		
pparα	F:	ATGGTGACTATCCGGAGCCCG	216	AY590300
	R:	ACTTCAAGAGAGTCACCTGGTCAT		
pparα1	F:	GGATGACAGTGACCTGGCTC	211	MF566100
	R:	ATGCTCAGTGACTAGCTGCC		
scd	F:	CATCGACGGTGGAGGATGTT	155	FN868643.1
	R:	TGACGTCGATGCATTGGGAA		
srebp1	F:	CACTCCAAGTGGTGGTCCTC	189	FN677951.1
	R:	TTCTGGCTTGGACAGCAGAG		
srebp2	F:	CGTGTGACTTGGGAACCAGA	192	MF438039
	R:	AAGAGTGAGCCAGATTCGCC		
act-b	F:	GTGGCTACTCCTTCACCACC	181	AJ537421.1
	R:	CGGAACCTCTCATTGCCGAT		
ef1α	F:	GGAGTGAAGCAGCTCATCGT	199	AJ866727.1
	R:	AGCCCATCTTTACACTGCCC		
tuba1α	F:	CAAGAGGGCTTTCGTCCACT	115	FM001820.1
	R:	GTCAGTGCCCACCTCTTCAT		
rplp0	F:	CAGGCTCTGGGTATCACCAC	197	FM018449.1
	R:	AGCACCTCAGGGCTGTAAAC		

VI.3 Results

3.1 Proximate composition

Significant effects of dietary VO on the proximate composition of seabass juveniles were only observed in liver, which was associated with an increase in hepatosomatic index (HSI) from 2.3 to 3.5 in fish fed diets D6 and D1, respectively ($P < 0.001$). In liver, there was a significant increase in lipid content ranging between 27.6 and 44.9 % ($R^2 = 66.4$, $P < 0.001$), while there were significant decreases in moisture ($R^2 = 60.4$, $P < 0.001$), ash ($R^2 = 46.6$, $P = 0.0011$) and protein ($R^2 = 53.0$, $P < 0.001$). The total lipid content of the mid-intestine ranged between 3.9 and 10.6 %, but was not significantly affected by diet ($P > 0.05$). The lipid contents of whole fish and carcass were unaffected by dietary VO ($P > 0.05$), and protein and ash contents were not affected in whole body, carcass or viscera ($P > 0.05$). A summary of these data is presented in the Appendix, Table A4.

VI.3.1 Fatty acid composition of liver and mid-intestine

The fatty acid profiles of two major lipid metabolic sites, namely liver and mid-intestine, were determined. Firstly, PCA was used to reduce the dimensions of the data. In the liver, principal component 1 (PC1) and principal component 2 (PC2) explained 47.6 and 22.2 % of the variance, respectively (Figure VI.1). Fatty acids that were correlated to PC1 were 16:1n-7, 18:1n-7, 18:4n-3, 20:5n-3, 22:5n-3, 22:6n-3 and 20:4n-6, and fatty acids with negative correlations to PC1 included 18:1n-9, Iso 18:2n, 18:3n-3, 18:2n-6 and 18:3n-6. There was considerable overlap in the individual plot for hepatic fatty acid profiles and, interestingly, the diets (plotted in Figure VI.1A as supplementary to the analysis) separated from the liver profiles on both PC1 and PC2 (Figure VI.1).

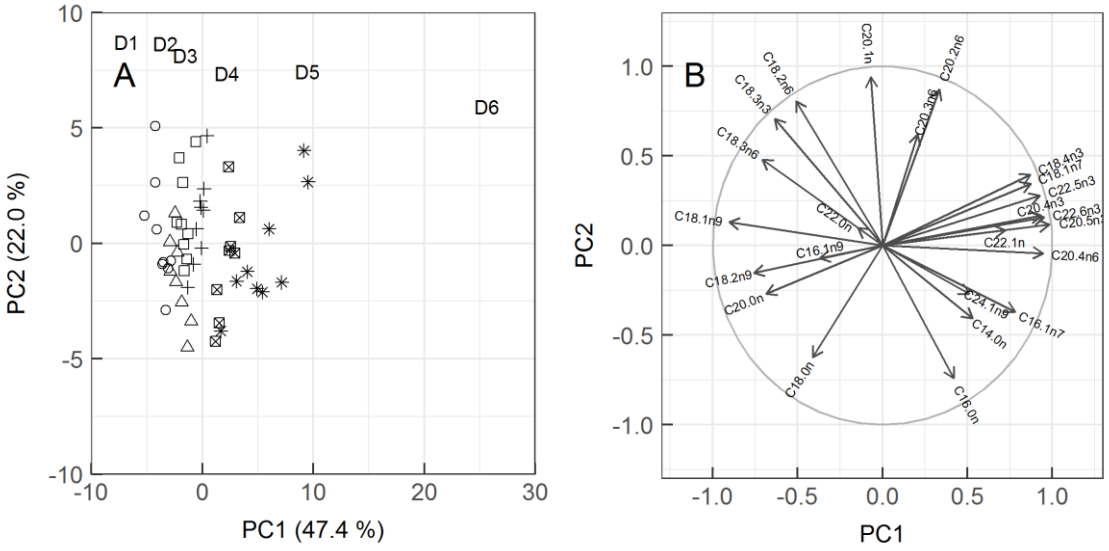

Figure VI.1. Graphical representation of principal components analysis (PCA) of fatty acid profiles from liver (n=54) of fish fed experimental diets (D1-D6). For explanation see section II.4.2.1. The profiles of the experimental diets (D1-D6) are plotted as supplementary to the analysis. Note the concentration of the points to the centre of the plot and the considerable overlap in the profiles of fish consuming D1-D4. The spread of the dietary PC scores, both on P1 and P2 indicates that the dietary fatty acid profiles were more variable than the livers'. Panel B shows all the variables (fatty acids) used to construct the PC. Note that the major fatty acids from FO are the biggest contributors to positive scores for PC1, with 18:1n-9 being the most important for negative scores. 18:3n-3 and 18:2n-6 point to the top left of the circle indicating that these fatty acids also make significant contributions to PC2. D1 = ○; D2 = ∆; D3 = □; D4 = +; D5 = ⊠; D6 = ✳.

In the mid-intestine fatty acid profiles, PC1 and PC2 accounted for 61.1 and 14.8 % of the variability, respectively. In addition to the fatty acids making strong contributions to PC1 in the liver, the saturated fatty acids (SFA) 14:0 and 16:0 were also important in forming PC1 in mid-intestine (Figure VI.2). As described in liver, negative contributions to PC1 were driven by levels of 18:1n-9, Iso 18:2n, 18:3n-6, 18:3n-3 and 18:2n-6. In mid-intestine, the dietary profiles aligned well on PC1, but were separated (negatively) on PC2 (Figure VI.2). The fatty acids making positive contributions to the formation of PC2 were 18:0, 16:1n-9, 20:2n-6 and 20:3n-6, these fatty acids having percentage levels in the tissue that would not be expected when considering simply their percentage in the experimental diets.

Absolute levels of n-3 LC-PUFA were strongly related to dietary levels in both the liver (R^2 = 87.6, $P < 0.001$) and mid-intestine (R^2 = 82.4, $P < 0.001$) (Figure VI.3). The mid-intestine showed higher proportions of n-3 LC-PUFA than liver. Absolute levels of SFA in liver were negatively influenced by dietary levels (R^2 = 36.4, $P < 0.001$) and ranged between 73.1 - 116.7 mg g^{-1}. However, in mid-intestine, SFA levels were not affected by diet ($P > 0.05$), with mean levels being 12.2 (± 3.2) mg g^{-1}. Absolute levels of MUFA in liver (R^2 = 59.2, $P < 0.001$) and mid-intestine (R^2 = 14.1, $P = 0.010$) increased with dietary supply, primarily dominated by the levels of 18:1n-9. This increase was particularly marked in liver (106.4 – 242.2 mg g^{-1}, derived from fitted values), but less so in the mid-intestine (11.5 – 15.9 mg g^{-1}) (Figure VI.4). Absolute levels of n-6 PUFA were affected by dietary level in liver (R^2 = 50.2, $P < 0.001$) and mid-intestine (R^2 = 20.8, $P = 0.0011$), with value ranges being 11.3-28.5 mg g^{-1} and 6.0-8.7 mg g^{-1}, respectively.

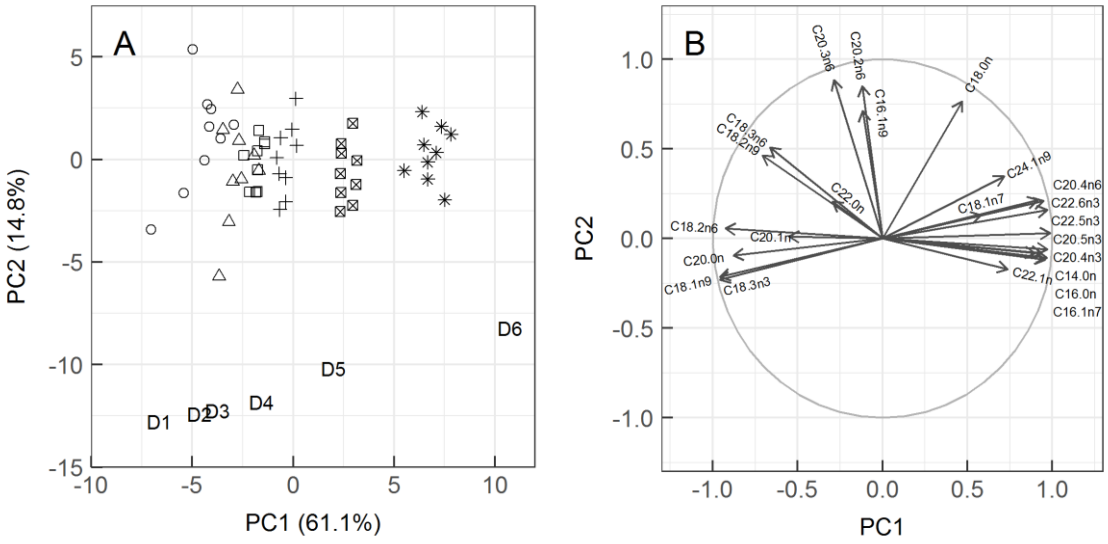

Figure VI.2. Graphical representation of principal components analysis (PCA) of fatty acid profiles from mid-intestine (n=54) samples of European seabass fed experimental diets (D1 - D6). Panel A is a biplot of the first two principal components (PC1 and PC2) of the mid-intestine fatty acid profiles. The fatty acid profiles of fish consuming diets D5 and D6 formed succinct groups to the right of the plot in Panel A. In the mid-intestine, the supplementary diets separate mainly on PC2 and not PC1 indicating that the fatty acids associated with PC2 are the variables separating the diet profiles from the mid-intestine fatty acid profiles. Note that the fatty acids important in assigning positive scores to PC2 included Iso 18:2n, 18:3n-6, 20:2n-6 and 20:3n-6. D1 = ○; D2 = ∆; D3 = □; D4 = +; D5 = ⊠; D6 = ✳.

The absolute levels of fatty acids in liver and mid-intestine are summarised in the Appendix, Tables A5 and A6, respectively. Fatty acid intermediates of LC-PUFA biosynthesis were found to be elevated in both tissues beyond their dietary concentration, particularly in the case of n-6 PUFA intermediates. The tissue content of the Δ6 desaturation product 18:3n-6 showed an opposing gradient to that of the experimental diets (liver: R^2 = 54.3, P < 0.001; mid-intestine: R^2 = 38.4, P < 0.001). With regards to 18:4n-3, the n-3 pathway equivalent to 18:3n-6, there was no significant dietary gradient in the levels of this fatty acid in liver but there was in mid-intestine (liver: R^2 = 0.0, P =

0.545; mid-intestine: R^2 = 65.1, P < 0.001). Despite not being present in the diets, another Δ6 desaturation product, Iso 18:2n, was detected in both liver and mid-intestine, and its levels were positively related to dietary VO (liver: R^2 = 56.7, P <0.001; mid-intestine: R^2 = 21.8, P <0.001). It is interesting to note that 20:3n-3 was not detected in these tissues, but the n-6 pathway equivalent (20:2n-6) was found in both tissues at proportions higher than that of the diet. The levels of 20:2n-6 (liver: R^2 = 12.9, P <0.001; mid-intestine: R^2 = 0.8, P <0.266) and 20:3n-6 (liver: R^2 = 0.6, P = 0.31; mid-intestine: R^2 = 0.9, P = 0.013) remained relatively constant across the experimental diets in both tissues. The high lipid level in the liver diluted the key LC-PUFA, (EPA, DHA and arachidonic acid, ARA 20:4n-6), yet the mid-intestine retained these fatty acids in fish fed VO diets (D1-D3).

VI.3.2 Gene expression

Candidate genes were analysed for their level of expression using relative qPCR, with the results presented as $\log_2$ expression ratios in the relevant Tables and Figures. In liver, the expression levels of only *fads2* and *igf1* were found to be influenced by dietary VO. The effects on *fads2* and *igf1* expression were only moderate (R^2 = 18.1 and 11.2, respectively), with *fads2* presenting a positive relationship and *igf1* a negative relationship to dietary VO (Table VI.2).

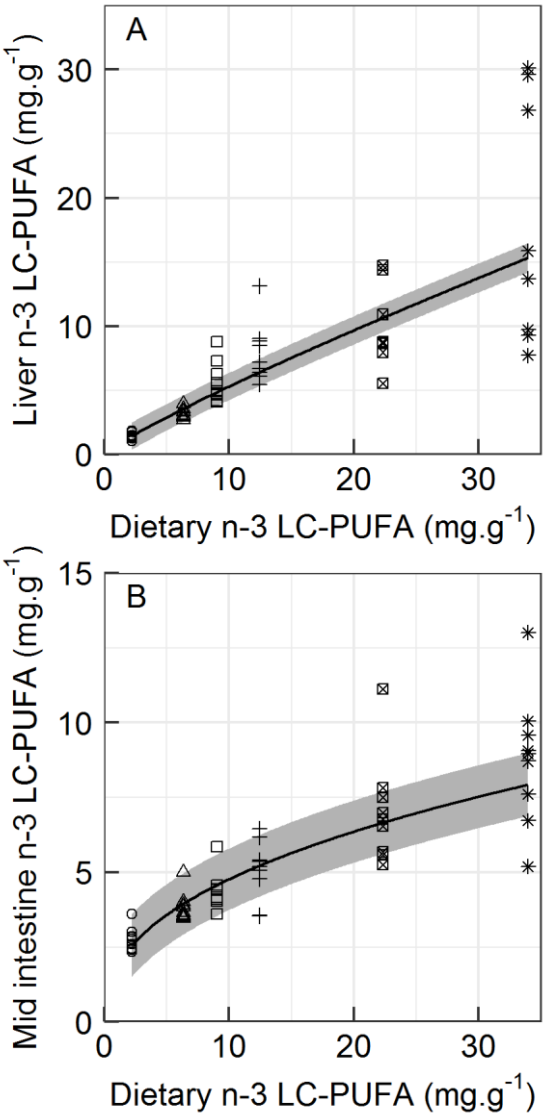

Figure VI.3. Levels of n-3 long-chain (≥ C_{20}) polyunsaturated fatty acids (LC-PUFA) in European seabass liver (A) and mid-intestine (B) against the dietary levels of n-3 LC-PUFA. The black line is a model derived by robust linear regression analysis (n=54) and the standard error of the model's fitted values shaded in grey. Natural logarithm (ln) transformation was applied to the data, but the data are plotted in their real dimensions. Liver, ln(y) = 0.870ln(x) – 0.339 and mid intestine, ln(y) = 0.416ln(x) + 0.603. Diet 1 = ○; Diet 2 = ▵; Diet 3 = □; Diet 4 = +; Diet 5 = ⊠; Diet 6 = ✳.

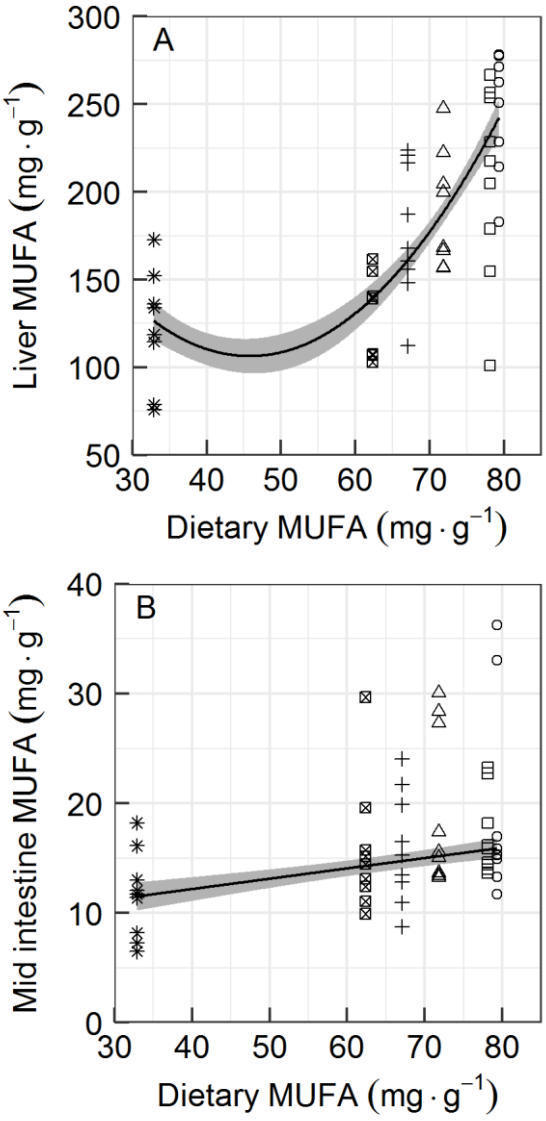

Figure VI.4. Levels of monounsaturated fatty acids (MUFA) in European seabass liver (A) and mid-intestine (B) against the dietary levels of MUFA. The solid lines are the model fits, with the standard error shaded in grey. Best fits were found to be a quadratic and linear function for the liver ($y = 359.81 – 11.069x + 0.121x^2$) and mid-intestine ($y = 8.401 + 0.094x$) data, respectively. Diet 1 = ○; Diet 2 = ▵; Diet 3 = □; Diet 4 = +; Diet 5 = ⊠; Diet 6 = ✳.

Table VI.2. Statistical treatment of liver and mid-intestine gene expression data using robust linear regression

Tissue	Gene	Intercept	Standard error	Gradient	Standard error	R^2	Pr(>F)
Liver	*pparα*	-0.529	± 0.253	0.031	± 0.024	1.688	0.201
	cpt1α	-0.455	± 0.185	0.019	± 0.016	1.368	0.237
	fabp1	-0.205	± 0.089	0.001	± 0.010	-1.949	0.935
	srebp1	0.004	± 0.224	0.009	± 0.024	-1.684	0.712
	srebp2	-0.539	± 0.331	0.014	± 0.034	-1.457	0.690
	fas	0.078	± 0.227	-0.019	± 0.024	-0.605	0.439
	scd	-0.147	± 0.244	0.010	± 0.026	-1.514	0.709
	fads2	-0.844	± 0.136	0.059	± 0.014	18.078	<0.001
	elovl5	-0.242	± 0.473	-0.041	± 0.044	0.372	0.350
	igf1	0.418	± 0.171	-0.045	± 0.016	11.215	0.008
Mid intestine	*pparα*	0.163	± 0.143	-0.028	± 0.013	9.705	0.030
	pparα1	0.282	± 0.206	0.033	± 0.019	8.822	0.094
	cpt1α	0.232	± 0.244	-0.029	± 0.022	1.550	0.200
	scd	-0.167	± 0.236	0.009	± 0.022	-1.637	0.680

In mid-intestine, most tested genes were found to be influenced by the experimental diets. However, *pparα, pparα1, cpt1α* and *scd* were not influenced by dietary VO (Table VI.2). Three of the genes, namely *srebp1*, *srebp2* and *fas*, were best fitted by quadratic functions (Figure VI.5; panels A, B and C), displayed similar relationships with dietary VO, and their expression correlated with each other (*srebp1:srebp2*: r = 0.76, P <0.001; *srebp1:fas*: r = 0.73, P <0.001; *fas.srebp2*: r = 0.71, P <0.001, Figure VI.5, panel D). Furthermore, the expression level of *srebp1* was found to be negatively correlated (r = -0.55, P <0.001) with the tissue level of n-3 LC-PUFA (Figure VI.5, panel E). The expression of *fads2* (R^2 = 53.7, P <0.001) and *elovl5* (R^2 = 17.8, P <0.001) in mid-intestine increased with dietary VO. The best fit for *fads2* was achieved with a single term quadratic function and *elovl5* a linear model (Figure VI.6; panels A and B). The expression of *fads2* and *elovl5* were correlated (r = 0.71, P < 0.001, Figure VI.6, panel C), and a weak correlation was found between *fads2* expression and two desaturation

products including Iso 18:2n ($r = 0.43$, $P = 0.0013$) and 18:3n-6 ($r = 0.47$, $P < 0.001$). No correlations between *elovl5* expression and its potential elongation products were found.

VI.4 Discussion

Replacement of FO has been an important area of research, particularly with the growth of aquaculture (Turchini et al. 2010), and increasing proportions of VO have been used in feeds of farmed fish species as pressure is placed on limited marine fisheries resources (National Research Council 2011, Glencross 2009, Tocher, Glencross 2015). In the present trial, juvenile European seabass were fed high fat (21 %), low FM (12.5 %) feeds with a dietary gradient of FO and, therefore, LC-PUFA, and we report the impact this had on tissue composition and gene expression. During the trial, all experimental groups increased in weight despite the provision of diets with very low levels of LC-PUFA (Skalli, Robin 2004), however, only fish fed 100 % VO showed statistically lower weight than fish fed the other diets. The SGR values reported in the present study are above those reported in several previous studies indicating a strong performance of the fish was attained (Montero et al. 2005, Mourente, Bell 2006, Richard, Mourente et al. 2006), although they were below a recent study, albeit with smaller seabass for a shorter time period (Torrecillas et al. 2017).

The impact of dietary VO on proximate compositions was an increase in lipid content in liver (27.6 – 44.9 %), which was accompanied by an increase in HSI. These data agree with a recent study in European seabass juveniles with a body weight of 40 – 60 g fed a similar gradient of LC-PUFA (Torrecillas et al. 2017). The dietary effect of VO on the hepatic lipid level was the same in gilthead seabream (18.6 – 31.8 %) (Houston et al. 2017), but in all dietary treatments the lipid level was approximately 10 – 13 % higher in

seabass livers. In wild European seabass, the liver's lipid content was dependent on season and ranged between 4 – 28 % (Yıldız, Şener et al. 2007), therefore the data in the present study are mostly higher than these values (except in D5 and D6). The higher range in hepatic lipid values reported in gilthead seabream (Houston et al. 2017) may suggest that seabream are more sensitive to dietary VO. However, an alternative explanation is that the dietary effects, in seabass, could be obscured by an effect of high dietary lipid.

Diet had a strong influence on the fatty acid compositions of both tissues as observed in previous studies (Mourente, Bell 2006, Montero et al. 2005). Oleic acid dominated hepatic fatty acid composition, but levels of 18:2n-6 and 18:3n-3 also increased nearly three-fold with increasing dietary VO. The PCA gives each fatty acid equal weight and, when analysed in this way, the fatty acid profiles of the liver were separated by diet, but in diets D1-D4 there was considerable overlap. The diets did not align with the liver fatty acid profiles indicating that the diet fatty acid profiles were more heterogeneous. The key LC-PUFA in the seabass livers appeared to be heavily diluted by other fatty acids in all diets (indicated by the higher spread of the diets along PC1 than the seabass liver samples). The mid-intestine samples aligned well with their respective diets (along PC1) indicating that the fatty acid composition more closely resembled the respective dietary composition. It should be reiterated that in the liver there was a total lipid effect.

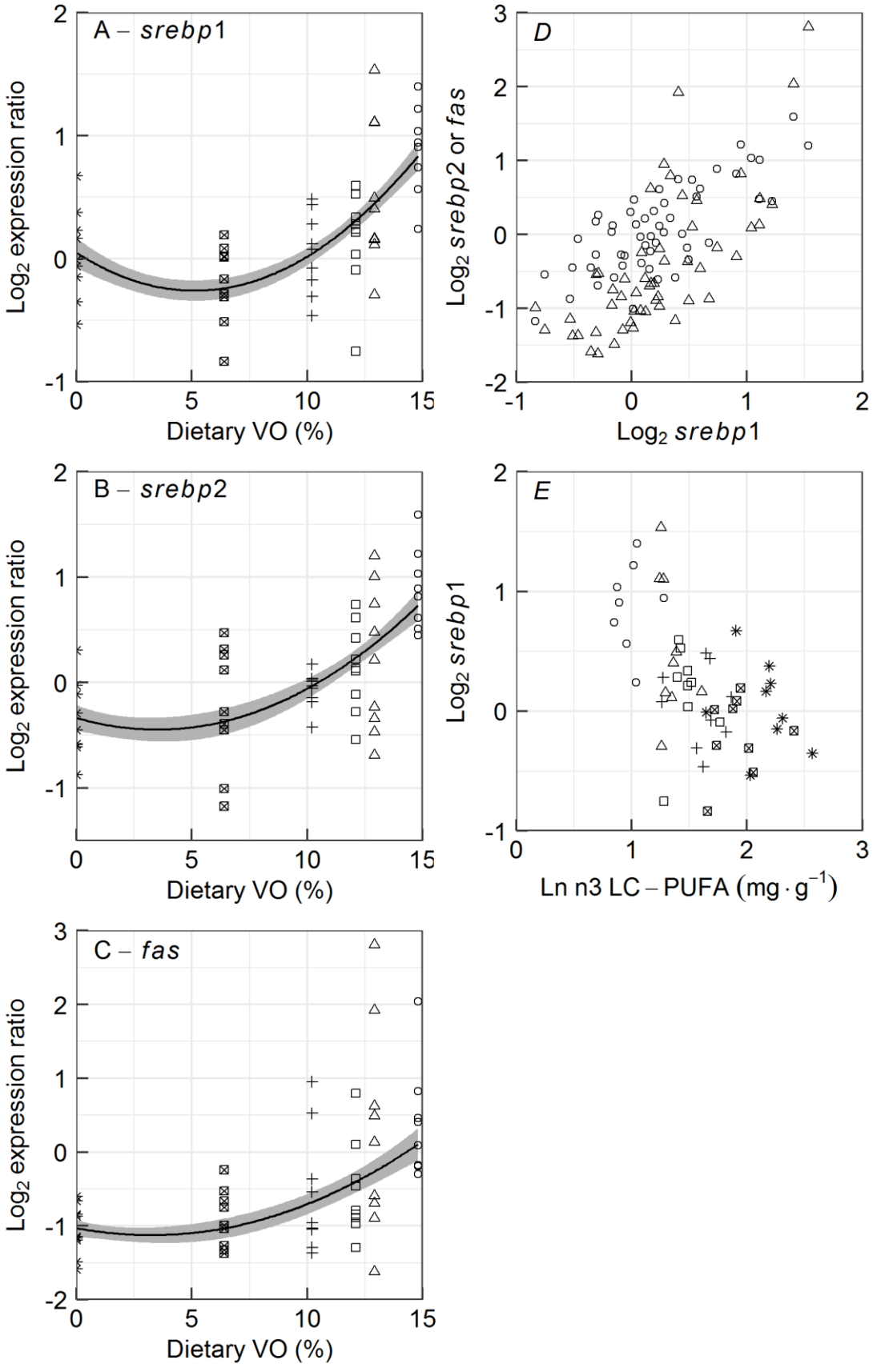
A – srebp1
Log2 expression ratio
Dietary VO (%)
B – srebp2
Log2 expression ratio
Dietary VO (%)
C – fas
Log2 expression ratio
Dietary VO (%)
D
Log2 srebp2 or fas
Log2 srebp1
E
Log2 srebp1
Ln n3 LC – PUFA (mg · g−1)

Figure VI.5. Expression of srebp1 (A), srebp2 (B) and fas (C) against dietary vegetable oil (VO) in the mid-intestine of European seabass fed experimental diet D1-D6. Data are log2 expression ratios normalised to the expression of four reference genes and then to the calibrator sample. The fitted lines are quadratic functions describing the relationship between dietary VO and log2 expression ratio (srebp1, $y = 0.0500 – 0.1205x + 0.0117x2$; srebp2, $y = -0.3341 – 0.0643x + 0.0092x2$ and fas, $y = -1.0311 – 0.0593x + 0.0092x2$). Standard errors of the model's fitted values are highlighted in grey. Panel D shows the high level of correlation between the expression of srebp1 (x axis), fas (○) and srebp2 (△). Panel E shows the correlation between srebp1 expression and the tissue content of n-3 long-chain (≥ C20) polyunsaturated fatty acids (LC-PUFA). Diet 1 = ○; Diet 2 = △; Diet 3 = □; Diet 4 = +; Diet 5 = ⊠; Diet 6 = ✳.

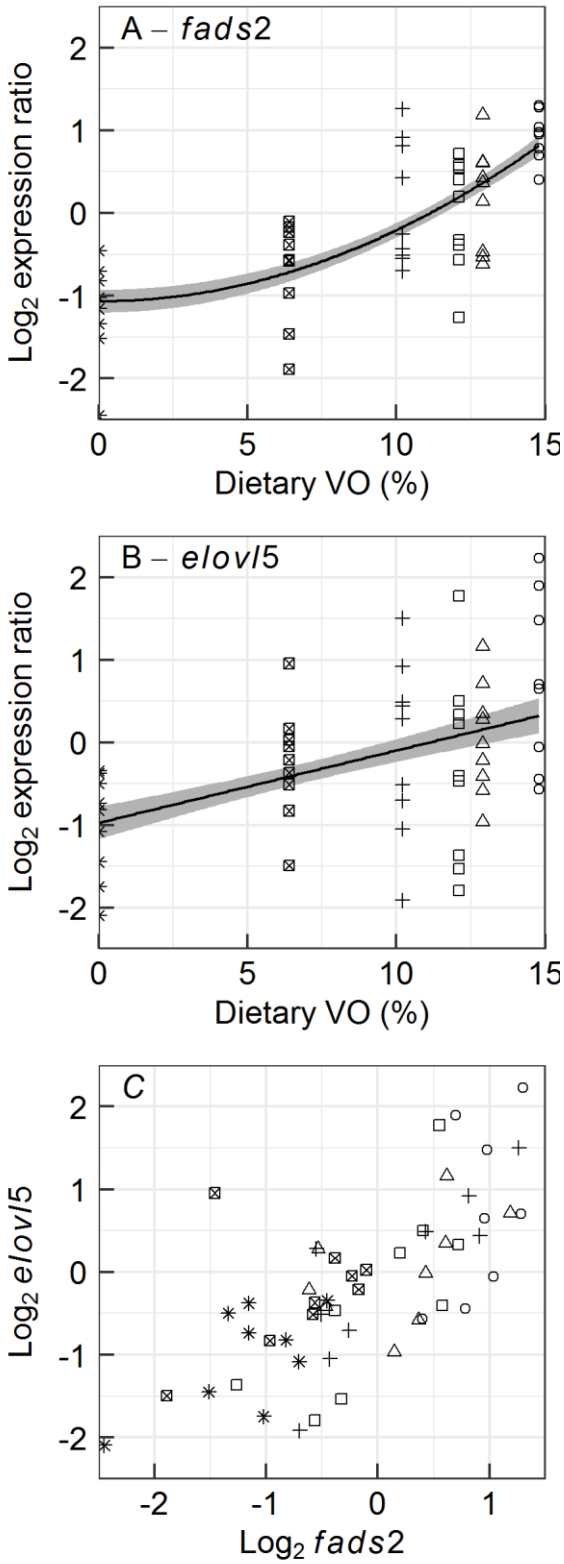

Figure VI.6. Expression of *fads2* and *elovl5* in the mid-intestine of European seabass fed experimental diets, D1-D6, plotted against dietary vegetable oil (VO). Data are $\log_2$ expression ratios normalised to the expression of four reference genes and then to the calibrator sample. The fitted line for *fads2* is a single term quadratic function ($y = -1.0686 + 0.0086x^2$) and, for *elovl5* a linear function ($y = -0.9728 + 0.0876x$). The standard errors of the model fitted values are shaded in grey. Panel C illustrates the correlation between *fads2* and *elovl5* expression. Diet 1 = ○; Diet 2 = ▵; Diet 3 = □; Diet 4 = +; Diet 5 = ⊠; Diet 6 = ✳.

There is evidence (pathway intermediates) in the fatty acid data of stimulation of the LC-PUFA biosynthesis pathway by dietary VO, particularly in n-6 PUFA (18:3n-6, 20:2n-6 and, to a lesser extent, 20:3n-6), and Iso 18:2n was also observed despite its absence in the diets, as previously observed in European seabass (Torrecillas et al. 2017). This points to increased desaturase and elongase activity in liver and mid-intestine (Mourente, Dick 2002). Interestingly, evidence of significant production of 20:3n-3 and 20:4n-3 is absent in the seabass data reported in the present study, and previously (Torrecillas et al. 2017), suggesting a possible preference in elongation activity towards n-6 substrates. Conversely, in recently reported data in European seabass, 18:4n-3 was increased more so than n-6 PUFA, however the diets in that study used linseed oil, which was richer in 18:3n-3 than 18:2n-6 (Torrecillas et al. 2017). Taken together these results suggest that Fads activity in European seabass shows no major preference towards n-3 or n-6 substrates *in vivo*. Indeed, in the related Japanese seabass (*Lateolabrax japonicus*), when dietary 18:3n-3 was supplied at a higher level than 18:2n-6, the Δ6-desaturase product 18:4n-3 appeared in liver at a higher proportion than in the diet (18:3n-6 was not reported) (Xu, Dong et al. 2014). These conclusions contrast with previous study in gilthead seabream where production of 18:4n-3, 20:3n-3 and 20:4n-3 was observed, revealing an important difference in LC-PUFA biosynthesis in these two species (Chapter V). There was no evidence in the present study for the Δ-5 desaturation necessary to form ARA or EPA, supporting previous work that European seabass is unable to biosynthesise these fatty acids in measurable quantities (Mourente et al. 2005, Almaida-Pagán, Hernández et al. 2007, Vagner, Santigosa 2011, Tocher 2003). Two studies have described the function of European seabass Fads2 (fatty acid desaturase) expressed in yeast, and both found that 18:3n-3 and 18:2n-6 were suitable substrates, but one found a

preference for 18:3n-3 (González-Rovira et al. 2009) while the other found no preference for either substrate (Santigosa et al. 2011). The *in vivo* data reported here, agree with the latter findings.

In the present study, the expression of several genes related to lipid metabolism is reported and, previously, some of these genes were also assayed in gilthead seabream using similar methodologies and experimental design (Chapter V). In both species, expression ratio data were variable and, where a dietary relationship was found, often R^2 values were low, indicating that dietary VO was not the sole source of variability in the data. In the liver of seabass, only two genes responded to the dietary gradient of VO, *igf1* and *fads2*. This was surprising as we previously observed trends in most of the genes analysed here in gilthead seabream liver (Chapter V). The moderate negative relationship between *igf1* and dietary VO suggests that VO may suppress circulating levels of insulin-like growth factor 1 (IGF1). The IGF signalling system is involved in vertebrate growth (Wood, Duan et al. 2005), and previous work in fish has usually associated this response with dietary protein or fasting, for example in barramundi (*Lates calcifer*) (Matthews, Kinhult et al. 1997) and coho salmon (*Oncorhynchus kisutch*) (Duan, Duguay et al. 1993). Use of probiotics was shown to increase *igf1* expression and this was associated with higher weight gain in European seabass juveniles (Carnevali, de Vivo et al. 2006). However, the effect of dietary VO on *igf1* expression, to our knowledge, has not been previously published. The increase in *fads2* expression, and stability in *elovl5* expression, is consistent with the fatty acid compositional data discussed above. Previous studies of *fads2* and *elovl5* found upregulation of both transcripts in response to dietary VO (C. Castro, Corraze et al. 2015), however we did not observe an increase in *elovl5* expression. The *fads2* response to diets with low levels of dietary LC-PUFA is well known (Vagner,

Santigosa 2011, Xu et al. 2014) and the weak linear relationship found in the liver in the present study suggests that the response of this transcript to diet is concentration-dependent. The moderate response of *fads2* in the liver partially agrees with results obtained for desaturation activity in European seabass hepatocytes, which showed no influence of 60% replacement of dietary FO with VO (Mourente, Dick 2002). Two further dietary patterns in gene expression were observed in seabream liver, but not in seabass liver. Firstly, lipogenic transcripts (*fas* and *srebp1*) were stimulated by dietary VO and showed very similar responses to that of *fads*2 (Chapter V). Secondly, there was evidence that dietary VO downregulated several transcripts involved in β-oxidation (*pparα, cpt1α*) (Chapter V). A previous study examining the dietary regulation of *pparα* in Japanese seabass found that dietary VO tended to reduce the expression of *ppara2* with mixed results for *pparα1* (Dong et al. 2017). These same authors, also compared their data to two other species, rainbow trout (*Oncorhynchus mykiss*) and yellow croaker (*Larimichthys crocea*), and this gene responded differently in all three species (Dong et al. 2017). This indicates that the transcriptional response of *pparα* (1 and 2) is highly species specific. Overall, the transcriptional response of the European seabass liver to dietary VO was less pronounced than that of gilthead seabream.

In the European seabass mid-intestine, several lipogenic genes were found to be responsive to diet. Some upregulation was observed in *elovl5* and *fads2*, with the pattern observed in *fads2* suggesting activation at a certain dietary threshold, but the data for *elovl5* were too variable to comment in this regard. However, there was reasonable correlation between these two transcripts suggesting that they were responding to the same stimuli. The different dietary effects on these transcripts observed between the liver and mid-intestine may be attributed to the lipid micro-environment of the different

tissues. There was high variability in the fatty acid profiles of livers of fish consuming diets D1-D4, if these transcripts are responding according to this lipid micro-environment (which is variable) then gene expression will be highly variable too. Interestingly, it had been previously reported that, albeit still at low levels, desaturation activity is higher in European seabass enterocytes (from pyloric caeca) than in hepatocytes, and the gene expression data reported here are consistent with this finding (Mourente et al. 2005).

The lipogenic gene transcript levels were also stimulated by dietary VO in seabass mid-intestine (*srebp1, srebp2* and *fas*), furthermore their expressions were highly correlated with one another. This pattern of response to diet was very similar to observations in seabream mid-intestine (Chapter V). It is interesting to note that stearoyl-CoA-desaturase (SCD) did not respond to diet, possibly suggesting that its regulation is by separate mechanisms to the *fas* transcript. Furthermore, the high correlations between *srebp1*, *srebp2* and *fas*, suggest a response to the same dietary stimuli. The response of *srebp1* and *srebp2* points to a stimulation of fatty acid and cholesterol synthesis. The experimental diets were balanced for cholesterol, so the upregulation of *srebp2* may be related to the presence of dietary phytosterols originating from VO (Leaver et al. 2008). Alternatively, *srebp2* transcription may be coupled to the stimulation of *srebp1*. Stimulation of cholesterogenic transcripts, *srebp2* targets, has been reported previously in European seabass (Geay, Ferraresso et al. 2011), in a study where the diets were not corrected for cholesterol.

In summary, the proximate composition of European seabass was unaltered by the experimental diets, although the lipid content of liver was increased. Tissue fatty acid compositions were modified towards that of the experimental diets, but there was

considerable variability observed between individual fish. The fatty acid profiles of liver were dominated by oleic acid, which diluted the PUFA. The mid-intestine on the other hand, retained higher proportions of PUFA, a likely reflection of the lower triacylglycerol level of the tissue. Parts of the LC-PUFA biosynthesis pathway were stimulated by dietary VO, however there was no evidence of the critical Δ5 desaturation necessary to produce EPA or ARA. Effects on hepatic lipid metabolism were limited to a moderate upregulation of *fads2* but, in the mid-intestine, more lipogenic transcripts were responsive to diet. No evidence was found of any dietary influence on transcripts involved with β-oxidation. Reasons for differences between gilthead seabream and European seabass are unclear but point to a higher sensitivity of gilthead seabream to dietary VO than European seabass. These findings provide data and insights that are relevant to the application of VO in the diets of European seabass.

VII. General Discussion and Conclusions

VII.1 Introduction

The present study has re-evaluated EFA requirements of gilthead seabream and European seabass, which are important cultured species in the Mediterranean Sea. Over recent years, aquafeed formulations for finfish have reduced the quantity of FO and increased the energy density of the diet by the addition of VO, and thus it was necessary to reconsider the question of EFA requirements (National Research Council 2011). Using a dose-response design, a gradient of dietary EPA and DHA was delivered to both species for four months with a mid-point sampling, at the change of pellet size (3 mm to 4.5 mm). High growth rates were achieved in both species; however, the seabass grew slower than the seabream. The analysis of biometric data in Chapters III and IV revealed the body weight dependent nature of the EPA+DHA requirement, which has not been previously documented in similar studies. Furthermore, the effect of the dietary EPA+DHA gradient on the composition of body compartments, fatty acid composition and expression of lipid metabolism genes was investigated in Chapters V and VI. The following text discusses the key findings of the project with an emphasis on comparisons between the two species and uniting the first (Chapters III and IV) and second (Chapters V and VI) halves of the thesis.

VII.2 EPA+DHA requirements, growth and FCR

The present study has shown that the current published EFA requirements for both species are too low (Kalogeropoulos et al. 1992, Skalli, Robin 2004). In both species, analysis of the growth and FCR data showed that the requirement was higher during the first period of the trials (3 mm pellet, 25 – 80 g fish) and, depending on the selected model,

a similar recommendation of 1.30 - 1.50 % EPA+DHA can be advanced for both species. Overall, for fish of > 80 g (4.5 mm pellet) the requirement can be reduced to 1.20 – 1.25 % EPA+DHA for seabream and 1.10 – 1.20 % EPA+DHA for seabass. These figures suggest that the EFA requirement appears to be falling more rapidly in the seabass, though it should be noted that this species grew slower, which may explain this difference. These requirement estimates are based on biometric data derived from the 3 mm pellet and over the whole trial. If the 4.5 mm pellet data is considered, the fish have diverged in weight during the first half of the experiment. Therefore, it is not sensible to derive a requirement from these data. The 4.5 mm pellet data was presented to demonstrate the temporal effects. The requirement estimates for the 4.5 mm are in line with the current published levels of ~ 1.0 % LC-PUFA (Kalogeropoulos et al. 1992, Coutteau et al. 1996, Skalli, Robin 2004, National Research Council 2011). In seabass, the effect on growth almost diminished completely, and the value seems to be ~ 0.8 % EPA+DHA.

In the discussion section of Chapter III, it was postulated that the form of the relationship between fish mass and the dietary requirement for EPA+DHA would likely take the form: requirement = $-a \cdot$ fish mass $(Kg)^b$, similar in nature to the relationship of dietary protein to fish mass (Lupatsch et al. 2003b). Although this thesis, does not provide sufficient data for formal analysis there are potentially three geometric mean weights that could be considered to examine this relationship. Figure VII.1 shows the available EPA+DHA requirement data (3 mm, 4.5 mm and over the trial) plotted over the geometric mean weight of the fish during these experimental feeding periods. For comparative purposes the suggested dietary protein levels, for both species have been plotted using data for gilthead seabream (Lupatsch 2003) and European seabass (Lupatsch 2005). Assuming other nutrients follow (destined for somatic growth, not maintenance) these

parallel trajectories with dietary protein, we can begin to speculate that it is more sensible to define the ratio of all nutrients with dietary protein (which is dependent on somatic growth rate) because it would seem this is constant over fish mass. The energy demand of fish (and animals) increases as they grow and causes them to consume more feed, therefore satisfying the animal's protein, and in our case EFA, requirements with lower proportions of the latter nutrients in the diet (Glencross, Bermudes 2012). In the author's mind, expressing a nutrient demand for EPA+DHA per unit energy complicates the reasoning, as the declining relative requirements are a consequence an animal's increasing demand for energy. The data presented in this thesis, suggest that nutrients supplied with the metabolic fate of somatic growth are supplied in a similar ratio with one another across animal weights, therefore, why express them per unit energy? Why not define their ratios for each species and dilute this basal diet with components (lipid/more protein) to satisfy the rising energy demand? This line of reasoning reverses the current process of diet formulation, where feeds are formulated after the energy content has been determined for an animal size (pellet size). To provide an alternative phrasing, give the animal the building blocks it needs to build more animal and, as it grows boost this base formulation with additional oil and, or protein to supply rising maintenance requirements.

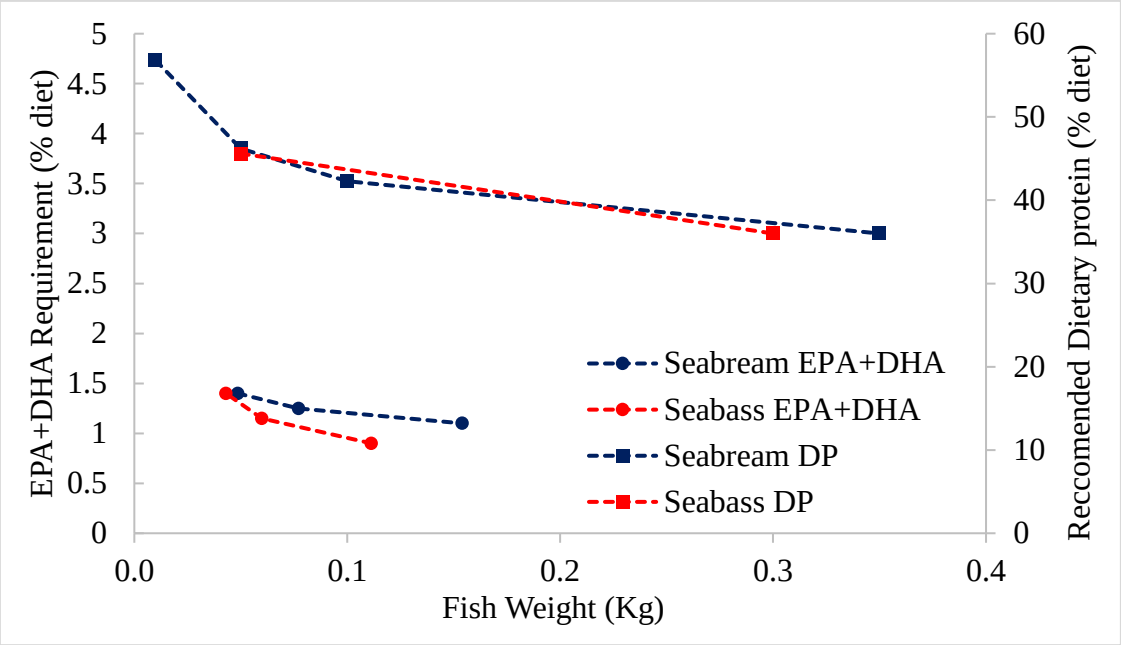

Figure VII.1. The requirements for EPA+DHA over geometric weight of gilthead seabream and European seabass. Dietary protein (DP) recommendations for these species a plotted for comparison. Note there appears to be a consistent ratio (parallel relationships) between dietary protein and the EPA+DHA requirements.

The FCR data revealed important differences between the two species. Firstly, the effect of the diets on FCR in seabream were stronger than in seabass. In fact, only the seabass FCR data over the whole trial could be used to suggest a requirement in terms of FCR and this was 1.04 % EPA+DHA. There was an effect on FCR for the 3 mm pellet but it could only be modelled with a straight line. The seabream FCR data displayed an unusual shape that could only be accommodated by the FPL model. The cause of the unusual shape of the FCR data is the lower than expected FCR of fish consuming D1 and this is possibly explained by the slower growth of this group meaning a lower level of feed intake. For the 3 mm pellet, the EFA requirement derived from FCR data was 2.0 % EPA+DHA, and this fell to 1.5 % EPA+DHA over the whole trial. Therefore, EFA are much more important in terms of determining feed efficiency in seabream than seabass. In both species the dietary impact on FCR diminished as the fish increased in weight, but

the effect of dietary EPA+DHA was stronger in gilthead seabream. It is widely accepted that larval diets should contain high levels of EFA, especially DHA, to ensure good survival, growth and vitality (Izquierdo 1996, Sargent et al. 1997, Tocher 2010). The present findings extend this view to juvenile fish and EFA are most important in rapidly growing larval and juvenile stages. The practical implications of this are that as the fish grows the FO content of the diet can be reduced to preserve the use of this expensive and limited raw material (Shepherd, Jackson 2013, Tacon, Metian 2015).

There are two important caveats that should be acknowledged. Firstly, the experimental design treated EPA and DHA as though they represent a single nutrient. This was the result of selecting commercial oils to formulate the experimental diets. The DHA:EPA ratio is also known to be important to optimise fish performance (Rainuzzo et al. 1997, Sargent et al. 1999). In the dietary design the DHA:EPA ratio was 0.87 – 0.69, D1 having the highest ratio and D6 the lowest. Studies into the ideal DHA:EPA ratio for juvenile seabream indicate that it is about 0.5 (Ibeas et al. 1996), yet in larvae the ideal ratio is 2.0 (Rodriguez et al. 1998). Given the present findings it is reasonable to conclude that this ratio also declines as larvae/juveniles increase in weight but the herein experimental design does not allow comment on the ideal DHA:EPA. It should be acknowledged that a higher ratio may have reduced the requirement estimates, particularly for the 3 mm pellet size. A South American FO was used, so the DHA:EPA ratios in these trials represented the most widely used commodity in aquafeeds.

Secondly, ARA was not controlled in this experiment and exhibited a gradient in parallel to the other LC-PUFA, but at lower levels (D1 – D6: 0.07 – 1.01 g Kg^{-1} ARA per diet). ARA has been shown to be important in stress resistance and survivability in larval

stages of gilthead seabream (Bessonart, Izquierdo et al. 1999, Koven, Barr et al. 2001) and European seabass (Atalah, Hernández-Cruz et al. 2011). A recent study in juvenile seabass (14 – 45 g) suggested a requirement, in terms of growth, for ARA between 0.1 – 0.2 g Kg^{-1} dry diet (Torrecillas, Román et al. 2017). Therefore, D1 and D2 in the present study may have also been deficient in ARA. Therefore, it must be conceded that dietary ARA may have had an influence in the data attained for both trials (Fountoulaki, Alexis et al. 2003). The question of juvenile ARA requirements warrants investigation with a dose response experiment in both species.

The cost-benefit analysis for gilthead seabream was conducted to explore the economic implications of the experimental diets in terms of economic gain over feed (Chapter III). D4 (1.1 % EPA + DHA) was the highest performing diet in this regard. Considering the other conclusions of the chapter, it is reasonable to conclude that the best feeding strategy in terms of economic gain would be achieved by feeding EFA at the requirement levels of the fish then reducing EFA to D4 levels when the requirement had fallen to the level of ~1.0 % EPA+DHA. Indeed, the growth trajectory of D4 will have been overestimated because these fish were fed a diet that was deficient in EFA for the 3 mm pellet but at some fish weight during the latter part of the experiment (4.5 mm pellet) this diet had sufficient EFA to support growth at the level observed in D5 and D6 (these fish had growth rates that accelerated during the trial). However, this analysis was conducted in terms of laboratory-reared fish at their thermal optimum, so is highly theoretical. The cost-benefit analysis for European seabass was not informative so it was not presented due to the small dietary effect on FCR.

An important law in nutritional studies is the Liebig principal, which states that a plant or animal's performance will be limited by the first deficient nutrient. In animal production, performance is related not only to nutrition but also the rearing environment and genetic characteristics of the population. As modern intensive aquaculture is still younger than terrestrial livestock farming, the potential gains available in fish breeding are as high as 12% per generation (Gjedrem, Robinson 2014). Future improvements in stock selection will need to be balanced with new definitions of nutrient requirements as any gains in animal performance will have nutritional consequences. Nutrient requirements are dynamic and interrelated and need to be evaluated periodically in line with cross-disciplinary developments that improve culture performance.

VII.3 Composition of the fish and key tissues

In Chapters V and VI, the composition of body compartments and fatty acid profiles of the liver and mid-intestine of the fish after consuming the experimental diets for four months was presented. Generally, the proximate composition of the whole body and eviscerated carcass was unaffected by the diet in both species. The liver lipid level increased in both species with the addition of dietary VO, from 18.6 to 31.8 % in seabream and 27.6 to 44.9 % in seabass. Visceral lipid deposition declined with increasing VO in seabream from 53.5 to 45.2 %, although this relationship was not as strong as in the liver. There was no effect on visceral lipid levels in seabass. When seabream and seabass are compared, level of lipid in liver and viscera was markedly higher across all dietary groups in seabass. High liver lipid (or steatosis) is a known characteristic in marine fish fed diets deficient in EFA (Spisni, Tugnoli et al. 1998) and high energy diets (Vergara et al. 1999). Therefore, the levels of dietary lipid need also to be considered in the context of the high

energy content of the diets. In seabream, but not seabass, the lipid level of the mid-intestine was also influenced by dietary VO. In these trials, dietary VO appeared to have more of an impact on overall lipid deposition in seabream than seabass.

The fatty acid composition of the liver and mid-intestine was modified toward the composition of the diets in both species, supporting previous findings (Kalogeropoulos et al. 1992, Torrecillas et al. 2017, Wassef, Wahbi et al. 2012, Montero et al. 2005). The main fatty acids that increased with dietary VO, in decreasing order, were: oleic acid, LOA and ALA. This is the expected order from the experimental diets. The SFA were balanced in the dietary design and therefore increased where an effect on the total lipid content of the tissue was found, mainly the liver.

In the liver of both species, there was evidence for the stimulation of the LC-PUFA biosynthesis pathways. In liver of seabream, there was significant production of n-3 (18:4n-3; 20:3n-3; 20:4n-3 and 22:5n-3) and n-6 (18:3n-6; 20:2n-6 and 20:3n-6) intermediate fatty acids. In liver of seabass, only 18:4n-3 appeared at a higher level than in D1 in fish that consumed this diet, interestingly 20:3n-3 was not detected, and n-6 (18:3n-6; 20:2n-6 and 20:3n-6) intermediates were found to be produced. When compared, the levels of 18:3n-6 production by seabream and seabass are similar, but elongation products only occurred at a very low level in the seabass liver. Therefore, the response of the LC-PUFA biosynthesis pathway to diets with low LC-PUFA was more pronounced in seabream, especially when elongation activity is considered. Furthermore, the n-6 LC-PUFA biosynthetic pathway was more apparent in seabass. Recently, a study with linseed oil found that n-3 intermediates were produced in juvenile seabass livers, but linseed oil is richer in 18:3n-3 than 18:2n-6 (Torrecillas et al. 2017). Taken together, LC-

PUFA synthesis in seabass showed no preference for n-3 or n-6 substrates. There was little evidence of activity of LC-PUFA biosynthesis in the mid-intestine of seabream, with levels of most fatty acid intermediates explained by their respective levels in the diets. The seabass mid-intestine fatty acid profiles indicated that production of LC-PUFA intermediates was similar both qualitatively and quantitatively to the seabass liver. In both species, Iso 18:2n was detected in both tissues despite the absence of this fatty acid in the diet, this being a Δ6 desaturase product of 18:1n-9. However, its elongation product, Iso 20:2n was only observed in seabream. Whence important differences exist between the two species and the two tissues in the operation of this pathway. Supporting previous work, there was no evidence that either species can perform the critical Δ5 desaturation required to produce EPA or ARA (Tocher 2010). The levels of LC-PUFA were negatively affected by dietary VO in both tissues (liver and mid-intestine) and species. The higher accumulation of other fatty acids (16:0 and 18:1n-9) in livers, diluted these important fatty acids. The mid-intestine, however, retained LC-PUFA at higher proportions than would be expected from their proportions in the diet.

VII.4 Lipid metabolic gene expression

A range of genes involved in lipid metabolism were assayed in samples of the liver and mid-intestine taken at the end of the trials and presented in Chapters V and VI. The data were variable indicating that where dietary effects were observed, diet was not the sole source of variation and other factors not controlled by the experiment influenced the level of expression, in agreement with previous gene expression studies (Morais et al. 2011, Jordal et al. 2005, Panserat, Hortopan et al. 2009). The regression design employed in this study allowed individual variability to be appreciated while describing the dietary

effects. Nevertheless, some patterns in gene expression were observed and it was possible in some genes to correlate this to the fatty acid micro-environment of the tissue. The biological processes selected for study were β-oxidation, lipogenesis, intra-cellular transport and LC-PUFA biosynthesis. Evidence for dietary modulation of β-oxidation was only found in the seabream liver, where a subtle downregulation in *pparα, cpt1α* and *fabp1* was observed in response to dietary VO. Downregulation of *pparα* in response to dietary VO has been observed in Atlantic salmon (Morais et al. 2011), but results in other species (rainbow trout, Japanese seabass and large yellow croaker) have been mixed (Dong et al. 2017). Indeed, no effect of diet was found in seabass liver and mid-intestine samples. If downregulation of transcripts involved in β-oxidation in liver equates to lower catabolism of fatty acids, this may be a contributing factor toward the increased fat deposition observed in the liver.

In the seabream liver and mid-intestine of both species, VO led to an upregulation of transcripts involved with lipogenesis. Therefore, lipogenic processes may be increased in the liver and mid-intestine in seabream and, the mid-intestine in seabass. It has been advanced in fish that the presence of dietary DHA downregulates lipogenic processes in fish (Leaver et al. 2008, Thomassen, Rein et al. 2012). In seabream liver, the effect was only measurable in D1 and D2, two diets that were severely deficient in EFA, the nonlinear nature of the relationship with dietary VO suggests that the upregulation of *srebp1* and *fas* expression occurs at a dietary threshold. However, the high variability prevents speculation as to what this level may be. The dietary pattern of *fads2* and *fas* expression were remarkably correlated to *srebp1* expression and, in turn, *srebp1* expression was negatively correlated to the tissue levels of n-3 LC-PUFA in the liver of seabream and the mid-intestine of seabass. On its own, this does not imply causation.

However lines of evidence in salmon head kidney cell line (SHK-1) indicate that *fads2* and *fas* are targets of Srebp1, but interestingly, *elovl5* was not, indicating that fish *elovl5* may be regulated by different mechanisms compared to mammals (Minghetti et al. 2011, Carmona-Antoñanzas et al. 2014). Interestingly, at least in mammals, Srebp1 is known to regulate its own transcription, therefore alteration of the expression of *srebp1* suggests its activation (Daemen et al. 2013). An increase in the synthesis of *de novo* fatty acids would also be expected to contribute to increased lipid content of the liver, and perhaps the lipid content of the mid-intestine that was found in seabream. Upregulation of the *srebp2* transcript with dietary VO was limited to the mid-intestine in both species. Dietary VO has been shown to affect cholesterol metabolism in Atlantic salmon (Leaver et al. 2008) and European seabass liver (Geay et al. 2011). Cholesterol formed part of the micronutrient supplement in the dietary design so that it was balanced across the experimental diets. The upregulation of *srebp2* may therefore be the result of phytosterols introduced to the diets with dietary VO. In mammals, phytosterols are not absorbed by the gut and inhibit cholesterol uptake (Ostlund Jr 2004), this effect has not been demonstrated in fish, but may explain the upregulation of *srebp2* observed here. The absence of a dietary effect in the liver suggest that the overall cholesterol status was not strongly affected by the diets.

Two genes involved in LC-PUFA biosynthesis, *fads2* and *elovl5*, were assayed in the liver and mid-intestine. *Fads2* was upregulated in both liver and mid-intestine of both species by dietary VO. In seabream the strongest response was found in the liver and a moderate increase observed in the intestine. In seabass this was reversed and a stronger response of *fads2* was observed in mid-intestine, with a more modest effect observed in liver. Only a subtle positive relationship was found with *elovl5* and dietary VO in the

seabass mid-intestine. The *fads2* data are in line with the statement made in the previous section that LC-PUFA biosynthesis was more strongly affected in the seabream liver. Furthermore, correlations were able to be made between the level of *fads2* expression and the level of Fads2 products (e.g. Iso 18:2n and 18:3n-6) in the liver of seabream, but only in the mid-intestine in seabass. However, despite the absence of a dietary effect on *elovl5*, elongation products were still found in the fatty acid composition, suggesting an underlying activity of this protein with little dietary influence.

Chapters III and IV demonstrated that EFA requirements are higher in smaller fish and decline as the fish grows. Chapters V and VI related dietary compositional changes to the transcriptional response in liver and mid-intestine. Therefore, it must be conceded that the gene expression samples taken in these trials were taken at the point when the requirement for EFA was the lowest. For this reason, it is difficult to use the gene expression data to complement the requirement estimates for EFA. Overall, the lipid metabolic genes seemed to be more responsive to diet in the seabream liver, but the mid-intestine was more responsive to diet in the seabass. *Srebp1*, *fas* and *fads2* responded in similar ways and this response was negatively correlated with the tissue content of LC-PUFA.

VII.5 Key conclusions

The requirement for EPA+DHA declines as fish weight increases.

Based on growth, the current published EFA requirements are too low for both species to attain optimum or maximum performance using modern diet formulations.

At a size range of 24 – 80 g, the period when a 3 mm pellet is consumed, the optimum requirement for growth is 1.3 – 1.5 % EPA+DHA, for both species.

Beyond ~80 g (4.5 mm pellet) seabream require 1.20 – 1.25 % EPA+DHA, whereas seabass require 1.10 – 1.20 % EPA+DHA.

Seabream of 24 – 80 g require 2.0 % EPA+DHA to minimise FCR. Above 80 g this falls to 1.5 % EPA+DHA.

Taking 95% of the asymptote is not a satisfactory method of deriving a requirement level, it is sensitive to parameter selection (WG or DGI).

The effect on FCR in seabass diminishes quickly, but is present in P1, but no breakpoint was found. The OV estimate of EPA+DHA requirements for FCR was lower than the estimates for growth, so the growth data should be used to inform nutrient specification, not FCR.

The nutrient response curve for EFA changes shape as the fish grow, particularly in seabream, changing from a curve to a more angular relationship as growth accelerates in fish that satisfy their requirement over time feeding. This required different functions to fit the data.

Therefore, no requirement should be viewed as a static dietary percentage, but rather as an amount that supports a certain level of animal performance at a certain animal mass.

Proximate composition of whole fish and eviscerated carcass were unaffected by diet in both species.

Lipid content in liver was increased in both species, with seabream also exhibiting lower visceral lipid deposition. Lipid level of the mid-intestine was also elevated in seabream but not seabass. This must be interpreted in the context of the high energy diets.

Fatty acids in both liver and mid-intestine were modified towards dietary profiles.

The stimulation of LC-PUFA biosynthesis modulated fatty acid profiles. The seabream showed preference for n-3 substrates, the seabass did not exhibit this preference. The mid-intestine in seabream appeared to be less active in this regard, but both tissues in seabass were sites of desaturation and elongation.

The transcriptional machinery of lipid metabolism was more responsive to dietary VO in seabream, especially in liver, where a subtle downregulation of β-oxidation and stimulation of lipogenesis and LC-PUFA biosynthesis was observed. The mid-intestine of seabass was more responsive to dietary VO than the liver.

The transcriptional response could be correlated to the fatty acid profile of the tissue; expression of *srebp1* was negatively correlated with tissue LC-PUFA, and *fads2* was positively correlated to tissue 18:3n-6 and Iso 18:2n.

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Appendix

Table A1. Proximate composition of body compartments in *S. aurata* presented as wet weight (g Kg^{-1}), means and standard deviations (N=3). Superscript letters indicate Tukey's HSD[1] after ANOVA ($P < 0.05$).

Whole Bodies	**Initial**	**D1**		**D2**		**D3**		**D4**		**D5**		**D6**		**Pr(>F)**
Protein wet	148.7	154.8	± 9.7	159.1	± 5.3	157.0	± 7.4	157.1	± 4	155.3	± 2.6	164.1	± 9.4	0.782
Lipid	103.5	181.2	± 11.7	172.7	± 9.3	188.4	± 15.1	176.1	± 6.5	164.6	± 6.4	166.9	± 16.4	0.371
Ash wet	27.4	14.3	± 1.6	16.3	± 0.7	16.6	± 1.8	15.5	± 0.7	16.3	± 0.5	17.3	± 1.3	0.264
Moisture	692.8	627.3	± 9.2	628.7	± 2.3	623.2	± 0.9	631.4	± 15.2	633.9	± 2.2	637.4	± 7.1	0.562
Carcass														
Protein wet		179.2	± 11.7	173.7	± 2.2	171.2	± 1.7	182.6	± 10.7	172.7	± 3.1	160.7	± 6.5	0.123
Lipid		162.7	± 7.3 a	149.7	± 9.1 ab	151.8	± 9.6 ab	129.5	± 8.8 b	154.6	± 10 ab	139.9	± 10.1 ab	0.044
Ash wet		32.1	± 2.9	27.3	± 4.7	26.2	± 4.3	31.2	± 4.4	29.5	± 4.5	36.1	± 6.1	0.361
Moisture		612.9	± 15.2	639.4	± 2.5	625.3	± 10.6	630.7	± 2.6	623.8	± 21.8	622.3	± 7.1	0.425
Viscera														
Protein wet		69.2	± 8.9	62.9	± 3.3	64.4	± 6.2	51.4	± 10.3	62.8	± 11.1	63.1	± 4.9	0.419
Lipid		448.8	± 23.2	450.9	± 14.4	452.6	± 36	502.2	± 59.9	514.5	± 45.6	525.1	± 47.8	0.261
Ash wet		6.7	± 0.5	7.3	± 1.1	8.9	± 0.1	6.6	± 0.7	8	± 1.7	6.4	± 0.4	0.114
Moisture		475.3	± 15.9	478.9	± 16.3	474.1	± 32.6	439.8	± 50.4	414.6	± 33.6	405.5	± 43.4	0.186
Liver														
Protein wet		101.4	± 13.4	95.3	± 2.7	103.3	± 5.6	107.0	± 11.1	99.3	± 1.6	101.9	± 5.9	0.776
Lipid		323.7	± 6.3 a	317.8	± 31.8 ab	311.0	± 22.8 ab	233.1	± 30.7 bc	220.1	± 22.8 c	186.2	± 31.8 c	< 0.001
Ash wet		6.4	± 1	6.1	± 1.9	6.9	± 0.8	7.6	± 0.9	7.8	± 0.8	8.2	± 1.7	0.517
Moisture		473.2	± 7.2 d	486.5	± 23.1 bcd	498.7	± 19.8 bd	550.9	± 16.6 abc	554.6	± 24.3 ab	568.8	± 24.1 a	0.0014

[1] Honest significant differences

Table A2. Lipid and fatty acids in the liver of *S. aurata* as mg g^{-1} fatty acid per tissue. Data are summarised as mean and standard deviation (N=3·3). Superscripts indicate Tukey's HSD[1] after ANOVA (P < 0.05).

	D1			D2			D3			D4			D5			D6			Pr(>F)
Total lipid																			
Lipid (%)	29.9	± 0 6	a	26.8	± 4.5	a	25.4	± 4.7	a	29.9	± 5.1	a	24.4	± 6.5	a	13.1	± 4.4	b	< 0.001
Fatty acid																			
14:0	2.8	± 0.7	b	2.6	± 0.6	b	2.8	± 0.7	b	3.8	± 0.8	ab	4.5	± 1.3	b	3.8	± 1.5	ab	0.001
16:0	38.6	± 9.1	a	34.5	± 6.5	a	34.2	± 7.4	a	40.1	± 5.7	a	33.6	± 9.6	a	18.4	± 6.4	b	< 0.001
18:0	12.7	± 2.8	a	11.5	± 2.3	a	10.7	± 1.9	a	12.5	± 1.6	a	10.5	± 3.6	ab	6.8	± 2.6	b	< 0.001
20:0	0.4	± 0.1	a	0.4	± 0.1	a	0.4	± 0.1	a	0.5	± 0.1	a	0.4	± 0.1	a	0.2	± 0.1	b	< 0.001
22:0	0.2	± 0.1	ab	0.3	± 0.1	a	0.3	± 0.0	a	0.3	± 0.0	a	0.2	± 0.1	a	0.1	± 0.0	b	< 0.001
16:1n-9	2.0	± 0.6	a	1.4	± 0.3	bc	1.4	± 0.3	bc	1.5	± 0.3	ab	1.0	± 0.3	cd	0.5	± 0.2	d	< 0.001
16:1n-7	4.3	± 1.2	bc	4.2	± 0.9	c	4.6	± 1.2	bc	6.5	± 1.2	ab	7.0	± 2.0	a	5.7	± 2.2	abc	< 0.001
18:1n-9	109.0	± 25.6	a	90.9	± 18.6	ab	87.6	± 19.7	ab	96.7	± 15.5	a	63.4	± 19.1	b	20.9	± 9.2	a	< 0.001
18:1n-7	8.1	± 1.9	a	7.3	± 1.5	a	7.1	± 1.7	ab	8.9	± 1.2	a	7.4	± 2.3	a	4.5	± 1.8	b	< 0.001
20:1n	3.4	± 1.0	a	3.6	± 0.9	a	3.5	± 1.0	a	4.6	± 1.3	a	3.1	± 1.2	a	1.5	± 0.7	b	< 0.001
22:1n	1.0	± 0.4	ab	1.0	± 0.3	ab	1.0	± 0.2	ab	1.4	± 0.4	a	1.1	± 0.4	ab	0.7	± 0.2	b	0.003
24:1n-9	1.0	± 0.2		0.9	± 0.2		0.9	± 0.2		1.0	± 0.4		0.9	± 0.3		0.6	± 0.2		0.061
Iso 18:2n	2.7	± 1.0	a	1.5	± 0.6	b	1.2	± 0.4	b	0.9	± 0.3	bc	0.4	± 0.2	cd	0.2	± 0.1	c	< 0.001
18:2n-6	33.4	± 8.4	a	29.9	± 7.6	a	29.9	± 7.5	a	33.7	± 8.1	a	23.9	± 6.6	a	9.7	± 3.7	b	< 0.001
18:3n-6	2.4	± 1.0	a	1.3	± 0.7	b	1.2	± 0.5	bc	0.8	± 0.3	bcd	0.4	± 0.2	cd	0.2	± 0.1	d	< 0.001
Iso 20:2n	2.2	± 0.7	a	1.6	± 0.4	b	1.4	± 0.4	b	1.3	± 0.4	b	0.7	± 0.2	cd	0.2	± 0.1	c	< 0.001
20:2n-6	1.3	± 0.4	ab	1.3	± 0.4	ab	1.3	± 0.4	ab	1.6	± 0.5	a	0.9	± 0.3	bc	0.4	± 0.2	c	< 0.001
20:3n-6	1.2	± 0.4	a	0.9	± 0.3	a	0.9	± 0.3	a	0.8	± 0.2	a	0.5	± 0.2	b	0.2	± 0.1	b	< 0.001
20:4n-6	0.1	± 0.1	d	0.3	± 0.0	c	0.4	± 0.0	c	0.6	± 0.1	b	0.7	± 0.2	ab	0.8	± 0.2	a	< 0.001
18:3n-3	6.7	± 1.9	ab	6.3	± 1.8	ab	6.2	± 1.7	ab	7.3	± 2.1	a	4.8	± 1.3	b	1.3	± 0.5	c	< 0.001
18:4n-3	0.7	± 0.2	ab	0.6	± 0.2	b	0.6	± 0.2	b	0.8	± 0.3	ab	1.1	± 0.3	a	1.1	± 0.5	a	0.0019
20:3n-3	0.4	± 0.1	ab	0.4	± 0.1	ab	0.4	± 0.1	ab	0.6	± 0.2	a	0.3	± 0.1	b	0.1	± 0.1	c	< 0.001
20:4n-3	0.5	± 0.2	c	0.5	± 0.1	c	0.6	± 0.2	bc	1.0	± 0.2	a	1.0	± 0.3	a	0.9	± 0.4	ab	< 0.001
20:5n-3	1.2	± 0.5	c	2.2	± 0.4	c	2.8	± 0.5	bc	4.9	± 1.1	b	7.1	± 2.0	a	7.5	± 2.7	a	< 0.001
22:5n-3	0.7	± 0.3	b	1.3	± 0.4	b	1.7	± 0.4	b	3.5	± 1.0	a	4.5	± 1.4	a	4.7	± 2.0	a	< 0.001
22:6n-3	1.6	± 0.5	c	2.7	± 0.6	c	3.2	± 0.6	c	6.1	± 1.3	b	8.4	± 2.3	ab	9.6	± 3.1	a	< 0.001

[1] Honest significant differences.

[2] Sum of 20C or 22C monounsaturates (n-7 + n-9 +n-11).

Table A3. Lipid content and fatty acids in the mid-intestine of *S. aurata* presented as mg g^{-1} fatty acid per tissue. Data are summarised as mean and standard deviation (N=3·3). Superscripts indicate Tukey's HSD[1] after ANOVA (P < 0.05).

Diet	D1			D2			D3			D4			D5			D6			Pr(>F)
Total lipid																			
Lipid (%)	16.4	± 3.6	a	13.8	± 2.2	ab	11.1	± 3.5	bc	10.5	± 1.9	bc	11.1	± 2.4	bc	9.5	± 1.5	c	< 0.001
Fatty acid																			
14:0	0.9	± 0.2	b	1.3	± 0.3	b	1.2	± 0.5	b	1.5	± 0.3	b	2.6	± 0.8	a	3.3	± 0.8	a	< 0.001
16:0	19.4	± 3.9	a	17.8	± 3.4	ab	15.7	± 4.5	ab	14.1	± 2	b	15.5	± 3.6	ab	13.6	± 2.5	b	0.01
18:0	5.7	± 1.2	a	5.4	± 0.8	ab	4.3	± 1.1	bc	4.1	± 0.6	c	4.1	± 0.8	bc	3.9	± 0.5	c	< 0.001
20:0	0.4	± 0.1	a	0.4	± 0.1	ab	0.3	± 0.1	abc	0.3	± 0	cd	0.3	± 0.1	bcd	0.2	± 0.0	d	< 0.001
22:0	0.2	± 0.1	a	0.2	± 0.1	ab	0.2	± 0.1	abc	0.1	± 0	bc	0.1	± 0.0	bc	0.1	± 0.0	c	< 0.001
16:1n-9	0.5	± 0.1	a	0.4	± 0.1	ab	0.3	± 0.1	bc	0.3	± 0.1	bc	0.3	± 0.1	bc	0.2	± 0.1	c	< 0.001
16:1n-7	1.2	± 0.3	b	1.6	± 0.4	b	1.5	± 0.6	b	1.9	± 0.3	b	3.1	± 1.0	a	3.5	± 0.9	a	< 0.001
18:1n-9	63.4	± 17.3	a	45.3	± 10.8	b	32.3	± 14.7	bc	28.3	± 7.2	c	23.5	± 6.5	c	9.1	± 2.0	d	< 0.001
18:1n-7	4.3	± 1.1	a	3.6	± 0.8	ab	2.7	± 1.1	bc	2.5	± 0.5	bc	2.8	± 0.7	bc	2.1	± 0.4	c	< 0.001
20:1n[2]	1.3	± 0.4	a	1.1	± 0.2	ab	0.9	± 0.4	ab	0.8	± 0.1	b	1.0	± 0.3	ab	0.9	± 0.2	b	0.007
22:1n[2]	0.4	± 0.1	ab	0.4	± 0.2	ab	0.3	± 0.2	b	0.3	± 0.1	b	0.5	± 0.2	ab	0.6	± 0.1	a	0.004
24:1n-9	0.6	± 0.2	a	0.5	± 0.1	ab	0.4	± 0.2	ab	0.4	± 0.1	b	0.5	± 0.2	ab	0.5	± 0.1	ab	0.022
18:2n-6	24.3	± 6.4	a	18.5	± 3.8	ab	14.3	± 5.6	bc	12.7	± 2.7	bc	11.9	± 3.2	cd	6.4	± 1.2	d	< 0.001
20:2n-6	0.4	± 0.1	a	0.3	± 0.1	ab	0.3	± 0.1	bc	0.2	± 0.0	c	0.2	± 0.1	cd	0.1	± 0.0	d	< 0.001
20:4n-6	0.1	± 0.1	d	0.2	± 0.0	c	0.2	± 0.0	bc	0.3	± 0.0	b	0.5	± 0.1	a	0.5	± 0.1	a	< 0.001
18:3n-3	5.4	± 1.7	a	3.9	± 0.9	b	2.9	± 1.4	bc	2.6	± 0.7	bc	2.3	± 0.7	cd	0.8	± 0.2	d	< 0.001
18:4n-3	0.0	± 0.0	c	0.1	± 0.1	bc	0.2	± 0.1	bc	0.2	± 0.1	b	0.5	± 0.2	a	0.6	± 0.2	a	< 0.001
20:4n-3	0.0	± 0.0	c	0.0	± 0.1	c	0.1	± 0.1	bc	0.2	± 0.0	b	0.3	± 0.1	a	0.3	± 0.1	a	< 0.001
20:5n-3	0.6	± 0.1	d	1.2	± 0.1	cd	1.4	± 0.4	cd	1.9	± 0.2	c	3.5	± 0.8	b	4.6	± 1.0	a	< 0.001
22:5n-3	0.3	± 0.1	c	0.5	± 0.1	bc	0.5	± 0.1	bc	0.7	± 0.1	b	1.0	± 0.3	a	1.3	± 0.3	a	< 0.001
22:6n-3	1.1	± 0.2	e	2.0	± 0.2	d	2.4	± 0.5	cd	3.1	± 0.2	c	4.7	± 0.7	b	6.7	± 1.1	a	< 0.001

[1] Honest significant differences

[2] Sum of 20C or 22C monounsaturates (n-7 + n-9 +n-11).

Table A4. Results of proximate analysis of European seabass (*Dicentrarchus labrax*) body compartments (N=3) after 18 weeks feeding on experimental diets D1 - D6. Values are reported as g kg^{-1} (± SD). The results of statistical treatment by ANOVA (different lower-case letters denote Tukey's HSD[1] tests).

Diet		**D1**			**D2**			**D3**			**D4**			**D5**			**D6**			**Pr(>F)**
Liver	Moisture	371.7	± 2.1	a	426.0	± 61.0	abc	398.0	± 26.6	ab	439.7	± 18.6	abc	469.3	± 4.5	bc	491.3	± 17.6	c	0.003
	Ash	5.2	± 0.2	a	6.1	± 1.2	ab	5.4	± 0.5	ab	6.6	± 0.5	ab	6.8	± 0.7	ab	7.3	± 0.8	b	0.022
	Protein	60.4	± 0.6	a	72.6	± 9.0	abc	66.2	± 6.2	ab	79.3	± 3.2	bc	79.0	± 2.2	bc	84.0	± 5.3	c	<0.001
	Lipid	485.2	± 7.6	d	416.7	± 75.3	bcd	427.0	± 35.4	cd	362.5	± 11.1	abc	322.3	± 15.1	ab	302.4	± 20.5	a	<0.001
Carcass	Moisture	634.7	± 12.2		608.3	± 11.0		605.7	± 16.8		599.7	± 6.1		610.3	± 17.0		600.3	± 18.2		n.sig
	Ash	35.9	± 2.8		37.0	± 3.1		34.8	± 1.6		38.1	± 2.7		31.0	± 3.0		37.7	± 7.2		n.sig
	Protein	168.4	± 1.4		170.9	± 0.2		171.9	± 9.2		170.3	± 0.2		169.4	± 3.6		168.3	± 3.2		n.sig
	Lipid	143.7	± 17.7		170.0	± 17.8		181.4	± 11.9		174.8	± 7.8		173.0	± 11.7		165.6	± 13.9		n.sig
Viscera	Moisture	203.7	± 15.3		187.0	± 35.1		211.7	± 7.6		181.0	± 12.5		199.3	± 29.3		207.7	± 10.3		n.sig
	Ash	3.1	± 0.1		3.2	± 0.5		3.4	± 0.2		2.9	± 0.5		3.0	± 0.3		3.4	± 0.2		n.sig
	Protein	31.5	± 8.3		32.9	± 7.9		31.2	± 3.3		32.1	± 5.6		36.6	± 6.6		38.3	± 5.2		n.sig
	Lipid	708.0	± 14.0		724.6	± 36.3		705.0	± 44.3		718.8	± 19.7		691.1	± 53.4		729.6	± 33.0		n.sig
Whole	Moisture	565.7	± 10.2		566.3	± 10.1		565.3	± 5		566.8	± 19.0		566.0	± 10.8		570.0	± 6.2		n.sig
body	Ash	33.7	± 2.5		35.4	± 7.3		34.8	± 2.7		34.9	± 2.2		32.6	± 4.8		36.0	± 3.2		n.sig
	Protein	154.2	± 6.8		154.1	± 6.9		155.8	± 5.8		157.1	± 3.8		153.6	± 0.9		158.1	± 2.7		n.sig
	Lipid	205.5	± 24.9		219.7	± 0.7		201.9	± 14.0		223.6	± 14.6		209.6	± 15.6		201.0	± 22.9		n.sig

[1] Honest significant differences

Table A5. Tissue total lipid (%) and mean levels (N = 3 · 3) of fatty acids (mg g^{-1} ± SD) in the liver of European seabass (*D. labrax*) after 18 weeks feeding on the experimental diets, D1 - D6. Results of statistical treatment by ANOVA (lower case letters indicate Tukey's HSD[1]).

Diet:	**D1**		**D2**		**D3**		**D4**		**D5**		**D6**		**Pr(>F)**
Total Lipid	50.21	± 5.32 c	38.95	± 6.88 abc	43.68	± 10.14 bc	39.20	± 7.55 ab	29.39	± 4.75 a	31.35	± 8.99 a	<0.001
Fatty acid													
14:0	6.11	± 1.19 b	4.66	± 1.07 ab	4.61	± 1.02 a	4.39	± 0.73 a	3.48	± 0.62 a	4.05	± 1.33 a	<0.001
16:0	85.12	± 13.33 b	70.42	± 14.01 ab	73.07	± 15.78 ab	68.98	± 12.48 ab	55.29	± 7.72 a	55.77	± 15.74 a	<0.001
18:0	32.05	± 6.61 c	24.98	± 7.24 bc	25.66	± 6.72 bc	21.78	± 5.51 ab	16.71	± 3.61 a	16.70	± 4.46 a	<0.001
20:0	1.01	± 0.16 d	0.73	± 0.18 c	0.79	± 0.21 c	0.64	± 0.13 bc	0.45	± 0.07 ab	0.43	± 0.11 a	<0.001
22:0	0.25	± 0.06 b	0.20	± 0.06 ab	0.20	± 0.06 ab	0.24	± 0.10 b	0.10	± 0.02 a	0.13	± 0.17 a	<0.001
16:1n-9	4.29	± 0.58 c	2.93	± 0.72 b	3.03	± 0.60 b	2.65	± 0.43 ab	2.01	± 0.27 a	1.95	± 0.46 a	<0.001
16:1n-7	11.87	± 3.07	9.41	± 2.44	9.76	± 2.28	9.84	± 1.71	8.89	± 1.35	10.48	± 3.39	n.sig
18:1n-9	217.45	± 28.67 d	158.40	± 37.21 c	180.97	± 48.19 cd	152.49	± 34.13 bc	107.67	± 21.48 ab	102.71	± 26.81 a	<0.001
18:1n-7	8.00	± 1.22	6.36	± 1.25	7.73	± 2.12	7.41	± 1.64	5.71	± 1.15	7.29	± 2.69	n.sig
20:1n[2]	4.65	± 0.84 c	3.25	± 0.79 ab	4.20	± 1.29 bc	3.52	± 0.74 abc	2.38	± 0.67 a	2.53	± 0.90 a	<0.001
22:1n[2]	0.51	± 0.15	0.44	± 0.19	0.60	± 0.21	0.50	± 0.11	0.43	± 0.09	0.55	± 0.24	n.sig
24:1n-9	0.73	± 0.31	0.65	± 0.21	0.71	± 0.27	0.54	± 0.14	0.53	± 0.14	0.60	± 0.23	0.033
Iso 18:2n	7.37	± 1.02 d	4.68	± 1.36 c	4.97	± 1.10 c	3.85	± 0.68 bc	2.84	± 0.47 ab	2.50	± 0.51 a	<0.001
18:2n-6	28.31	± 6.18 c	19.41	± 8.37 ab	22.95	± 7.68 bc	19.07	± 4.33 b	10.80	± 3.44 a	9.45	± 4.42 a	<0.001
18:3n-6	2.38	± 0.44 c	1.27	± 0.37 b	1.54	± 0.43 b	1.10	± 0.22 b	0.65	± 0.13 a	0.55	± 0.2 a	<0.001
20:2n-6	0.86	± 0.21 ab	0.67	± 0.24 ab	0.91	± 0.36 b	0.77	± 0.17 ab	0.53	± 0.16 a	0.58	± 0.27 ab	0.0083
20:3n-6	0.09	± 0.07	0.08	± 0.05	0.10	± 0.07	0.12	± 0.12	0.06	± 0.05	0.08	± 0.07	n.sig
20:4n-6	0.16	± 0.03 a	0.20	± 0.09 ab	0.26	± 0.07 cb	0.36	± 0.05 dc	0.47	± 0.09 de	0.67	± 0.18 e	<0.001
18:3n-3	5.38	± 1.35 d	3.99	± 2.29 bc	4.61	± 1.67 cd	3.72	± 0.92 c	1.90	± 0.63 ab	1.18	± 0.58 a	<0.001
18:4n-3	0.92	± 0.17 ab	0.69	± 0.22 a	1.11	± 0.54 ab	0.98	± 0.25 ab	0.91	± 0.24 ab	1.41	± 0.70 b	0.022
20:4n-3	nd		nd		0.12	± 0.07 a	0.12	± 0.08 a	0.19	± 0.09 a	0.38	± 0.22 b	0.0013
20:5n-3	0.76	± 0.12 a	2.01	± 0.88 b	3.10	± 0.92 bc	4.12	± 1.04 cd	5.09	± 1.41 d	8.49	± 3.95 e	<0.001
22:5n-3	nd		0.26	± 0.30 a	0.37	± 0.15 b	0.49	± 0.26 b	0.57	± 0.28 bc	1.15	± 0.76 c	<0.001
22:6n-3	0.68	± 0.14 a	1.72	± 1.18 b	2.01	± 0.82 bc	3.19	± 1.12 cd	4.11	± 1.43 de	7.38	± 4.16 e	<0.001
16:n	0.50	± 0.08 a	0.60	± 0.07 ab	0.79	± 0.23 bc	1.07	± 0.44 cd	0.96	± 0.28 cd	1.64	± 0.71 d	<0.001

[1] Honest significant differences.

[2] Sum of 20C or 22C monounsaturates (n-7 + n-9 +n-11).

Table A6. Total lipid (%) and mean levels (N = 3 · 3) of fatty acids (mg g^{-1} ± SD) in the mid-intestine of European seabass (*D. labrax*) after 18 weeks feeding on the experimental diets, D1 - D6. Results of statistical treatment by ANOVA (lower case letters indicate Tukey's HSD[1]).

Diet	**D1**		**D2**		**D3**		**D4**		**D5**		**D6**		**Pr(>F)**
Total Lipid*	6.83	± 1.53	7.12	± 1.46	6.51	± 1.15	6.53	± 1.56	6.88	± 1.56	6.43	± 1.67	n.sig
Fatty acid													
14:0	0.38	± 0.14 d	0.53	± 0.20 cd	0.57	± 0.13 c	0.69	± 0.23 bc	1.12	± 0.42 ab	1.46	± 0.52 a	<0.001
16:0	7.67	± 2.91	8.32	± 2.35	8.23	± 1.60	8.05	± 2.00	8.99	± 2.81	8.56	± 2.62	n.sig
18:0	2.80	± 0.68	2.85	± 0.42	2.95	± 0.43	2.88	± 0.49	3.04	± 0.59	3.02	± 0.58	n.sig
20:0	0.16	± 0.07 a	0.15	± 0.05 a	0.14	± 0.03 a	0.13	± 0.03 ab	0.13	± 0.04 ab	0.10	± 0.03 b	0.0064
22:0	0.08	± 0.04	0.07	± 0.02	0.07	± 0.01	0.07	± 0.01	0.06	± 0.02	0.06	± 0.02	n.sig
16:1n-9	0.19	± 0.06	0.18	± 0.05	0.17	± 0.03	0.16	± 0.03	0.18	± 0.06	0.16	± 0.04	n.sig
16:1n-7	0.52	± 0.18 d	0.74	± 0.30 cd	0.72	± 0.19 cd	0.88	± 0.31 bc	1.42	± 0.58 ab	1.78	± 0.64 a	<0.001
18:1n-9	16.64	± 8.35 b	16.40	± 6.11 b	14.18	± 3.11 b	12.97	± 4.24 b	11.94	± 4.63 ab	7.65	± 2.64 a	<0.001
18:1n-7	0.87	± 0.42	1.12	± 0.35	1.07	± 0.2	1.06	± 0.28	1.19	± 0.40	1.14	± 0.35	n.sig
20:1n[2]	0.68	± 0.31	0.65	± 0.23	0.60	± 0.12	0.57	± 0.17	0.61	± 0.22	0.52	± 0.16	n.sig
22:1n[2]	0.15	± 0.08	0.15	± 0.07	0.15	± 0.05	0.16	± 0.05	0.21	± 0.07	0.22	± 0.08	n.sig
24:1n-9	0.11	± 0.03	0.11	± 0.02	0.12	± 0.02	0.11	± 0.03	0.14	± 0.02	0.14	± 0.03	n.sig
Iso 18:2n*	0.23	± 0.08 a	0.19	± 0.05 ab	0.18	± 0.03 abc	0.16	± 0.05 abc	0.15	± 0.06 bc	0.13	± 0.04 c	0.0014
18:2n-6	9.13	± 3.48 b	8.70	± 2.49 b	8.20	± 1.38 b	7.48	± 1.81 ab	6.89	± 1.98 ab	5.04	± 1.62 a	<0.001
18:3n-6	0.20	± 0.08 c	0.17	± 0.04 bc	0.17	± 0.03 bc	0.15	± 0.03 abc	0.12	± 0.03 ab	0.10	± 0.03 a	<0.001
20:2n-6	0.29	± 0.07	0.27	± 0.07	0.27	± 0.04	0.28	± 0.07	0.27	± 0.07	0.24	± 0.05	
20:3n-6	0.10	± 0.03 b	0.08	± 0.02 ab	0.08	± 0.02 ab	0.07	± 0.01 ab	0.07	± 0.01 a	0.07	± 0.02 a	0.018
20:4n-6	0.17	± 0.02 a	0.27	± 0.02 b	0.30	± 0.05 bc	0.35	± 0.05 c	0.46	± 0.08 d	0.55	± 0.08 e	<0.001
22:5n-6	0.04	± 0.01 a	0.06	± 0.01 ab	0.07	± 0.01 b	0.08	± 0.01 b	0.10	± 0.02 c	0.12	± 0.03 c	<0.001
18:3n-3	1.62	± 0.83 b	1.64	± 0.65 b	1.42	± 0.27 b	1.28	± 0.41 b	1.10	± 0.39 b	0.59	± 0.20 a	<0.001
18:4n-3	0.11	± 0.04 c	0.13	± 0.05 bc	0.13	± 0.03 bc	0.17	± 0.07 b	0.29	± 0.13 a	0.41	± 0.17 a	<0.001
20:4n-3	0.03	± 0.01 c	0.04	± 0.01 b	0.04	± 0.01 b	0.06	± 0.02 b	0.10	± 0.04 a	0.14	± 0.05 a	<0.001
20:5n-3	0.85	± 0.15 d	1.34	± 0.25 c	1.51	± 0.22 bc	1.86	± 0.44 b	2.81	± 0.83 a	3.66	± 1.08 a	<0.001
22:5n-3	0.23	± 0.04 d	0.28	± 0.04 cd	0.32	± 0.04 c	0.34	± 0.07 bc	0.45	± 0.13 ab	0.54	± 0.16 a	<0.001
22:6n-3	1.65	± 0.21 d	2.17	± 0.21 c	2.53	± 0.37 bc	2.80	± 0.48 b	3.67	± 0.78 a	4.43	± 0.94 a	<0.001
16:n	0.16	± 0.02 b	0.16	± 0.02 b	0.16	± 0.02 b	0.17	± 0.02 b	0.29	± 0.15 a	0.41	± 0.22 a	<0.001

[1] Honest significant differences.

[2] Sum of 20C or 22C monounsaturates (n-7 + n-9 +n-11).