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Innovative feeds based on novel omega-3 sources to improve the production of Mediterranean fish

Marta Ribeiro Carvalho

Thesis for the degree of Doctor of Philosophy
by the University of Las Palmas de Gran Canaria, 2022

Supervisors:
Prof. María Soledad Izquierdo López
Prof. Daniel Montero Vítores

PhD programme in Sustainable Aquaculture and Marine Ecosystems

Institute of Aquaculture and Sustainable Marine Ecosystems (ECOAQUA Institute)

Aquaculture Research Group (GIA)

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Grupo de Investigación en Acuicultura

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El/la Director/a, El/la Codirector/a El/la Doctorando/a,

To my grandmother, who I am sure is the proudest person of this achievement of mine, wherever she is.

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LIST OF ABBREVIATIONS

ACC, acetyl-CoA carboxylase

AI, atherogenic index

ANOVA, analysis of variance

ARA, arachidonic acid

ATP, adenosine 5'-triphosphate

BDNF, brain-derived neurotrophic factor[1]

BHT, butylated hydroxytoluene

CAT, catalase[1]

cDNA, complementary DNA

CL, cholesterol

CF, condition factor

CM, chylomicrons

COX, cyclooxygenase[1]

CPT, carnitine palmitoyl transferase [1]

CVD, cardiovascular disease

DB, double bend

DGI, daily growth index

DHA, docosahexaenoic acid

DPA, docosapentaenoic acid

DW, dry weight

EFA, essential fatty acid(s)

EF1α, elongation factor 1 alpha[1]

EGR1, early growth response 1[1]

ELOVL, fatty acyl/fatty acid elongase[1]

EPA, eicosapentaenoic acid

ER, endoplasmic reticulum

FA, fatty acid(s)

FABP, fatty acid binding protein

FADS, fatty acyl/ fatty acid desaturase[1]

FAMES, fatty acid methyl esthers

FAS, fatty acid synthase[1]

FBL, final body length

FBW, final body weight

FCR, feed conversion ratio

FI, feed intake

FM, fish meal(s)

FO, fish oil(s)

FOH, fatty alcohol

[1] *Note: Also presented along the text as genes and written in italic*

GLC, gas-liquid chromatography

GM, genetic modified

GPX, glutathione peroxidase[1]

G6P, glucose-6-phosphatase[1]

h, hypocholesterolemic fatty acids

H, Hypercholesterolemic fatty acids

HDL, high density

H&E, haematoxylin and eosin

HPI, hypothalamus–pituitary–interrenal axis

HSI, hepatosomatic index

HSP, heat shock protein(s)[1]

IDL, intermediate-density lipoprotein

IGF-1, insulin-like growth factor 1

IL, interleukin

IUPAC, International Union of Physical and Applied Chemists

KO, krill oil

LA, linoleic acid

LC-PUFA, long-chain polyunsaturated fatty acid(s)

LDL, low density lipoproteins

LER, lipid efficiency ratio

LNA, linolenic acid

LOX, lipoxygenase[1]

LP, lysophospholipids

LPL, lipoprotein lipase[1]

LT, leukotrienes

LX, lipoxins

MAG, monoacylglycerol

MaR, maresin

MUFA, monounsaturated fatty acid(s)

NEUROD6, neurogenic differentiation factor 6[1]

NL, neutral lipid(s)

NOS, nitric oxide synthase[1]

OA, oleic acid

PC, phosphatidylcholine

PCA, principal components analysis

PCR, polymerase chain reaction

PD, protectin D

PE, phosphatidylethanolamine

PER, protein efficiency ratio

PG, prostaglandins

PI, peroxidation index

PI, phosphatidylinositol

PL, polar lipid(s)

PPAR (α), peroxisome proliferator-activated receptor (alpha)[1]

PO, poultry oil

PS, phosphatidylserine

PUFA, polyunsaturated fatty acid(s)

ROS, reactive oxygen species

RPL27, ribosomal protein L27[1]

RvD, resolvin D

S, sterol

SB, single bend

SCD, stearoyl-CoA desaturase[1]

SFA, saturated fatty acid(s)

SGR, specific growth rate

SREBP, sterol-regulatory element-binding protein[1]

TAG, triacylglycerols/ triglycerides

TCA, tricarboxylic acid cycle

TGC, thermal growth coefficient

TI, thrombogenic index

TL, total lipid(s)

TNF- α, tumour necrosis factor α

TX, thromboxanes

VLDL, very low-density lipoproteins

VM, vegetable meal(s)

VO, vegetable oil(s)

β-actin, beta-actin[1]

Δ(x), desaturase x, x being the number code of the desaturase

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CHAPTER 1. GENERAL INTRODUCTION

Aquaculture is one of the most fast-growing food industries in the world, representing nowadays more than 50% of total fish supply for human nutrition (FAO, 2020). Traditionally, sea food consumption has been a major source of nutrients for human nutrition, including protein, vitamins, minerals, lipids, and omega-3 fatty acids (FA), with important functions in vertebrates' health (Tocher, 2015). The good nutritional quality of artificial diets for fish used in aquaculture guarantee the optimal nutritional quality of fish products for consumers since fish composition usually reflects their feeds. Therefore, the research and choose of high-quality ingredients for aquafeeds is necessary to enhance not only aquaculture productivity and fish welfare but also to make aquaculture products an excellent food for human population. At the same time, in a context of increasing awareness for biological resources and environment, aquafeed ingredients must be "eco/bio-friendly" and optimize aquaculture economic viability to the maximum possible. This is an issue that has been more easily solved regarding protein source ingredients, but more difficult when lipid ingredients are concerning. Thus, this thesis will focus its attention on lipid nutrition for marine aquaculture.

1.1 BIOCHEMISTRY OF LIPIDS AND FATTY ACIDS

1.1.1 LIPIDS

Lipids are a diverse group of chemical substances, which are poorly soluble or insoluble in water, but soluble in nonpolar organic solvents such as chloroform, hydrocarbons, alcohols, or ethers. There are two main groups of lipids found in nature: neutral lipids (NL), which include triacylglycerols (TAG), diacylglycerols, wax esters, steroids, and free FA; and polar lipids (PL), which include mostly phospholipids, but also sphingolipids, glycolipids and sulpholipids.

Briefly, the TAG, also called triglycerides, are composed of three FA esterified to a glycerol group. TAG are the main lipids in foods and serve as the main energy source in the body, that can be readily used for energy production or stored in body tissues. Indeed, TAG are important macronutrients of fish bodies. Waxes are composed of a single fatty acid combined with a single alcohol group and live organisms use them for several reasons, particularly as water and contaminant-proofing molecules. Steroids are components of the cell

membranes, helping to support its structure, transport, and permeability. The most common steroid in animal tissues is cholesterol. Free FA serves also as energy source and participates in a wide number of functions, from receptor signalling to gene expression and control of homeostasis (Kimura et al., 2019).

In contrast, PL are characterized by a common backbone of phosphatidic acid, composed of L-glycerol-3-phosphate, and containing two esterified fatty acids. This phosphatidic acid can be esterified to the choline, ethanolamine, serine, or inositol and, forming, consequently, the PL phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), respectively. These types of lipids are the major constituents of the bilayer cell membranes in living organisms. In fish tissues, PC is the most abundant PL, followed by PE, the latter being highly present in neural tissues. Sphingolipids are at high quantities particular in nervous system and play a role in the cell differentiation and proliferation, cell recognition and adhesion, as well as act as receptor for other molecules (Olsen and Færgeman, 2017). Glycolipids also participates as receptors for pathogens and regulate membrane and interactions (Kirschbaum et al., 2021).

1.1.2 FATTY ACIDS

Fatty acids are the main and the most important components of lipids and are composed of a carboxylic acid with a long aliphatic chain. According to the International Union of Physical and Applied Chemists (IUPAC), FA nomenclature is defined based on the number of carbon atoms in the chain, the number of unsaturated bonds in the chain, and the position of those bounds relative to the methyl terminus. Therefore, based on their number of unsaturated bounds, there are basically three important groups of fatty acids: saturated fatty acids (SFA), with no unsaturated bounds; monounsaturated fatty acids (MUFA), with one single unsaturated bound; and polyunsaturated fatty acids (PUFA) with two or more unsaturated bounds. Additionally, PUFA with 20 or more carbon atoms and three or more double bounds are commonly defined as long-chain PUFA (LC-PUFA). The latter is the most important group of fatty acids for vertebrate nutrition for the reasons that will be described later in this thesis.

In animal lipids, palmitic acid (16:0) and stearic acid (18:0) are the most abundant SFA. MUFA and PUFA are predominantly derived from three important series: n-9, n-6, and n-3, although FA derived from n-1, n-4, n-5, n-7, and n-11 families are also present. Thus, the predominant MUFA in animal lipids are usually oleic acid (OA, 18:1n-9) and palmitoleic acid (16:1n-7), although in fish lipids, particularly in TAG, eicosaenoic (20:1n-9) and cetoleic

(22:1n-11) acids are also abundant (Ackman, 1989). There is a diversity of PUFA in fish tissues, mostly from n-3 series followed by n-6 series. Indeed, the marine environments, particularly the phytoplankton, are the primary producers of n-3 LC-PUFA, including the eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), with important functions in vertebrates, as it will be discussed detailly throughout the present thesis. They are produced through their metabolic precursor α-linolenic acid (LNA, 18:3n-3). In contrast, the most predominant n-6 PUFA in fish tissues is linoleic acid (LA, 18:2n-6) and its active derivative arachidonic acid (ARA, 20:4n-6), also with important functions in vertebrates.

1.2 BENEFITS OF OMEGA-3 FOR HUMAN HEALTH: THE CONTRIBUTION OF FARMED FISH

The n-3 LC-PUFA (the so called “omega-3”) benefits in human health and well-being are closely associated to their important roles in cell function and health, as it will be discussed in the following sections (Section 1.3). Their basic physiological functions are shared among all vertebrates. Briefly, in humans, n-3 LC-PUFA consumption is necessary for babyhood vision and neural development. Furthermore, beneficial effects of n-3 LC-PUFA, in particular of DHA, are also reported in the treatment of several neurological or behavioural disorders, including depression and attention deficit hyperactivity (Ortega et al., 2012). Thy are also benefice for the prevention of aging-neuronal pathologies, for instance Alzheimer’s disease, by reducing the neuronal loss and improving cognitive function (Hooijmans et al., 2012).

Additionally, due to their lowering effect in blood cholesterol and TAG synthesis, these fatty acids are beneficial for the prevention of cardiovascular diseases (CVD) (Calder, 2006). Therefore, many worldwide health institutions recommend up to 500 mg per day of EPA and DHA to reduce the risk of CVD, and until 2 g per day in patients with CVD (ISSFAL, 2004; EFSA, 2012), which is equivalent to the consumption of 2 oily fish meals a week.

There is also evidence that n-3 LC-PUFA may be beneficial in inflammatory diseases, such as Crohn's disease, although the daily doses required are much higher (3 g per day). Even though, these positive effects are closely related with the attenuation of the production of pro-inflammatory molecules, such as a wide range of eicosanoids, as well as the production of resolvins and maresins with anti-inflammatory effects (Serhan and Petasis, 2011).

Finally, many studies reported a protective role of these FA in several types of cancers, including that of breast, colorectal and prostate (Gerber, 2012), as well as in reducing the side-effects and muscle loss associated to chemotherapy, helping patients to handle more cycles

(Murphy et al., 2011). The mechanisms underlying these positive effects are still poorly understood but are possibly related with their known role in inflammatory and immune functions.

Humans have evolved consuming a diet high in n-3 PUFA, with a n-6/n-3 ratio of 1. Nowadays, modern diets have a n-6/n-3 PUFA ratio of 25:1, suggesting a deficiency intake of n-3 PUFA and an excessive intake of n-6 PUFA, being very different than the genetic patterns that were established for the species (Simopoulos, 2011). Therefore, increasing the intake of n-3 PUFA is mandatory for achieving a balanced and healthy diet. Indeed, a few recent studies have focused on estimating the supply of n-3 LC-PUFA in human diets around the world and all of them denoted a gap between demand and supply (Salem et al., 2015; Tocher, 2015, 2019). This indicates that our diets are currently incapable of satisfying minimum recommended requirements of 250 mg/day of EPA+DHA. Furthermore, the ever-increasing growth of the human population, that is estimated to reach 9.6 billion people in 2050, exacerbates this problem.

Fish consumption is the major source of n-3 LC-PUFA for human nutrition and health (United Nations, 2020). However, 90% of the total consumption of fish comes from fisheries, directly or indirectly, through the consumption of fish meal (FM) and oils (FO) derived from fisheries captures (Tocher, 2019; Colombo and Turchini, 2021). This represents a huge environmental, economic, and nutritional problem worldwide for two main reasons: (1) it is well known that wild fish stocks are over-exploited and that fisheries catch levels have stagnated for the last decades, without expectation of increasing in the near future. Therefore, demand for aquatic foods has been filled by aquaculture, representing nowadays more than 50% of total fish supply for human nutrition (FAO, 2020). However, (2) the traditional feeds for aquaculture fish contained large amounts of FM and FO, rich in n-3 LC-PUFA, but derived from wild fishery stocks. Furthermore, these finite resources, besides from paradoxical and unsustainable, had also increased their prices during the last decades, making them an unattainable economic option for inclusion in farmed fish feeds. Therefore, aquaculture is the best and possibly only option to meet the global demand of n-3 LC-PUFA in the future and its sustainable expansion is of foremost importance.

1.3 FUNCTIONS OF LIPIDS AND FATTY ACIDS IN FISH

1.3.1 ENERGY PRODUCTION

Lipids are one of the main nutrients presented in fish as well in their diets, as they play an important role as metabolic energy substrates, having the highest total energy per unit weight (9.5 Kcal / g). Furthermore, they are also a source of essential fatty acids (EFA) as well as carrier of vitamins and certain micronutrients of liposoluble characteristics (Tocher, 2003, 2010; Glencross, 2009). Fatty acids are the major source of metabolic energy for growth, swimming activity and reproduction in fish, providing ATP through their oxidation in cells (Frøyland et al., 2000; Tocher, 2002). The order in which each FA is utilized for ATP production is dependent on several factors and determines the rate of FA deposition in fish tissues, which is particularly important from the nutritional point of view for the consumers. In general, most marine species present a selective catabolism of FA, in particular oxidising MUFA and SFA over PUFA (Sidell et al., 1995). Therefore, SFA or MUFA are readily used for energy whereas the latter are deposited in fish tissues. Furthermore, a similar selective catabolism appears to exist among LC-PUFA, with EPA being usually oxidised faster than DHA or ARA, which are accumulated in tissues. This is particularly due to the vital structural functions of DHA for plastic purposes, as it will be discussed below, which is preferentially retained in PL and serves, therefore, as a poor substrate for oxidation compared to other FA and the different affinities of enzymes to the different FA (Tocher, 2002).

1.3.2 MEMBRANE STRUCTURE AND CELL FUNCTION

Fatty acids in the PL play important roles in cell function, maintaining membrane integrity and fluidity, protein function and controlling membrane signal (Sargent et al., 1995; Izquierdo, 2005). Furthermore, PL are also substrates for the formation of second messengers, such as diacylglycerols, lysophospholipids or other bioactive molecules (Calder, 2016). DHA is the major component of cell membranes in all vertebrates, including in teleost fish (Izquierdo, 2005), evidencing its structural and functional importance in cells. This is particularly driven by its three-dimensional shape, which maintains membrane function and fluidity, and improves cell responses to signals (Calder, 2016). Noteworthy is that fish cells present higher EPA and DHA contents in PL compared to other animal cells, explaining why fish is considered one of the healthiest foods in the world (Tocher, 2010).

1.3.3 NEURAL DEVELOPMENT, FUNCTION AND BEHAVIOUR

The role of n-3 LC-PUFA for brain health and neural tissue development and function of vertebrates is widely recognized, particularly in early stages of life (Izquierdo, 2005). In mammals, n-3 LC-PUFA are needed for neural growth and correct developed of nervous system in infants, showed by the large increase of their brain contents after birth (Lauritzen et al., 2001; Marszalek and Lodish, 2005). Indeed, DHA is the most important fatty acid with physiological significance for brain function, both in mammals and fish (Bourre, 2004; Marszalek and Lodish, 2005). Neural cell membranes contained approximately 30–40% of DHA (Innis, 1991; Lauritzen et al., 2001) and this FA is also largely present in membranes of retina cells, being associated with the development of vision and central neural system. The role of DHA in neural cells has been explained by the high flexibility of DHA lipid molecules, allowing the interaction with membrane proteins, and increasing neurotransmission signals (Innis, 2007). Furthermore, unesterified DHA in the brain seems to influence predominantly neural gene expression and ion channels function as well as it serves as substrate for the formation of neuroprotective metabolites (Innis, 2007). For instance, in mammals, DHA has been shown to control neurogenesis, neuronal migration and to prevent peroxidation of lipids in brain cells through the production of neuroprotective molecules, avoiding neuronal loss (Salem et al., 2001).

Despite being less studied, EPA may also play important roles in neural function early in life in vertebrates, including fish, by enhancing brain development (Furuita et al., 1998; Izquierdo et al., 2001) or by producing neuroprotective metabolites (Kidd, 2007). Additionally, in mammals, whereas DHA is essential to pre- and postnatal brain development, EPA seems to affect behaviour and mood (Kidd, 2007). Indeed, the brain is the primary organ in anticipating a stimulus or stressor and traducing it in a behavioural action. Animal behaviour is thus a strong indicator of health and welfare. Therefore, changes in FA composition of neural cells could directly affect neural perception, signals, and behaviour. For instance, chronically deficient DHA brains have been shown to increase n-6 docosapentaenoic acid (DPA, 22:5n-6) in neural PL, inducing deficits in behaviour in animal models (Moriguchi et al., 2000; Lim et al., 2005; Innis, 2007). This effect has been associated with an imbalance of brain activity, particularly in serotonergic and dopaminergic neurotransmission (Zimmer et al., 1998, 2002; Takeuchi et al., 2002; Aïd et al., 2003; Kodas et al., 2004). Decreased EPA and DHA brain levels were also linked to other behavioural disorders in humans, such as bipolar disorder, schizophrenia, and borderline personality disorder, whereas low EPA was closely associated with Huntington disease that affects movement, mood and thinking (Kidd, 2007). In fish, the studies of the role of n-3 LC-PUFA are scarcer, but previously dietary n-3 LC-PUFA,

particularly DHA and EPA, were also linked with fish behaviour and brain function. The first evidence of this important role in fish was shown by studies in larvae (Benítez-Santana et al., 2012, 2014). Those studies reported that DHA-deficient diets greatly reduce DHA incorporation in fish larval brain and, consequently, delayed escape response to a sound stimulus (Benítez-Santana et al., 2012, 2014). The escape response in fish is activated by Mauthner cells, two pairs of specific neurons in the hindbrain and it is involved in the normal behaviour of fish, for avoiding natural or artificial stressors (Zottoli and Faber, 2000; Eaton et al., 2001; Korn and Faber, 2005). Briefly, this response consists of a C-type fast-start, which is characterized by a rapid unilateral contraction of trunk musculature and head and tail movement, leading into a C-shape. Then, fish display subsequent movements leading to forward propulsion of the centre of mass to escape from the stressor stimulus (Foreman and Eaton, 1993; Domenici and Blake, 1997). The unsuccess or alterations in fish behaviour response, including in escape response, was associated either with a failure on the neuronal activity or a low perception of the stimulus. Interestingly, in fish larvae abnormal escape response was associated with a n-3 LC-PUFA deficiency (Benítez-Santana et al., 2012, 2014). Short and long-term dietary n-3 LC-PUFA deficiency also caused behaviour alterations in pikeperch (*Sander lucioperca*) larvae (Lund et al., 2014). Additionally, in fish juveniles, recent studies with rainbow trout (*Oncorhynchus mykiss*) also linked dietary n-3 LC-PUFA to feeding behaviour, as well as to inflammatory, oxidant and stress status of fish brains (Roy et al., 2020). Swimming performance of Atlantic salmon (*Salmo salar*) juveniles in seawater was also affected when fed diets containing low n-3 LC-PUFA (Wagner et al., 2004). Therefore, the compiled data, both in mammals and fish, suggest that the brain is sensitive to a lack of PUFA, particularly in early life stages, and that a deficiency in n-3 LC-PUFA, particularly in DHA, can affect neural function and health (Lauritzen et al., 2001).

1.3.4 IMMUNE FUNCTION, EICOSANOIDS PRODUCTION AND STRESS RESPONSE

One of the major roles of LC-PUFA in membranes is their controlled oxidation to produce eicosanoids, which are bioactive molecules, hormone–like compounds with a short half-life, that play important actions in blood clotting, immune response, inflammatory response, cardiovascular tone, renal function, neural function, and reproduction (Calder, 2006; Tocher, 2010). They include cyclic derivatives such as prostaglandins (PG) and thromboxanes (TX), that are formed by the action of cyclooxygenases enzymes (COX) trough C_{20} PUFA, particularly ARA and EPA, as well as linear derivatives produced by action of lipoxygenases (LOX), including hydroperoxy- and hydroxy fatty acids, leukotrienes (LT), and lipoxins (LX) (Yaqoo, 2004; Calder, 2006; Tocher, 2010). Eicosanoids are produced in all fish tissues during

stressful situations and this process is part of a natural mechanism to return to homeostasis. Their production is made through activation of cell surface receptors and release of the FA, via phospholipase A_2 activity, which stimulates COX and LOX to produce such derivatives.

Despite ARA, EPA and DHA being the preferable substrates for COX and LOX, dihomo-gamma-linolenic acid (20:3n-6) and eicosatetraenoic acid (20:4n-3) can also serve as precursors to produce eicosanoids (Ghioni et al., 2002). Therefore, since all these FA can compete as substrates for these enzymes, the ratios among them, determines the eicosanoid cascade. In general, it is recognised that n-3 FA-derived metabolites decrease the production of those derived from n-6 FA (Calder, 2009). Both EPA and ARA generate pro-inflammatory metabolites, with those derived from n-6 fatty acids as ARA (2-series PG and 4-series LK), being, generally but not always, more biologically active than those derived from EPA (3-series PG and 5-series LK) (Tocher, 2010). Indeed, EPA has a great importance as a precursor of PG in marine fish (Ganga et al., 2005). And, in mammals, PGE_2 was also reported to decrease the production of inflammatory mediators, for instance cytokines and leukotrienes suggesting that ARA-derived eicosanoids are not only highly pro-inflammatory (Calder, 2009). Additionally, EPA along with DHA, are also substrates for COX and LOX to produce pro-resolving derivatives with an important anti-inflammatory role, particularly resolvins (E-resolvins and D-resolvins, respectively), protectins and maresins (Marcheselli et al., 2003; Serhan, 2006, 2014). These mediators are relatively novel, and, therefore, they are less studied in fish compared to mammals. In brain cell cultures of rainbow trout, di- and tri-hydroxy-containing bioactive products, including the protectin D1 (PD1), resolvin D5 (RvD5), resolvin D1 (RvD1) and resolvin D2 (RvD2), derived from DHA, were found to have a potent anti-inflammatory response (Hong et al., 2005). In the same study, 14S-hydroxy-docosanoids acid and17S-hydroxy-docosanoids acid, from the conversion of DHA by LOX, were also identified (Hong et al., 2005). Derived from these effects on eicosanoids production and others, LC-PUFA boosts immune function in fish (Montero and Izquierdo, 2010).

Furthermore, n-3 LC-PUFA are also known to increase fish resistance to stress. Indeed, fish welfare received much attention since it was recognized that fish feel pain in a similar way to mammals (Rose, 2002; Chandroo et al., 2004; Sneddon et al., 2018). Nociception and stressful situations are processed by fish central nervous system that converts the information into a behavioural and/or physiological alteration, which in most cases enable fish to avoid harmful stimuli (Ashley et al., 2009). Therefore, the stress response can be considered as an adaptive strategy for coping with threats and a way to return to homeostasis. Short-term exposure to stressors may be beneficial for the adaptation of fish to stress (Pickering, 1998; Huntingford et al., 2006). However, prolonged, chronic stress conditions or repeated exposures to acute stressors cause depletion of energy reserves and

hormone imbalances, and could lead to immunosuppression, increasing susceptibility to infectious diseases and, eventually, death (Montero and Izquierdo, 2010).

Stress response comprises, therefore, endocrine, neural, haematological, metabolic, transcriptomic, antioxidant, immune and behaviour alterations (Barton and Iwama, 1991; Tort, 2011). This response is initiated at a first moment by alterations in neural cells, stimulating fish hypothalamus–pituitary–interrenal cells axis (HPI), and leading to the release of cortisol into the bloodstream (Tort, 2011). In fish juveniles, n-3 LC-PUFA regulate the increase in cortisol levels during stressful conditions (Montero et al., 1998) or the expression and activity of stress-related enzymes, such as that of heat shock proteins (Benedito-Palos et al., 2016). In fish larvae, n-3 LC-PUFA also increase resistance to acute thermal or handling stressors (Liu et al., 2002). Indeed, cortisol production is modulated by adrenal corticotrophin hormone in the head kidney, which in turn is regulated by PUFA-derived mediators through LOX (Ganga et al., 2006).

Stress is also recognised to stimulate the production of the reactive oxygen species (ROS), resulting from fish normal aerobic processes, and which, in excess, are known to be deleterious. To counterbalance ROS and protect against oxidation damage, fish have an antioxidant defence system, which is composed by vitamins (α-tocopherol) and enzymes, including the catalase (CAT) and the glutathione peroxidase (GPX) as the main antioxidant enzymes in fish (Halliwell, 1996). Usually, they lead with ROS and allow the animal to return to homeostasis preventing the oxidative damage. However, when the production of ROS surpasses organism detoxification and antioxidant capacity, cell membrane oxidation may lead to tissue damage (Olsvik et al., 2011). Moreover, despite fish having a specific requirement for n-3 LC-PUFA, they are also more susceptible to oxidation (Halliwell and Chirico, 1993). Therefore, dietary nutrients, in particular lipids and PUFA, can affect fish oxidative stress and their response to counterbalance it (Mourente et al., 1999, 2000, 2002). This is particularly important under stressful conditions since the release of stress hormones glucocorticoids and catecholamines can increase ROS production and, consequently, the risk of oxidative damage (Liu and Mori, 1999). For instance, in meagre juveniles, deficient dietary n-3 LC-PUFA induced an increase in glucocorticoid and serotonergic response, as well as altered oxidative stress-related genes, possibly increasing the oxidative stress damage (Carvalho et al., 2019).

In summary, n-3 LC-PUFA are essential not only for adequate perception and anticipation of the stressor by fish neural system, as well as for dealing with the biochemical alterations associated to it, being determinant for fish to return to homeostasis.

1.3.5 CONTROL OF LIPID HOMEOSTASIS

Lipid metabolism homeostasis is achieved by equilibrium between anabolic and catabolic pathways. Several genes are involved in lipid metabolism, which are regulated at transcriptional level by different fatty acids, particularly PUFA, either directly or indirectly, through the modification of membrane composition, eicosanoid production, gene expression or transcript factors (Tocher, 2010). Therefore, it is well recognised the role of n-3 LC-PUFA on lipogenesis and lipolysis by stimulating or depressing several key enzymes for *de novo* lipid synthesis or catabolism (Pierron et al., 2007; Zheng et al., 2013). Peroxisome proliferator-activated receptors, lipoprotein lipase (LPL) and carnitine palmitoyl transferase (CPT) are some of the enzymes targeting FA oxidation (lipolysis). In contrast, fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD) and sterol-regulatory element-binding protein (SREBP) regulates lipogenic pathway. Both processes mainly occur in fish liver and will be detailed in the following section (Section 1.4).

1.4 METABOLISM OF LIPIDS AND FATTY ACIDS

1.4.1 DIGESTION, ABSORPTION AND TRANSPORT OF LIPIDS AND FATTY ACIDS

Digestion processes of dietary lipids in fish (Figure 1.1) are like those in mammals. Briefly, dietary lipids need to be first hydrolysed for their correct absorption. This involves the activity of lipases, namely TAG lipases, phospholipases and, possibly, other lipolytic enzymes, which are mostly secreted by fish pancreatic or hepatopancreatic tissue. In fish digestive tract, lipolysis of the dietary lipids occurs mainly in the proximal intestine and pyloric caeca, although it can be extended, at a lesser intensity, throughout the entire length of the intestine (Tocher, 2003). Pancreatic lipase, non-specific lipase and phospholipase have all been detected in the digestive tract of fish (Izquierdo et al., 2000; Arantzamendi et al., 2019). The action of lipases leads to the release of free fatty acids, lysophospholipids, glycerol, monoglycerides, fatty alcohols or sterols depending on the lipid type (Tocher et al., 1989; Higgs and Dong, 2000; Rust, 2002).

The short-chain FA (less than 12 carbon atoms) and glycerol are directly absorbed by the enterocytes through the intestinal wall by passive transport, while the longer-chain FA (more than 12 carbon atoms) need to be emulsified by bile salts to form aggregates called micelles, which are then absorbed by the enterocytes of the intestinal wall by passive diffusion

(Rust, 2002). Absorption in fish occurs mainly in the proximal part of the intestine, also coincident with the highest lipolytic activity.

Once inside the enterocyte, free fatty acids are again esterified with glycerol, partial acylglycerols, and lysophospholipids in the endoplasmic reticulum, to produce TAG and PL, respectively, which can then be exported from the intestine and transported to other body tissues to be deposited for further utilization (Tocher et al., 1989).

Since extracellular fluids in animals are mostly aqueous, extracellular transport of lipids and FA need to be carried out through lipoproteins. There are two different lipid transport processes in fish, as in mammals: exogenous and endogenous (Figure 1.1). Exogenous pathway involves the association of lipids with proteins (apolipoproteins), inside the enterocytes, to form chylomicrons (CM), which are exported from the intestine. Liver is responsible for the synthesis and release of other lipoproteins into the bloodstream, including very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high density (HDL) that transport lipids to the body tissues (Rust, 2002; Tocher, 2003). Lipoproteins differed on their density due to their different TAG, phospholipid, free FA, cholesterol, and apolipoprotein composition, and, therefore, their distribution and abundance can be influenced by dietary composition (Caballero et al., 2006). Exogenous lipids absorbed by the intestine are mostly integrated into chylomicrons and VLDL, and transported to the liver (Caballero et al., 2003, 2006). Indeed, the liver is the link organ between exogenous and endogenous transports. In the liver, VLDL and CM are cleaved by lipoprotein lipases (LPL) into free lipid components and more high dense lipoproteins, for instance LDL and possibly an intermediate-density lipoprotein (IDL), are formed. The endogenous transport transfers lipids in VLDL, LDL and HDL to storage tissues, for instance the adipose tissue (Sheridan, 1988; Iijima et al., 1995). VLDL, mainly transport TAG, whereas LDL mainly transports cholesterol to the cells. In contrast, HDL removes the excessive remaining lipids from tissues resulting from lipid hydrolysis (Sheridan, 1988).

Although many FA can actively pass-through membrane cell organelles, there is evidence of the presence of a protein-binding transport. Therefore, intracellular transport of FA and derived products, including the eicosanoids, is carried out through tissue-specific fatty acid-binding proteins (FABP) (Schulz, 2008). These cytosolic enzymes are tissue specific and have been well characterized in mammals (Veerkamp and Maatman, 1995). In fish, many FABP have been described as homologous to the mammalian ones.

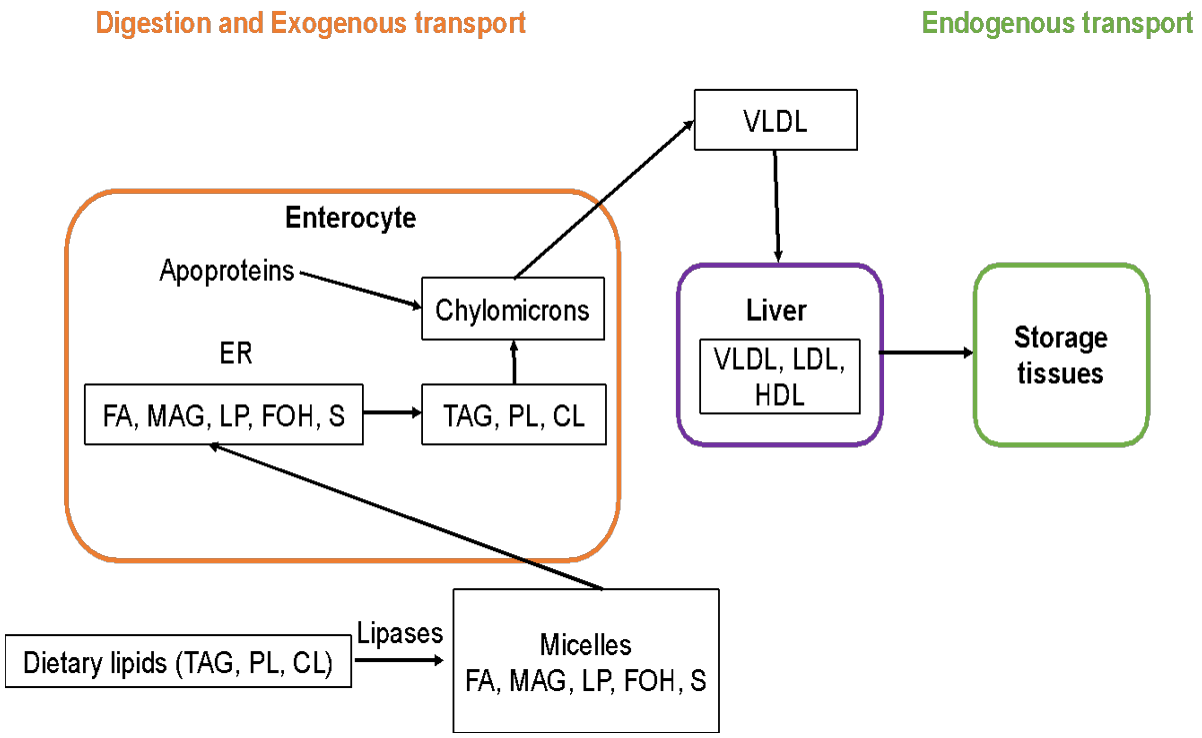

Figure 1.1. Digestion and extracellular transport of lipids (exogenous and endogenous pathways).

CL, cholesterol; ER, endoplasmic reticulum; FA, fatty acids; FOH, fatty alcohol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LP, lysophospholipids; MAG, monoacylglycerol; PL, phospholipid; S, sterol; TAG, triacylglycerol; VLDL, very low-density lipoprotein.

1.4.2 CATABOLISM OF LIPIDS AND FATTY ACIDS

Lipolysis is the process that catabolises stored TAG into free FA and glycerol, which involves three lipase enzymes similar between fish and mammals. Particularly, the above-mentioned LPL is a key enzyme involved in lipolysis (Tian et al., 2013), since its main function is to breakdown lipoproteins, and control FA uptake by different tissues, being, therefore a rate-limiting enzyme that determines whether FA are used in tissues for storage or utilization (Greenwood, 1985; Saera-Vila et al., 2005). Dietary FA have been shown to regulate the expression of LPL in a tissue-specific manner in fish (Liang et al.; 2002).

When the fate of the free FA released from lipid molecules is energy production, they enter in mitochondria or peroxisomes of the cells and are catabolized by β-oxidation, due to the breakdown of FA beginning on third carbon (β–carbon) and resulting in FA that lacks two carbon atoms producing acetyl-CoA. Then, acetyl-CoA can be used by gluconeogenesis or ketogenesis to produce glucose or ketone bodies and delivering energy for fish. CPT system mostly controls the mitochondrial β-oxidation (Figure 1.2). Briefly, in the cytosol, FA are activated to form fatty acyl-CoAs, which reacts with carnitine in the outer mitochondrial

membrane, forming fatty acylcarnitine, a reaction that is catalysed by CPT1. Fatty acylcarnitine passes to the inner membrane, where it again forms fatty acyl-CoA, catalysed by CPT2, which enters the mitochondrial matrix for oxidation.

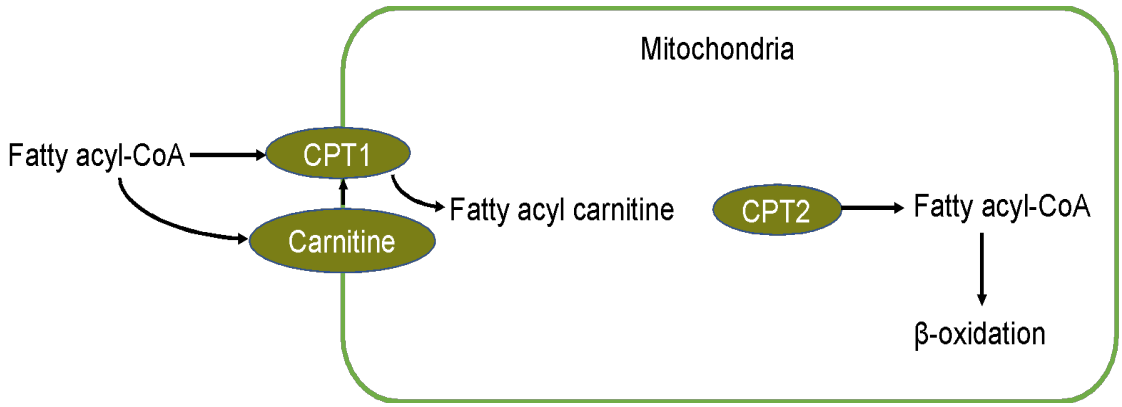

Figure 1.2. Mitochondrial fatty acid β-oxidation controlled by carnitine palmitoyl transferase (CPT) system.

Although mitochondrial oxidation seems to be more abundant and accepts a wide range of FA as substrates, peroxisomal oxidation (Figure 1.3) is the most relevant for LC-PUFA oxidation in fish, and both pathways are independently controlled. Peroxisomal FA oxidation is regulated by the activity of peroxisome proliferator-activated receptors (PPAR), which are of the most studied lipolytic enzymes in fish. All the three PPAR existing in mammals were also identified in fish: PPAR-α is mostly expressed in liver and heart, and, along with β, which is expressed in a broad range of tissues, are involved in the activation of genes and enzymes responsible for FA oxidation (Dressel et al., 2003; Varga et al., 2011; Li et al., 2015). In contrast, PPAR-γ is mostly expressed in adipose tissue and is involved in adipocyte function and differentiation, lipid storage and glucose responsiveness (Francis et al., 2003). FA are, therefore, activators of PPAR in teleost fish, particularly PUFA, with different affinities (Colliar et al., 2011). For instance, PPAR-α presents higher affinity for n-3 PUFA than for SFA, MUFA or n-6 PUFA (Desvergne and Wahli, 1999).

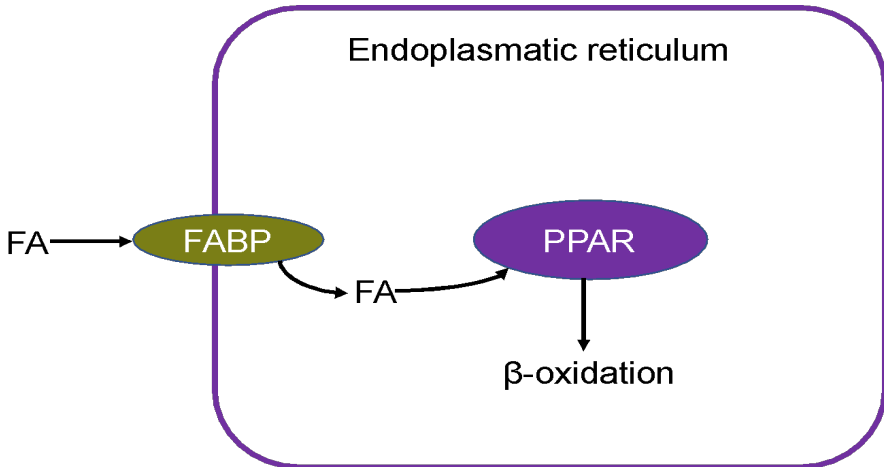

Figure 1.3. Peroxisomal fatty acid β-oxidation controlled by peroxisome proliferator activated receptors signalling pathway.

FABP, fatty acid binding protein; PPAR, peroxisome proliferator-activated receptors.

1.4.2 Synthesis of lipids and fatty acids

In the opposite way, lipogenesis involves *de novo* synthesis of lipids, which in fish occurs preferentially in liver, muscle or adipose tissue depending on species-preferred tissue for lipid deposition (Schulz, 2008). This process is catalysed by several enzymes (Figure 1.4). The first of them to act is acetyl-CoA carboxylase (ACC), which catalyses the conversion of acetyl-CoA, generated from tricarboxylic acid cycle (TCA) in mitochondria and transported to cytosol, to malonyl-CoA. ACC is regulated by CPT enzymes, particularly CPT1, as well as by other lipogenic enzymes, including SREBP, which is a key enzyme in regulating fatty acid synthesis (Minghetti et al., 2011). Whereas SREBP-1 directly targets lipogenic enzymes for *de novo* synthesis of lipids and fatty acids, SREBP-2 seems to be more involved in cholesterol synthesis (Jeon and Osborne, 2012). Additionally, FA are synthetized through FAS, that catalyses the conversion of acetyl-CoA and malonyl-CoA into palmitoyl-CoA, a precursor of palmitate (16:0), which can be either esterified with glycerol to form new TAG to be stored or used for the conversion into an unsaturated FA (Tian et al., 2013; Aysi et al., 2018). Indeed, SCD is an important enzyme for the first steps in the synthesis of unsaturated fatty acids (Ardiyanti et al., 2012). This enzyme is responsible for the conversion of palmitoyl-CoA (16:0) and stearoyl-CoA (18:0) into palmitoleoyl-CoA (16:1) and oleoyl-CoA (18:1), respectively (Hsieh et al., 2004). In turn, these MUFA are used for TAG and other lipid synthesis or go through desaturation and elongation processes for producing longer and more unsaturated FA.

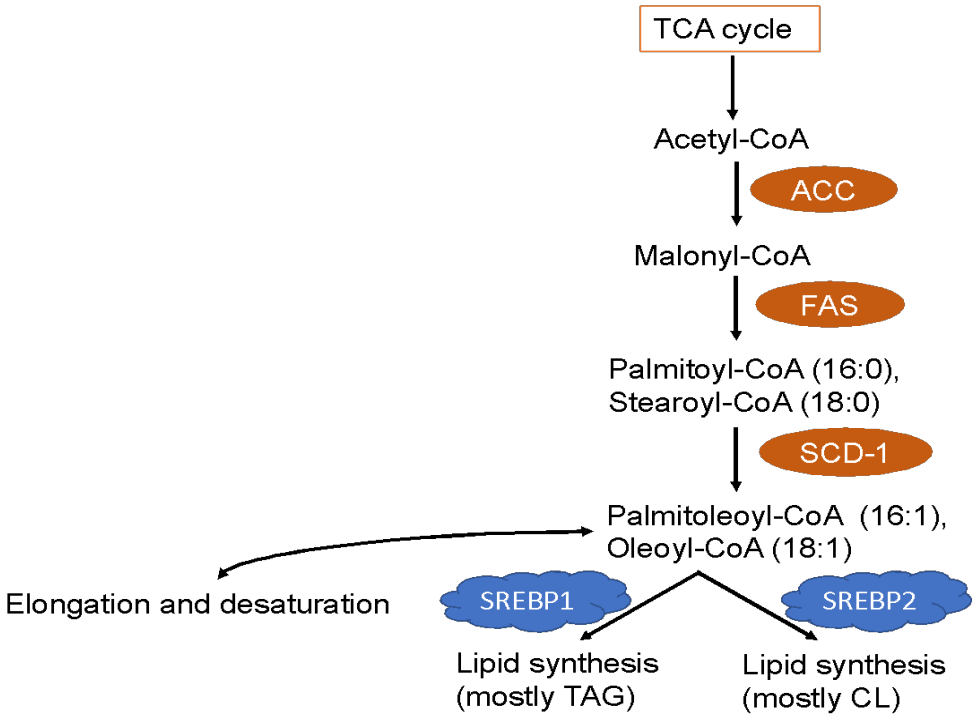

Figure 1.4. Lipogenesis and de novo fatty acid synthesis.

TCA, tricarboxylic acid cycle; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; SREBP, sterol-regulator element-binding protein; TAG, triacylglycerol; CL, cholesterol.

1.5 FATTY ACID REQUIREMENTS IN TELEOST FISH

Fatty acid requirements vary qualitatively and quantitatively along vertebrate subphylum. This is determined by the presence and activity of specific enzymes capable of producing *de novo* fatty acids independently from dietary supply. These enzymes are mainly fatty acyl desaturases (FADS) and elongases (ELOVL). Desaturation pathways comprises the removal of two hydrogen atoms from a fatty acid and the formation of a double bound between two carbon atoms to form more unsaturated fatty acids from less unsaturated precursors. Desaturases are commonly represented by the symbol Δ followed by the position where the double bound is introduced. In the present thesis, both FADS Δ terminology are used. In contrast, elongation is the process by which the fatty acid carbon chain is extended, by the addition of carbon atoms to the fatty acid chain (Tocher, 2003, 2015; Izquierdo, 2005).

1.5.1 QUALITATIVE REQUIREMENTS – INFLUENCE OF ENVIRONMENT, TROPHIC LEVEL AND PHYLOGENY

All living organisms can desaturate 16:0 and 18:0 into 16:1n-7 and 18:1n-9, respectively. This process occurs in the endoplasmic reticulum of cells of certain tissues through an aerobic via requiring NAD(P)H and catalysed by SCD-1, which have a Δ9 desaturase activity (Tocher, 2003). Further desaturation involves Δ12 and Δ15 desaturases to produce LNA and LA from their precursor OA, and these enzymes are missing in most vertebrates, including in fish (Tocher, 2006, 2010). Therefore, vertebrates are considered to have an essential requirement (EFA) for these specific PUFA. However, the most biologically active forms of these FA are generally their desaturation products, particularly 20:4n-6 (ARA), 20:5n-3 (EPA) and 22:6n-3 (DHA), that play important functions in development, metabolism, and health, as described above (Section 1.3).

In addition, among vertebrates there are different abilities to perform the conversion of 18:2n-6 and 18:3n-3 to longer and more unsaturated FA. These conversions (Figure 1.5) involve an enzymatic package that includes enzymes that includes FADS1 and FADS2 with Δ4, Δ5, Δ6 and Δ8 activities, and elongases (ELOVL5 and ELOVL2). In contrast to mammals, that have FADS1 and FADS2, with Δ5 and Δ6 desaturase activities, respectively, most teleost have lost the *fads1* gene and, therefore, *fads* genes were mostly identified as mammals *fads2* orthologues, with a greater diversification in its functionality among the different species, presenting from Δ6 activity only to combined Δ6/Δ8, Δ6/ Δ5 or Δ4/ Δ5 functional activities (Garrido et al., 2019). The functionality of FADS2 in fish is hypothesised to be driven by several factors, including the environment, trophic level, and phylogeny (Garrido et al., 2019). The environment in which fish evolved has conditioned the type and availability of food that fish have access to, which was determinant in the essentiality of nutrients in these organisms, in particular FA (Tocher, 2015). In marine environments, phytoplankton species are primary producers of n-3 LC-PUFA, which are uptaken by zooplankton and those by fish species. Thus, n-3 LC-PUFA are accumulated throughout the marine food chain and are highly available in marine environments. Therefore, marine teleost species had no evolutionary pressure to endogenously produce these FA and due to their low or nule production, n-3 LC-PUFA are considered essential for marine fish species and need to be supplied in the diet. The inability of most marine fish to synthesize these FA *de novo* in sufficient quantities to satisfy their requirement is mostly due to an impairment, deficiency, or low activity in the Δ4, Δ5 and Δ6 desaturases and elongases. In contrast, freshwater environments have low presence of n-3 LC-PUFA because freshwater phytoplankton are mostly producers of 18:2n-6 and 18:3n-3 and not of so much DHA, EPA, and ARA. Despite having moderate quantities of EPA, freshwater phytoplankton is characterised by low DHA contents. Consequently,

freshwater fish species maintained the evolutionary pressure to produce LC-PUFA from their precursors and, therefore, have higher activity of desaturases and elongases than marine fish (Tocher, 2010). In conclusion, whereas it is considered that in freshwater fish, the EFA requirements can be provided mainly by LA and LNA, in marine fish the requirements are provided by n-6 and n-3 LC-PUFA, particularly ARA, EPA and DHA, respectively (Watanabe, 1993; Bell and Sargent, 2003; Tocher, 2003).

Additionally, fish nutritionists have postulated that the trophic level might play a role in the qualitative EFA requirements of fish (Tocher, 2010). This issue has been raised with more attention when a study with the marine herbivore white-spotted spinefoot, *Siganus canaliculatus*, showed the activity of Δ4 and Δ5/Δ6 desaturases (Li et al., 2010). After this, more studies reported the existence of Δ4 desaturase in fish species from different habitats and trophic levels (Fonseca-Madrigal et al., 2014; Garrido et al., 2019; Kuah et al., 2015; Morais et al., 2012, 2015; Oboh et al., 2017). Therefore, it has been hypothesized that, irrespective of the environment in which fish evolved, low trophic level species (mostly herbivorous) would be capable of synthetize *de novo* LC-PUFA and, therefore, would have a specific dietary requirement for C_{18} PUFA. In contrast, higher trophic levels (mostly carnivorous) would be unable to synthesize LC-PUFA from C_{18} precursors and, therefore, would have an essential requirement for intact LC-PUFA. Even so, some exceptions to this generalisation need to be assumed, for instance species in the intermediary trophic levels, which may have an essential requirement for either C_{18} PUFA and LC-PUFA, depending on other aspects of their life history and ecology (Trushenski and Rombenso, 2020). Nevertheless, a recent study showed that the variability of FADS2 seems to be more influenced by species' phylogeny than the trophic level (Garrido et al., 2019). Therefore, the aspects driven PUFA biosynthesis capacity and functionality in fish are still controversial, not fully understood, which justifies their continuous study.

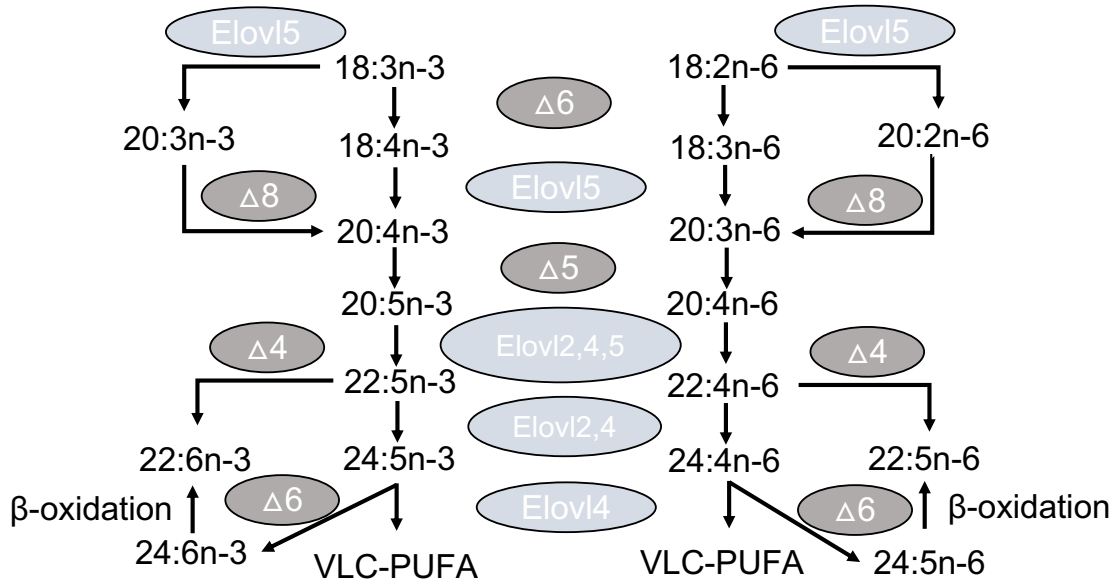

Figure 1.5. Desaturation and elongation pathways in de novo fatty acid synthesis.
Δ, delta desaturases; Elovl, elongases; VLC-PUFA, very-long chain polyunsaturated fatty acids (more than 24 carbons).

1.5.2 QUANTITATIVE REQUIREMENTS DURING ONTOGENY

It is difficult to quantify a single absolute quantitative requirement for EFA in fish since this requirement varies with developmental, physiological stage, nutritional condition, dietary lipid content and environmental conditions such as temperature, salinity, and light (Izquierdo, 2005). Furthermore, the determination of EFA requirements is also dependent on the criteria used to evaluate it.

Most fish nutritionists considered, at least, two requirement "levels". The first is the physiological/maintenance requirement that prevents deficiency symptoms and pathologies. The first studies that aimed to determine EFA requirements in fish reported that these requirements are relatively low, around 1% dry weight of the diet (reviewed in Tocher, 2010). However, many of those early studies were estimated with a low dietary lipid level, known to also influence EFA requirements (Izquierdo, 2005). Nowadays, modern fish diets include high energy and lipid contents, which raised the need to revalidate quantitative EFA requirements for farmed fish species. The second "level" of requirement could be the optimal requirement, where further supplementation above physiological requirement level leads to an improvement in survival, growth, health, or other productive parameters to the maximum potential.

Furthermore, this level also directly increases the deposition of FA in fish muscle, improving flesh nutritional lipid quality for human consumption.

Despite all these different perspectives and concepts, the quantitative EFA requirements are, however, mostly determined by life stage and physiological condition of fish. In general, fish in early life stages, namely in larval stage, have higher physiological requirements compared with juveniles and sub-adult fish due to the very high larval growth rate (Table 1.1; Izquierdo, 2005). This is particularly true for DHA in marine larvae, also due to its function in the development of neural and visual organs, as described above (Section 1.3.3). For instance, in gilthead sea bream (*Sparus aurata*) larvae, a minimum n-3 LC-PUFA requirement was estimated by several studies at 1.5% in dry weight, whether larvae are fed live preys (Rodríguez et al., 1998; Izquierdo 2005; Tocher, 2010) or microdiets (Salhi et al., 1999; Izquierdo, 2005; Tocher, 2010). This requirement was estimated based on a DHA/EPA ratio of 2 and it was further is increased up to 5.5% when the DHA/EPA ratio was reduced to 0.3 (Rodríguez et al., 1994), denoting that the relative proportions between EFA are very important for marine larvae, due to interactions between both FA. The increasing of EFA requirements with the decrease of DHA/EPA ratio is explained by the competition of EPA with DHA for the incorporation into PL, which can lead to the displacement of DHA from its normal position (Izquierdo et al., 2000; Izquierdo, 2005). High optimal n-3 LC-PUFA requirements to maintain growth throughout the life cycle were also estimated for gilthead sea bream larvae (more than 3%; Izquierdo, 2005) and red sea bream (*Pagrus major*) larvae (2.1%, with 1.0% being DHA) (Furuita et al., 1996). Fast-growing species, such as greater amberjack (*Seriola dumerili*) or meagre (*Argyrosomus regius*), also require a very high n-3 LC-PUFA contents (at least 4 and 3%, respectively) in their weaning diets (Izquierdo, 2005; El Kertaoui et al., 2015).

In contrast, for juveniles of marine species, n-3 LC-PUFA physiological requirements were estimated at up to 1%, including in turbot (*Psetta maxima L.*) or European sea bass (*Dicentrarchus labrax*) (Tocher, 2010). Other species, including gilthead sea bream or yellowtail (*Seriola quinqueradiata*) have a physiological requirement for n-3 LC-PUFA above 1%. Concretely in gilthead sea bream, 1.5-1.8% dry weight is required to optimize growth (Ibeas et al., 1994) and stress resistance (Montero et al., 1998), respectively, with a 12-15% dietary lipid level. However, 2.5% is recommended to optimize growth during the on-growing period with high lipid diets (Izquierdo, 2005). Similarly, in meagre juveniles an optimal requirement of 2.1% was estimated to maximize growth and hepatic function for only 30 days of feeding (Carvalho et al., 2018, 2019), whereas for greater amberjack around 2.5% is necessary (Izquierdo, 2005).

Although studies on the specific EFA requirements on broodstock are less available, it is considered that EFA demand can also increase during the reproduction season (Izquierdo, 2005). Indeed, studies reported that egg quality, hatching and fertilization rates, as well as survival of the progenies, were improved with increased levels of n-3 LC-PUFA, DHA:EPA ratio and ARA in gilthead sea bream (Izquierdo, 2005; Salze et al., 2005), establishing a n-3 LC-PUFA requirement of 1.5-1.6% in dry weight for optimal spawn quality (Fernandez-Palacios et al., 1995) or even up to 2.2% if an additional antioxidant source could be added to the diet (Izquierdo et al., 2001).

Regarding n-6 LC-PUFA, requirements per se are less studied, but many studies reported beneficial effects in fish performance along all fish life stages. For instance, dietary ARA supplementation up to 1% improved growth in gilthead sea bream larvae (Bessonart et al., 1999), as well as survival after stress (Koven et al., 2001). Furthermore, ARA is required at 0.8% in dry weight in flatfish juveniles, such as turbot (Castell et al., 1994) or at 2% in Atlantic halibut (*Hippoglossus hippoglossus*) broodstock (Broomage et al., 2001).

Table 1.1. N-3 long-chain polyunsaturated fatty acids requirements (%) of Mediterranean marine fish species

	Larvae	Juveniles	Broodstock
Sparus aurata	>3	2.5	2.2
Psetta maxima	3.2[2]	0.8[3]	n.d.
Dicentrarchus labrax	2.3[12]	>3.5[4]	2.7[13]
Seriola dumerili	4[1]	2.5[1]	1.7[14]
Pagrus pagrus	<3.4[5]	<5[1]	n.d.
Dentex	4[9]	n.d.	n.d.
Argyrosomus regius	>3[10]	2.1[11]	n.d.

Adapted and updated from Izquierdo (2005). [1]Izquierdo (own data); [2]Le Milinaire, 1984; [3]Gatesoupe et al., 1977; [4]Lanari et al., 1999; [5]Hernández-Cruz et al., 1999; [6]Radunz-Neto et al., 1993; [7]Corraze et al., 1933; [8]Takeuchi and Watanabe, 1976; [9]Mourente et al., 1999; [10] El-Kertaoui et al., 2015; [11] Carvalho et al., 2018; [12]Villeneuve et al., 2005; [13]Bruce et al., 1999; [14]Sarih et al., 2020; n.d., not determined.

1.6 METABOLIC CONSEQUENCES OF LIPID IMBALANCE AND EFA-DEFICIENCY IN FISH

In fish, the most obvious clinical signs of EFA-deficiency are poor growth and increased mortality. However, other pathologies are frequently observed, as myocarditis, fins erosion, shock syndrome, lordosis, bleeding, and disaggregation of gill epithelia, as well as increased sensibility to stressful situations, immune-deficiency, increased cortisol levels, and decreased reproductive capacity (Izquierdo, 2005; Glencross, 2009; Tocher, 2010). In addition, a common sign is the appearance of fatty livers (steatosis). This is because n-3 LC-PUFA increase β-oxidation, phospholipid synthesis and, consequently, lipoprotein formation avoiding hepatic lipid accumulation (Fukuzawa et al., 1971; Caballero et al., 2004; Kjaer et al., 2008). Consequently, an increase in dietary n-3 LC-PUFA has been shown to reduce hepatic lipid vacuoles by reducing lipogenesis, including triacylglycerols synthesis (Berge et al., 1999; Leaver et al., 2008). Therefore, when EFA-deficiency occurs, lipid mobilization is deficient and hepatic lipid infiltration surpasses liver storage capacity, negatively affecting its functionality, which in extreme cases, can result in fish death (Spisni et al., 1998; Caballero et al., 2004).

A few biochemical markers can be helpful to identify EFA-deficiency in fish. When the level of dietary EFA is insufficient, the production of mead acid (20:3n-9) in freshwater fish, and its ratio to DHA increase. Values of 20:3n-9/DHA higher than 0.4 are considered indicators of EFA-deficiency (Castell et al., 1972; Watanabe et al., 1974; Watanabe et al., 1989; Glencross, 2009). However, in marine fish, the production of mead acid is usually not a suitable indicator of EFA-deficiency, since marine species lack the enzymes necessary to produced it from its precursor OA (18:1n-9), as these enzymes are the same involved in the production of EPA and ARA (in this case the Δ6 desaturase), as mentioned in the section above (Section 1.5.1) (Tocher, 2010). Despite this, 18:2n-9 and 20:2n-9 have been reported sometimes in tissues of sea bream and turbot fed low EFA diets (Tocher et al., 1988; Kalogeropulos et al., 1992). However, using cell lines from marine or freshwater species origin, n-9 FA, including OA, were accumulated in cell lines grown in the absence of EFA and were reduced when they were supplemented (Tocher et al., 1989). Therefore, for marine fish species, the ratio OA/n-3 LC-PUFA have been considered a biochemical indicator of EFA-deficiency (Fujii et al., 1976; Kalogeropoulos et al., 1992; Montero et al., 2001).

1.7 SOURCES OF LIPIDS AND ESSENTIAL FATTY ACIDS IN MODERN FISH DIETS FOR THE FURTHER DEVELOPMENT OF AQUACULTURE

1.7.1 FISH OIL AS THE TRADITIONAL LIPID SOURCE – THE REAL PROBLEM

FO and FM were the primary traditional ingredients used in aquaculture feeds since the beginning of the intensive systems of production. This was mostly due to their high digestibility and palatability by fish and their balanced nutrient composition, meeting nutritional requirements of fish species, basically because they are the natural food for many fish in natural environments (NRC, 2011). FO and the lipids derived from FM, are the major source of n-3 LC-PUFA in aquafeeds. Worldwide production of FO and FM fluctuates with several factors, particularly the changes in wild stocks of pelagic species (the most used for FM and FO production), as well as with the occurrence of the El Niño, affecting species abundance. The most used species for FO (and FM) production are pelagic species, including anchovy, capelin, herring, cod, or menhaden, which differ in their fatty acid composition, with those produced from anchovy and menhaden oils being usually the richest in DHA and EPA (Figure 1.6).

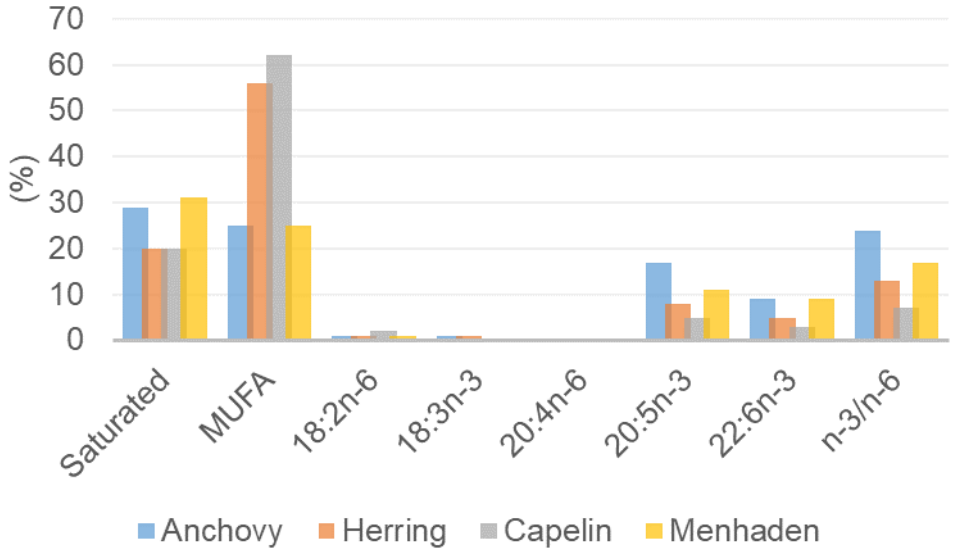

Figure 1.6. Fatty acid composition of fish oils commonly used in aquaculture feeds, extracted from different fish pelagic fish species.
(Data collected from Turchini et al., 2009; values presented are a mean between a specific range of fatty acids found for each species oil).

When aquaculture production started, these ingredients were largely available in the market at lower cost. In 1994, the use of fish to produce FO and FM reached its maximum peak with more than 30 million tonnes (FAO, 2020). Indeed, in 2006, the aquaculture industry used 87% of the global supply of FO in feeds (Tacon et al., 2006; Turchini et al., 2009). However, the unsustainable use of finite resources and the dramatic decrease of fish wild stocks led to the stagnation in the availability of these marine ingredients coming from fisheries, decreasing to 14 million tonnes in 2014 (FAO, 2020). This decrease in supply was accompanied with an increase in their market prices, surpassing the market prices of some plant raw materials, for instance derived from soy (soybean oil and meal) (Figure 1.7). Additionally, FO and FM deterioration is fast, turning rapidly rancid and needing the use of special and expensive measures to avoid it. Moreover, fish of poor quality yield malodorous oil with high contents of FFAs, sulphur, and the accumulation of persistent organic pollutants and heavy metal has been also concerned (NRC, 2011). These environmental and economical sustainability issues led to a decrease in the use of FO and FM in fish diets and their replacement by alternative sources in the last decades. Currently, their use in high quantities is almost limited to some specific phases of the production cycle, which require high amounts of n-3 LC-PUFA such as weaning or broodstock diets. In contrast, their use in feeds for the on-growing period is constantly decreasing, accounting for instance to only 10% of the diets in Atlantic salmon production (FAO, 2020). However, whereas great improvements in reducing and replacing FM in marine fish diets was accomplished in the last years, the total replacement of FO is still a major challenge (Colombo and Turchini, 2021). Consequently, the most recent data showed that aquaculture feed industry is still responsible for 75% of the use of the total FO production (Colombo and Turchini, 2021).

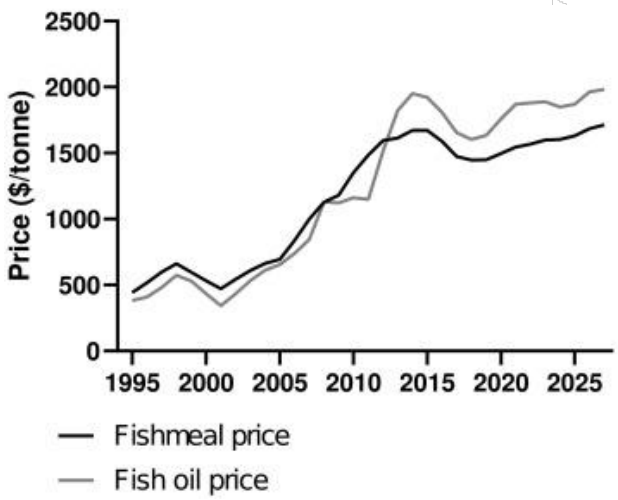

Figure 1.7. Real and expected fluctuations in market price of fish meal and fish oil from 1995 to 2025. Source: Kok et al. (2020).

1.7.2 VEGETABLE OILS AS ALTERNATIVE LIPID SOURCES

Some of the most studied alternatives to FO in fish feeds are oils derived from terrestrial plants. Contrary to FO, vegetable oils (VO) production showed an increasing tendency during the last decades, with palm oil, soybean oil and rapeseed oil on the top of the most produced VO, even the most produced oils in the world (Turchini et al., 2009). As consequence of their wide availability, their market prices are considerably lower than that of FO. Therefore, VO were considering attractive alternatives to FO, and intensive research during the past two decades was done to study their inclusion for fish diets and their effects on fish growth and health. The current knowledge shows that VO are rich in SFA and MUFA (Table 1.2) and are similarly used as energy source by fish as FO, being similarly catabolized for growth (Bell et al., 2001; Turchini et al., 2009). However, despite VO have an interesting price as lipid sources for fish, they are devoid of n-3 LC-PUFA, and consequently EPA and DHA levels in fillets are reduced when fish are fed a VO-based diet (Turchini et al., 2009). This impacts not only the final product for the consumer, diminishing the benefits of n-3 LC-PUFA intake through fish consumption, but could also affect fish production itself, particularly marine carnivorous species, for who's these FA are essential (Izquierdo, 2005). Therefore, although blends of VO are successfully used to mimic SFA, MUFA and PUFA composition of FO, their lack in LC-PUFA content, limit their use as total replacers of FO in marine carnivorous fish diets. This often causes an EFA-deficiency in fish, particularly when FM, that also contributes for the residual n-3 LC-PUFA dietary content, is concomitantly replaced with FO. Furthermore, VO often contain high n-9 and n-6 fatty acids, mainly OA and LA, respectively, which might cause an imbalance in FA, with inadequate n-3/n-6 ratios and, consequently, reduce fish health for

instance by altering eicosanoids synthesis (Fracalossi et al., 1994). High percentages of FO replacement by VO were also reported to cause growth depletion, immunosuppression, and reduced stress resistance due to an EFA-deficiency (Montero et al., 2003). Besides, lower digestion rates of some VO with very high SFA and MUFA contents were also reported because these FA are known to be digested and absorbed slower than PUFA (Ng et al., 2004; Francis et al., 2007). Indeed, FA digestibility is partly determined by their melting point, which increases with the decrease of unsaturated bounds in the FA chain. Therefore, the higher the melting point of the FA, the lower digestibility, although FA digestibility is also dependent on the pH, morphology, and length of the digestive tract, as well as the enzymatic capacity (Morais et al., 2005; Francis et al., 2007). Additionally, n-3 FA are better digested and absorbed in fish intestine than n-6 or n-9 FA (Izquierdo et al., 2000; Francis et al., 2007). Consequently, the typical high contents in n-6 and n-9 FA, SFA and MUFA and the low n-3 PUFA of many VO, are reflected in a lower digestibility of the feed by fish (Francis et al., 2007). Furthermore, feeding on VO can also affect feed intake of the fish by altering digestible energy and/or palatability of the feed. Indeed, some studies with rainbow trout reported that fish can distinguish from the different types of oils and show a preference for FO when compared with VO (Guerden et al., 2005, 2007).

Table 1.2. Main fatty acids (% total fatty acids) of conventional lipid sources used in modern diets for farmed fish

	SFA	MUFA	LNA	LA	ARA	EPA	DHA
FO (Anchovy)	29	25	1	1	0.1	17	9
Soybean oil	14	23	7	51	-	-	-
Palm oil	49	37	0.2	9	-	-	-
Rapeseed oil	5	62	12	20	-	-	-
Poultry oil	29	42	1	20	-	-	-
Tallow oil	48	41	0.6	3.1	0.4	-	-

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; LNA, linolenic acid (18:3n-3); LA, linoleic acid (18:2n-6); ARA, arachidonic acid (20:4n-6); EPA, eicosapentaenoic acid (20:5n-3); DHA, docosahexaenoic acid (22:6n-3); Adapted from Turchini et al. (2009).

1.7.3 TERRESTRIAL ANIMAL-DERIVED OILS AS ALTERNATIVE LIPID SOURCES

Oils and fats derived from by-products of terrestrial animals, including poultry, lard and tallow were also positioned as cost-effective and more sustainable sources than FO, due to their constant availability and low market prices, even cheaper than soybean or palm oil, for instance (Bureau and Gibson, 2004). Indeed, the worldwide animal rendering industry processes over 60 million tonnes annually, of which 12 million tonnes are produced in Europe (Tacon et al., 2008; Turchini et al., 2009). In 2001, the European Union banned the use of some rendered animal products (protein, fats, and other by-products) in all farmed animals feeds due to the risk of zoonotic transmission, particularly of transmissible spongiform encephalopathies (Jędrejek et al., 2016). However, with the reduced risk of these zoonotic diseases, on 1st June 2013, EU Commission, revised the strict rules applied to animal by-products and allowed the use of non-ruminant processed animal by-products for aquafeeds, upon meeting regulatory criteria regarding its source, production, and treatment (Jędrejek et al., 2016).

From the entire animal rendered fats, poultry oil (PO) represents 25% of the total production and, therefore, is the most used and studied terrestrial animal oil in aquaculture feed industry (EFPRA, 2021; Campos et al., 2019). The nutritional composition of rendered animal fats, in particular their FA composition, depends on the nutritional status of the original animal, species, stage of development, processing, etc. Therefore, a high variability can be observed in these types of sources. However, generally they are recognised for their high SFA and MUFA contents, being considered a good energy source for fish (Turchini et al., 2009). For instance, PO contains around 29% of SFA, whereas tallow oil can contain up to 48% (Table 2). PO also presents high contents of LA, particularly derived from feeding poultry with VO rich in this FA, but still lower when compared with soybean oil. However, terrestrial animal oils have very low n-3 PUFA contents, including only up to 1% of LNA, and thus n-3/n-6 ratios are very low. Furthermore, like VO, terrestrial animal fats lack n-3 LC-PUFA. Consequently, FO can only be completely replaced without negative consequences on survival, growth, or fillet organoleptic characteristics if the diet contains sufficient FM or other EFA source to cover n-3 LC -PUFA requirements of the target species (Monteiro et al., 2018; Campos et al., 2019; Carvalho et al., 2020).

In summary, the amount of FO that could be replaced by these conventional and mostly used alternative lipid ingredients, depends on the EFA requirements of the species and the stage of development. Therefore, 60-100% of replacement is possible without negative effects on fish productivity in most species, if EFA are met by the remaining FO and FM present in the diet. Although the simultaneous replacement of FO and FM in carnivorous fish diets is less

studied and needs further attention, the available studies point out that the lower the dietary FM content, the more challenging the FO replacement (Drew et al., 2007; Torrecillas et al., 2007a,b; Turchini et al., 2009). Furthermore, lipid metabolism is inherent to each species and also influences the degree of replacement, being more difficult to successfully replace in marine carnivorous species, such as gilthead sea bream or meagre, which have a high specific requirement for n-3 LC-PUFA, than in salmonids that have some desaturation and elongation capacity (reviewed in Turchini et al., 2009). Despite this, nowadays aquaculture feeds include high percentages of plant or other conventional ingredients to reduce the amount of FM and FO, being more environmentally and economically sustainable and, thus, more responsible. For instance, the use of FM and FO in Norwegian aquaculture, one of the top European aquaculture producers, and the most important world producer of salmon, decreased from 63% in 1990 to 25% in 2010. An opposite tendency was observed for the inclusion of plant proteins, which represented a 36% of aquaculture feeds in Norway (Nofima, 2011). However, FO was only reduced from 23% to 17% between 1990 and 2010, reflecting the challenge to replace FO with good alternative sources (Nofima, 2011). Therefore, whereas these numbers represent an achievement of 60% of replacement of FM, for FO they represent a much lower percentage (only 20% of replacement). Furthermore, although some conventional ingredients would not have a negative impact on fish performance, the marked reduction of EPA and DHA levels in fish fed VO, PO, or similar sources with poor n-3 LC-PUFA contents, remains to be solved, irrespectively of the species. To solve all these issues, a new era of research was opened in aquaculture nutrition in the recent years, for finding novel n-3 LC-PUFA-rich sources for aquafeeds, that do not compromise the cost-effective and sustainable potential of other conventional sources, while increase fish productivity and the nutritional value of the final product for the consumers.

1.7.4 Novel n-3 LC-PUFA sources

1.7.4.1 Fish by-products and by-catches

Fisheries produce annually 25% of fish wastes or by-products (Racioppo et al., 2021). These “wastes” include parts of the fish, mainly viscera, that are wasted in the processing industry before fish reaches the consumer, as well as fisheries by-catches that have a low market value due to small size, injuries or for being an untargeted product. However, they can represent a valuable resource as ingredients in fish feeds, by converting them into more sustainable FM or FO (Kim and Mendis, 2006; Ramírez-Ramírez et al., 2008; Afreen and Ucak, 2020). Furthermore, while these products are much more available and are usually

cheaper than the traditional FM and FO, mostly because they are not used for human consumption, they can further provide protein, lipids, or other essential nutrients, including n-3 LC-PUFA, for farmed animal feeds (Esteban et al., 2007). Indeed, in many species, viscera (liver), presents a high potential for oil production due to its high lipid and n-3 LC-PUFA content. However, the use of these "wastes" for FO production, is dependent on the species used, which represent some limitations since the by-catches include for instance varied species, not necessarily oily species that are the richest in n-3 LC-PUFA (Lekang and Gutierrez, 2007; Batista, 2007).

1.7.4.2 LOWER TROPHIC MARINE ORGANISMS

N-3 LC-PUFA are primarily produced in the lowest levels of the trophic chain by microalgae and are bio-transferred and bioaccumulated along the trophic chain. Therefore, marine organisms in the next trophic level, particularly zooplankton which fed on microalgae, are characterised by high PUFA contents and thus can be a valuable source of nutrients for feeds. Lower trophic species present larger biomasses, but their fishing still presents technical difficulties and high costs, limiting the market (Pauly et al., 1998). Moreover, it also relies on the question if they are really a sustainable source and if overfishing of these organisms, for instance krill, would negatively impact marine ecosystems since it serves as feed for many important species from high trophic levels. Most research on production of oils from lower trophic marine organisms have focused on Antarctic krill (*Euphasia superba*), which are, along with the calanoid copepods (*Calanus finmarchicus*), the only species being commercialised at large-scale production (Olsen et al., 2006). Copepods, produced as live feeds for fish larvae, accumulate high levels of lipids (50-70%) with high n-3 PUFA, but generally are rich in wax esters, which are less digestible. Antarctic krill is whether used to produce krill meal (KM) with protein, amino acids, and ash levels comparable to those of FM and highly palatable, or krill oil (KO) rich in DHA and EPA (Tou et al., 2007). Moreover, n-3 LC-PUFA from KO are predominantly in the PL form (40-80%), mainly in PC (45-99% of total PL) compared to the TAG form of FO, and it seems to exist an apparent higher bioavailability in the absorption of PL through intestinal wall when compared to TAG (Burri and Johnsen, 2015; Ulven, 2015). Therefore, it has been hypothesized that n-3 LC-PUFA from krill oil are more bioavailable than those coming from FO (Burri and Johnsen, 2015; Ulven, 2015; Kim et al., 2020). Indeed, many studies demonstrated that KO was more effective in enhancing n-3 LC-PUFA levels compared with FO (Ramprasath et al., 2013). For instance, in golden pompano (*Trachinotus Ovatus*), KO successfully replaced the total FO, without affecting growth performance and feed utilization, while increasing DHA and EPA in fish muscle (Xiuling et al., 2019). Furthermore,

KO is also rich in other micronutrients, particularly vitamins and antioxidants, such as astaxanthin, which are important functional nutrients for fish (Zhou et al., 2017). KM incorporation has been also associated with lower hepatic vacuolization in fish and better growth performance, partially also due to the high EFA contents of its lipid fraction, successfully replacing FM (Saleh et al., 2018; Torrecillas et al., 2021). However, despite the future high potential of krill raw materials as n-3 LC-PUFA sources, given the high costs of production, they are still being more used as “Premium” ingredients rather than primary sources of EFA. Consequently, they are being incorporated only in specific phases of fish grow-out cycle. For instance, in salmonids diets, KM is incorporated at 10% to increase growth rate during the seawater phase (Tocher, 2015).

1.7.4.3 GENETICALLY MODIFIED PLANTS

Another novel and promising alternative for aquafeeds are genetically modified (GM) plants, being GM canola (*Brassica napus*) and GM camelina (*Camelina sativa*) the most studied. The genetic modification comprises the introduction of genes responsible for the *de novo* production n-3 LC-PUFA into plants, which normally do not produce or contain LC-PUFA, as aforementioned (Section 1.7.2). For instance, in GM camelina, 5 to 7 desaturases and elongases genes originally from microalgae could be introduced, to produce oils with EPA (24% of total FA) or EPA+DHA (11% and 8% of total FA, respectively) (Ruiz-Lopez et al., 2014). Therefore, GM plants could represent a viable alternative to FO for fish feeds (Usher et al. 2015). Similarly, in canola genetically modified with the polyketide synthase gene from bacteria and other marine eukaryotes, EPA, and DHA levels of 0.7 and 3.7% of total FA can be obtained (Walsh et al., 2016). Polyketide synthase is an alternative pathway to the desaturase and elongation pathway that is used by a few organisms to produce unsaturated FA (Napier et al., 2015). The same mechanism was introduced in soybean, producing oil with 2.7% of DHA and 1.5% of EPA, although polyketide synthase mechanism is not so effective in increasing EPA and DHA levels as phytoplankton desaturases and elongases.

GM camelina oil, rich in EPA and DHA, allowed the total replacement of FO in Atlantic salmon diets without effects on growth, metabolism or health and doubled EPA and DHA levels in fish fillets when compared to fish fed a reference modern diet (blend of FO and VO) (Betancor et al., 2017). Given the promising results in salmon, studies on the replacement of FO by GM camelina oil rich in EPA and DHA were further carried out in other farmed species, including in gilthead sea bream and European sea bass, which reported similar growth

performance, metabolism, and health, as well as similar EPA and DHA levels in fish fillets, when compared to a control FO diet (Betancor et al., 2016a, 2021; Huyben et al., 2020).

Despite the high potential of GM plant oils, production of these organisms is still low. Although some products derived from GM plants have recently reached the market in the USA, the regulation of these products in Europe faces significant regulatory terms (Sprague et al., 2017). Furthermore, another challenge of incorporating high quantities of GM plants in human or animal feeds is directly related with consumer acceptance (Sprague et al., 2017). Thus, oils derived from GM organisms with enhanced EPA and DHA levels could potentially solve n-3 LC-PUFA demand for aquaculture nutrition in the future, once rules and laws become slight to cultivate these organisms and society accept more easily these products.

1.7.4.4 MICROALGAE

Microalgae are probably the most promising n-3 LC-PUFA source for the aquaculture feed industry. They are the primary natural producers of n-3 LC-PUFA in aquatic environments, which depend on light as energy source. They are not only important as natural feed for many aquatic species but also produce around 80% of global oxygen of the Earth (Enzing et al., 2014; Borowitzka, 2018; Deviram et al., 2020). Microalgae comprise a diverse group of organisms, from unicellular prokaryotic, such as cyanobacteria (belonging to bacteria but often included in microalgae taxonomy), to eukaryotic photosynthetic microorganisms (dinoflagellates, diatoms, and other brown and green algae). Being autotrophs or very simple heterotrophs, these microorganisms are considered more sustainable than other lipid sources for their low footprint and their less use of land and non-recycled water (Tibbetts, 2018; Shah et al., 2018). Algae biomasses are a rich source of nutrients such as amino acids, EFA, vitamins and natural antioxidants, positioning these products as promising ingredients in fish feed formulations. Indeed, the use of microalgae in aquaculture is not new, since they have been extensively used for over 40 years in hatcheries to: enrich live feeds or rotifers and Artemia for larval diets (Barclay and Zeller 1996; Benemann 1992); as "green water" and "pseudo green water" techniques, blooming or adding microalgae in fish larval rearing tanks (Shields and Lupatsch, 2012); or as microalgae concentrates in weaning diets for larvae (Becker, 2004; Patil et al., 2005; Shields and Lupatsch, 2012).

Several microalgae species, that are produced autotrophically or heterotrophically, have been tested in marine fish diets for their high EPA and/or DHA contents including those from the genus *Crypthecodinium* (Eryalcyn et al., 2015; Schafberg et al., 2018, 2020), *Nannochloropsis* (Eryalcyn et al., 2015; Metsoviti et al., 2018), *Phaeodactylum* (Ruyter et al.,

2016; Sørensen et al., 2016) and, particularly, *Schizochytrium sp.* (Metsoviti et al., 2018; Perez-Velazquez et al., 2018; Kosoulaki et al., 2020). The lipid contents and dietary fatty acid profiles of these microorganisms depend on the species and growth stage, but also on different aspects of the production and the processing technology employed. Therefore, dietary FA profiles of these lipid sources or their combinations can be tailored to match the EFA requirements of the different fish and crustacean species and life stages. For instance, products derived from the genus *Crypthecodinium, Thraustochytrium* or *Schizochytrium* are very high in DHA (Qiu et al., 2001; Ganuza and Izquierdo, 2007), whereas those from *Phaeodactylum* or *Nannochloropsis* are rich in EPA (Atalah et al., 2007; Eryalcyn et al., 2015). Thus, complete replacement of FO by a *Crypthecodinium* product in early weaning diets for gilthead sea bream allowed to cover the DHA requirements of this species (Eryalcyn et al., 2015). However, sea bream requirements for EPA were not covered by this microalga, and other EPA sources, such as the algae *Nannochloropsis gaditana,* had to be supplemented to sustain maximum growth (Eryalcyn et al., 2015). Furthermore, some species, for instance *Schizochytrium sp*., also presents high amounts of DHA, EPA and n-6 DPA (approximately 20% of the DHA produced), increasing the levels of these FA in fish fillets (Kousoulaki et al. 2015, 2016; Sprague et al. 2015). *Schizochytrium sp*. meals were also efficient in totally replace FO in diets for Atlantic salmon (Mizambwa, 2017), red drum (*Sciaenops ocellatus*) (Pérez-Velazquez et al., 2018), pacific white shrimp (*Litopenaeus vannamei*) (Wang et al., 2017) and Nile tilapia (*Oreochromis niloticus*) (Sarket et al., 2016) without affecting fish productive parameters.

Algal biomasses or oils can be used in aquafeeds. Whole-cell biomasses often show low digestibility due to the cell wall (Sprague et al., 2015). Extracting oil from algal biomass could potentially solve digestibility problems associated with cell walls in some species, but more technology needs to be employed for extracting oils, increasing the costs and, thus, whole-cell algae have been most used. Indeed, recently, oil extraction has been shown to be effective in supplying EFA for salmonids as well as in increasing the quality of the final product (Santigosa et al., 2021). Therefore, algal oils have also become commercially available in the recent years. In general, algal oils can be considered as LC-PUFA concentrates containing 40-60 % LC-PUFA in addition to SFA. The LC-PUFA fraction in an oil from fermentation of *Aurantiochytrium sp.* contains mainly DHA and n-6 DPA and minor amounts of EPA, whereas an oil from *Schizochytrium sp*. can contain both EPA and DHA and less n-6 DPA. Furthermore, compared to the biomass, the oils can more easily be combined with other oils to optimize the dietary fatty acid profile, thus providing higher flexibility in formulation and production of fish feeds. Despite their high potential, particularly as DHA sources in aquaculture feeds, feed

producers still need to face challenges, mostly because of their high costs of production, low market availability and the large variation in algae biochemical composition.

1.8 OBJECTIVES

The traditional marine ingredients used in aquaculture feeds (FM and FO) are no longer cost-effective and sustainable for fulfilling the n-3 LC-PUFA demand of aquaculture, which is in continuous expansion. Furthermore, aquaculture products are the primary source of n-3 LC-PUFA for human nutrition and thus providing adequate EPA and DHA levels in aquaculture products is of foremost importance. Given the consequences of using VO or PO as sole lipid alternatives to FO in modern marine fish feeds with less marine ingredients (Sections 1.7.2 and 1.7.3), new lipid sources rich in n-3 LC-PUFA are mandatory to guarantee an efficient fish production and increase the benefits of fish consumption for consumers. Within the potential ingredients studied in the last years, microalgae are one of the most promising alternatives as mentioned above (Section 1.7.4.4).

Thus, the main general objectives of the present thesis were:

1. To investigate the potential of the complete replacement of FO with commercial products from microalgae biomasses in diets for gilthead sea bream (*Sparus aurata*) larvae (Chapter 3).

2. To validate the potential of blending commercial microalgae oils with cheaper conventional alternative sources (PO and VO) for the complete replacement of FO, in practical diets for juveniles of two important marine species for the Mediterranean aquaculture: gilthead sea bream and meagre (*Argyrosomus regius*) (Chapters 4 and 5).

3. To understand the effects of the microalgal products on some indicators of fish health and welfare (Chapters 3, 5, 6 and 7).

4. To understand the effects of the microalgal products on fish lipid metabolism pathways (Chapters 3, 5 and 6).

5. To evaluate the effects of the long-term feeding with microalgal products on juveniles' behaviour and neural function (Chapter 7).

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1 GENERAL DESIGN AND OVERVIEW

Due to importance of lipids and fatty acids in the physiology of vertebrates, particularly the n-3 LC-PUFA, and being fish one of the few sources of n-3 LC-PUFA for human nutrition, there is a huge need for finding novel sustainable lipid and n-3 LC-PUFA sources for farmed fish. These sources should meet fish nutritional requirements while increasing the health benefits associated to fish consumption for consumers by increasing EPA and DHA levels on fish meat. Therefore, the present thesis focused on the evaluation of potential novel n-3 LC-PUFA sources from different microalgal products in diets for two important farmed marine fish species in the Mediterranean region (gilthead sea bream and meagre).

First, the potential of three commercial microalgal biomasses were evaluated as sole dietary sources of n-3 LC-PUFA, totally replacing FO in weaning diets for gilthead sea bream larvae. The larval stage was chosen because early stages of life are known to have higher nutritional requirements, including for EFA. The effects of the dietary formulations were assessed on larval performance, stress resistance, metabolism and the expression of some health-related genes were studied (Chapter 3).

Given the potential of microalgae products shown in Chapter 3 for larvae, the next step was to evaluate the potential of microalgae oils as n-3 LC-PUFA sources for sea bream juveniles. Microalgal oils were considered to facilitate the inclusion on fish diets because they avoid digestibility problems associated to microalgae cell walls. Furthermore, since microalgae are still high-costly resources for aquafeeds, oils allow more flexibility in the formulation by combining them with other cheaper lipid sources as a cost-effective strategy. Therefore, the total replacement of FO by two microalgal oils combinations with PO and VO were accessed in the growth performance of gilthead sea bream juveniles, n-3 LC-PUFA deposition and fillet lipid quality (Chapter 4). Besides, these combinations were assessed under two different FM replacement-approaches to evaluate the concomitant replacement of FO and FM.

The effects of the same dietary combinations were further validated in growth, health and flesh lipid quality of meagre juveniles, a more recent species for the diversification in aquaculture industry and known for its fast growth and its high requirement for n-3 LC-PUFA (Chapter 5).

Since the liver is the most key organ for lipid regulation, the hepatic lipid metabolism and health were assessed after feeding sea bream juveniles with practical diets that replaced FO by microalgal oils. For that, liver composition, hepatic indices, liver morphological evaluation as well as expression of lipid metabolism-related genes were determined (Chapter 6). Besides, these effects were assessed under two different FM replacement-approaches to evaluate the concomitant replacement of FO and FM.

Fish brain has a high demand of energy and EFA and it is a primordial organ in commanding fish behaviour, which is closely related to fish health and welfare, both in the natural environment and in captivity. Since n-3 LC-PUFA are particularly important for the correct function of neural circuits and their effect on fish juveniles are scarcely studied, the long-term feeding effects of using microalgal products in fish diets were evaluated in sea bream behaviour to a stimulus, brain composition and neural function-related gene expression (Chapter 7).

All trials and analytical analysis present in this section were conducted in ECOAQUA University Institute facilities (Canary Islands, Spain). Gilthead sea bream and meagre were produced from broodstocks belonging to ECOAQUA University Institute and reared using the established protocols until the target size for each experimental trial. All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA Institute from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were also approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (references given in each chapter).

2.2 FISH MODELS DESCRIPTION

2.2.1 GILTHEAD SEA BREAM (SPARUS AURATA)

Class: Osteidacy

Order: Perciformes

Family: *Sparidae*

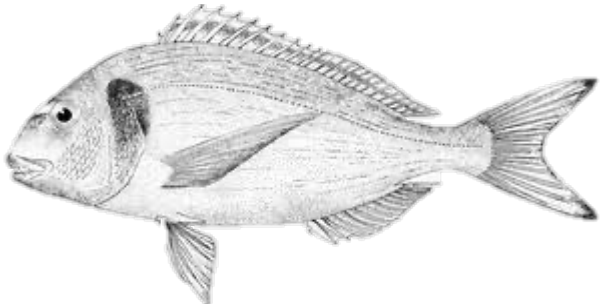

Figure 2.1. Gilthead sea bream (*Sparus aurata*)

2.2.1.1 BIOLOGICAL CHARACTERISTICS

Gilthead sea bream (*Sparus aurata*) is a marine teleost fish from *Sparidae* family (Figure 2.1). It has a silver-grey colour, an oval-shaped compressed body, and it is distinguished by its black spot that partially covers the operculum and the yellow band between its eyes. This species is common in the Mediterranean Sea, along the Eastern Atlantic coasts from Great Britain to Senegal. It is euryhaline and eurythermal, which means that it is often found in both marine and brackish water environments such as estuaries, particularly during the early stages of life. It is usually found on rocky and seagrass (*Posidonia oceanica*) meadows or sandy grounds. *Saparidae* family are typically carnivore fish. Wild sea bream feed on a variety of prey, including crustaceans, molluscs, polychaetes, teleosts, and echinoderms (Wassef and Eisawy, 1985; Andrade et al., 1996).

2.2.1.2 FARMING

Gilthead sea bream is a high-valuable species in Mediterranean aquaculture production, taking place in almost all countries of that region. Hatcheries produce eggs from breeding individuals under controlled conditions (2 million eggs of 1 mm in diameter per kilo of weight) (APROMAR, 2020). During their first month of life, the larvae feed on living organisms: rotifers and artemia, and from that moment to the juvenile phase they feed on artificial diets. The on-growing period can be carried out on floating pens at sea, concrete

tanks, or ponds on land. Each specimen takes between 18 and 24 months to reach commercial size since hatching (250-400 g, although it can go up to 2 kg) (APROMAR, 2020).

In Europe and the Mediterranean area, the total aquaculture production was 252 406 tonnes in 2019 (APROMAR, 2020). Top producer countries are Turkey with 85 000 tonnes, Greece with 65 300 tonnes, Egypt with 36 000 tonnes, Tunisia with 16 000 tonnes and Spain with 13 521 tonnes (Figure 2.2).

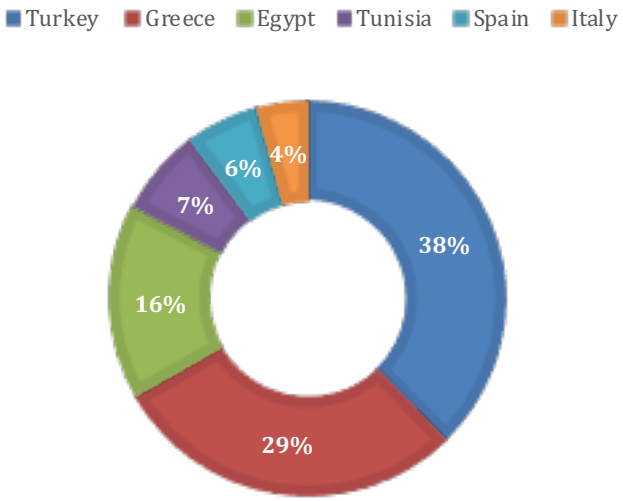

Figure 2.2. Top countries in the aquaculture production of gilthead sea bream (*Sparus aurata*) in 2019.

2.2.2 *Meagre (Argyrosomus regius)*

Class: Osteidacty

Order: Perciformes

Family: *Scienidae*

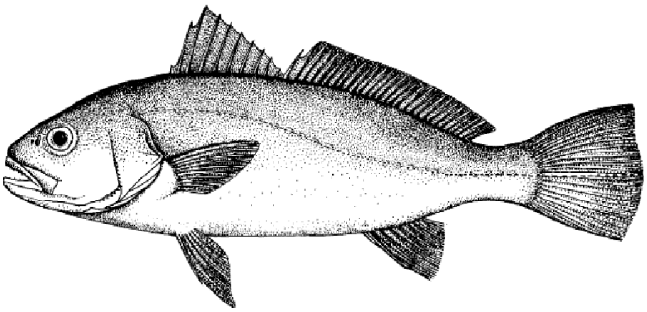

Figure 2.3. Meagre (*Argyrosomus regius*)

2.2.2.1 BIOLOGICAL CHARACTERISTICS

Meagre is a large, corpulent, and very agile fish (Piccolo et al., 2008) from *Sciaenidae* family, which can reach a total length of 2.3 meters (Maigret and Ly, 1986), weigh 103 kilograms (Quéro and Vayne, 1987) and reach 42 years old (Figure 2.3). It is found along the eastern Atlantic, from Senegal to the north of France, including the Canary Islands and the Mediterranean Sea (APROMAR, 2020).

Meagre is a nekton-benthic species established on rocky or sandy bottoms, from surface areas of 15 m to almost 300 m depth (Schneider, 1990; Quéméner, 2002). In addition, it is a euryhaline and eurythermal species, which allows its adaptation to very diverse environments (Lavié et al., 2008; Cárdenas, 2010). Growth is mainly achieved during summer and its feeding activity is reduced when sea temperatures drop below 13-15 ºC. The digestive tract is relatively short, typical of carnivorous species. In the wild, it feeds on Mysidacea, Decapode and Teleostei (Cabral and Ohmert, 2001).

2.2.2.2 FARMING

Aquaculture production of meagre is carried out in several Mediterranean countries. In captivity, natural spawns are not recorded, and viable eggs are only produced by artificial induction with hormone treatments. After hormones administration, the spawning occurs spontaneously without being required to strip or artificially fertilize the eggs (Duncan et al., 2008; Mañanós et al., 2008). Females produce more than 1 million eggs per year, with a diameter of less than 1 mm. Breeding techniques and larval protocols are very similar to those used for seabass and sea bream. Thus, larvae feed on rotifers and artemia during the first weeks of life and then they start with artificial diets. On-growing is carried out usually in floating pens at sea.

A great interest for meagre production has grown and this species constitutes one of the most promising species for the diversification of aquaculture, considering it as a priority species in the research and development programs of the Mediterranean countries (Chatzifotis et al., 2010). Meagre has several characteristics that support it as a suitable candidate for commercial production, such as easy adaptation to captivity (Cárdenas, 2010; Monfort, 2010), fast growth (around 1 kg per year) and excellent feed conversion rates (Calderón et al., 1997; Jiménez et al., 2005; El-Shebly et al., 2007). Its commercial size is between 1 and 4 kg.

In 2019, meagre derived from aquaculture in the Mediterranean area was estimated at 41 295 tonnes, with the top producer countries being Egypt (32 000 tonnes), Spain (3 650 tonnes), Turkey (2 600 tonnes) and Greece (1 800 tonnes) (Figure 2.4).

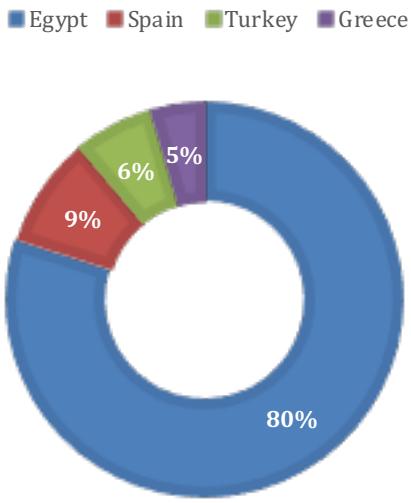

Figure 2.4. Top countries in the aquaculture production of meagre (*Argyrosomus regius*) in 2019.

2.3 NOVEL OMEGA-3 SOURCES FOR MARINE FISH USED AND DIETS PRODUCTION

Regarding the novel omega-3 sources used in the present thesis, three commercial microalgal biomass products were tested in weaning diets for sea bream larvae (Chapter 3): ALL G RICH™ (Alltech Inc., Nicholasville, KY, USA), DHA GOLD (DSM, Basel, Switzerland) and MO060 (Adisseo, France SAS). In contrast, for sea bream and meagre juveniles, two commercial microalgal oils from *Schizochytrium sp.* were selected: one rich in EPA and DHA (Veramaris algal oil; Veramaris, The Netherlands) and the other rich in DHA and n-6 DPA (DHA Natur Oil; Archer Daniels Midland, USA) (Chapters 4-7). The specific formulation of the experimental diets is detailed in the respective chapter. In general, the microalgal diets were compared to a positive control diet with FO and a negative control diet with OA (to mimic VO), or PO and VO in each experiment.

All diets were prepared following similar technical protocols. Briefly, ingredients were first grounded and mixed. For microdiets preparation, the mixed wet ingredients were dried for

24 h and then sieved below <250 µm. For juveniles' diets, mixed ingredients were pelletized to obtain 1-3 mm particle size and then dried. Microdiets were prepared at ECOAQUA University Institute feed unit facility, whereas pelletized diets for juveniles were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) and shipped to the ECOAQUA Institute laboratories.

2.4 FEEDING TRIALS

Feeding trials were conducted in an open system, continuously supplied with filtered sea water (37 mg L^{-1} salinity) and aeration. Water temperature and dissolved oxygen were recorded daily. Fish were stocked in 250 or 500 L fiberglass cylinder tanks, artificially or naturally illuminated, respectively. Artificial illumination was done with fluorescent lights placed above each tank at an intensity of 100 lx and programmed for 12 h light photoperiod (from 8 a.m. to 8 p.m.).

Each experimental diet was tested in quadruplicate (in larval trial) or in triplicate (in juveniles' trials). During the feeding periods, larvae were manually fed each 45 min from 8:00-20:00, whereas juveniles were manually fed until visual apparent satiety, 3 times a day, 6 days per week. Feed delivery to fish juveniles was calculated daily, and uneaten pellets were collected in a net by opening the water outlet 30 min after each meal, dried in an oven for 24 h and weighed to estimate feed intake (FI). Trials specificities, as fish size, stock densities, duration of the feeding period, dissolved oxygen and water temperature are detailed in the respective chapters.

2.5 SAMPLING PROCEDURES

Prior to each sampling, all fish were fasted for 24 h. Weight and length of fish were assessed at the beginning and the end of each feeding trial. For larvae, growth was determined by measuring body weight of 30 larvae per tank, dried at 105°C until constant weight. Additional 30 larvae per tank were removed, placed on ice, and transferred to a microscope slide with distiller water to measure individual total length (Profile Projector V-12A; Nikon, Tokyo, Japan). For juveniles, individual weight and length of each fish were recorded, after anaesthesia with clove oil (4 mL 100 L^{-1}). When necessary, sacrifice was carried out by painlessly immersion in iced water or with excess of anaesthesia with clove oil.

2.6 KEY PERFORMANCE INDICATORS

Fish performance and feed utilization were estimated using the following formulas:

Survival (%) = 100* (nº alive larvae at the end of the trial / nº total initial larvae)

Mortality (%) = 100* (nº dead fish / nº total initial fish)

Specific growth rate (SGR, % day^{-1}) = 100* (ln BW_1−ln BW_0) / nº days of the trial, where BW_0 and BW_1 corresponded to fish body weight (g) at the beginning and at the end of the trial, respectively (applicable also for the next equations).

Thermal growth coefficient (TGC, arbitrary units) = ($BW_1^{1/3}$−$BW_0^{1/3}$)/ Temperature (ºC)/days of the trial.

Daily growth index (DGI, % day^{-1}) = ($BW_1^{1/3}$−$BW_0^{1/3}$ / nº days of the trial) * 100.

Feed intake (FI, g feed g fish^{-1}) = Feed delivered (g feed fish^{-1}) / fish body weight (g).

Biological feed conversion ratio (FCR) = Feed delivered ($t_1 - t_0$)/ (Biomass t_1 – Biomass t_0 + $Biomass_{harvested}$ + $Biomass_{lost}$), where t_0 and t_1 corresponded at the beginning and end of the trial, respectively.

Condition factor (CF, %) = 100* (BW / TL^3), where BW and TL corresponded to fish body weight and length, respectively, at the end of the trial.

Allometric exponent (arbitrary units) = BW=a*TL^b, where b corresponded to the slope of the potential regression between the body weight (BW) and the total length (TL) of all fish.

Nutrient utilization and deposition were estimated based on the following equations:

Protein efficiency ratio (PER, arbitrary units) = (BW_1 - BW_0) / protein intake (g fish^{-1}).

Lipid efficiency ratio (LER, arbitrary units) = (BW_1 - BW_0) / lipid intake (g fish^{-1}).

Efficiency of protein retention (% of protein intake) =100* (BW_1 (g) * protein in whole-$body_1$ (%))– initial weight (g) * protein in whole-$body_0$ (%) / FI (g) * dietary protein (%).

Efficiency of lipid retention (% of lipid intake) =Efficiency of protein retention (% of lipid intake) =100* (BW_1 (g) * lipid in whole-$body_1$ (%))– initial weight (g) * lipid in whole-$body_0$ (%) / FI (g) * dietary lipid (%).

Efficiency of energy retention (% of energy intake) =Efficiency of energy retention (% of energy intake) =100* (BW_1 (g) * energy in whole-$body_1$ (%))– initial weight (g) * energy in whole-$body_0$ (%) / FI (g) * dietary energy (%).

Relative fatty acid retention efficiency (% FA intake) = 100* (BW_1 (g) * FA in whole-$body_1$ (g 100 g FA^{-1}) * whole-body $lipids_1$ (%)– initial weight (g) * FA in whole-$body_0$ (g 100 g FA^{-1}) * whole-body $lipids_0$ (%) / FI (g) * dietary lipids (%) * dietary FA (g 100 g FA^{-1}).

Additionally, lipid quality indices (arbitrary units) for human nutrition were calculated in fish fillets:

Hypercholesterolemic fatty acids (H) = 12:0 + 14:0 + 16:0.

Hypocholesterolemic fatty acids (h) = 18:0 + Σ MUFA + Σ PUFA.

Atherogenic index (AI) = (12:0 + 4 * 14:0 + 16:0) / (Σ MUFA + Σ n-3 PUFA + Σ n-6 PUFA).

Thrombogenic index (TI) = (14:0 + 16:0 + 18:0) / (0.5* Σ 18:1) + (0.5* Σ MUFA) + (0.5* Σ n-6 PUFA) + (3* Σ n-3 PUFA) + (Σ n-3 PUFA / Σ n-6 PUFA).

Peroxidation index (PI) = 0.025 × (Σ monoenoic fatty acids) + 1 × (Σ dienoic fatty acids) + 2 × (Σ trienoic fatty acids) + 4 × (Σ tetraenoic fatty acids) + 6 × (Σ pentaenoic fatty acids) + 8 × (Σ hexaenoic fatty acids).

2.7 STRESS AND BEHAVIOUR CHALLENGES

2.7.1 AIR-EXPOSURE STRESS TEST

To test sea bream larval stress resistance (Chapter 3), at the end of the feeding period, an air-exposure stress test was conducted with 20 larvae per tank that were placed in a scoop net out of the water for 2 min. Subsequently, larvae were allocated in a new tank supplied with clean seawater and aeration, and survival was determined after 24 h (Burbano et al., 2020).

2.7.2 BEHAVIOURAL RESPONSE TO AN EXTERNAL MECHANO-SENSORY STIMULUS

Nine gilthead sea bream juveniles per treatment were randomly selected for testing behaviour response to an external mechano-sensory stimulus. Fish were tested in three groups per treatment, of three fish each, using a white bottom-tank of 100 L, indirectly illuminated, and covered by a dark plastic canvas to avoid disturbance during acclimation and testing (Figure *2.5*). Each fish was gently tagged with a colour pearl attached to the base of the first ray of the dorsal fin, under anaesthesia with clove oil (4 ml 100 L^{-1}). Then, fish were let to recovered and acclimated in the testing tank for 1h, according to the acclimation time used in most of locomotor activity- related studies in several fish species (Melvin et al., 2017). After this acclimation period, fish were exposed to a mechano-sensorial vibrational stimulus, which consisted in an iron pendulum of 600 g that was dropped without any additional force against the tank wall from 71 cm (Figure 2.5). Fish behaviour was monitored by a video-camera (Xiaomi Mijia 4k) placed above the tank and recording at 200 fps. The first 60 seconds of recording prior to stimulation were used to measure fish basal activity level (cruise speed), while the first 300 milliseconds immediately after applying the stimulus were used for measuring the activity level (measured through swimming speed) in fish after stimulation. Furthermore, fish motion during the escape response (Domenici and Hale, 2019) was analysed when applying the stimulus using the software Kinovea for measuring specific points on the fish, i.e., the centre of mass of the fish (at 0.35 lengths, based on other fish species with similar body shape) (Webb, 1978; Dadda et al., 2010) and the tip of the head, as well as turning angles. The following variables were analysed:

(1) Responsiveness: responsiveness to the stimulation was defined as the number of fish that responded to the sensory-mechanical stimulus with an escape response (Domenici and Hale 2019); non-responders were considered individuals that did not display any movement in the moment of the stimulation.

(2) Escape type: Escape responses consists of a fast body muscular contraction, usually in C-shape form (stage 1), where a second contralateral contraction (stage 2) may or may not following (Domenici and Blake, 1997). Thus, escape type was defined as either single if escape response consisted of stage 1 alone (SB) or double bend (DB) if stage 2 followed stage 1 (Domenici and Blake 1997).

(3) Escape latency: Escape latency was measured as the time (in ms) from the frame F0 in which the stimulus started disappearing below the upper edge of the tank seen from the camera above until the first detectable movement by the fish.

(4) Turning rate: Turning rate was measured as the ratio between the turning angle observed in stage 1 and the duration of the stage 1 (degrees /s) (Domenici et al., 2008). The duration of this stage was measured between the frame 0 (prior to the first visible reaction) and the moment when a change in the direction of rotation of the head occurred (Domenici and Blake, 1997). Stage 1 turning angle was measured (in degrees) as the rotation of the line passing through the centre of mass and the tip of the head of each individual, during the whole duration of stage 1.

(5) Distance covered: Distance covered was defined as the distance (in cm) between the centre of mass of the fish at the frame before the first visible response and 70 ms later. The 70ms time-period was chosen as a shortest fixed time through which all fish had completed stage 2 (if DB) or stage 1 (if SB) (Meager et al., 2006; Turesson et al., 2009). This fixed time was chosen because stage 1 and 2 correspond to the period considered crucial for survival when avoiding stressors, for instance ambush predator attacks (Walker et al., 2005).

(6) Basal activity level: Basal activity level (in cm/s) was defined as the cruise speed (distance/ time) during the first 60 seconds prior to stimulation.

(7) Post-stimulation activity level: Post-stimulation activity level (in cm/s) was defined as the swimming speed (distance/ time) during the 300 ms immediately after the stimulation.

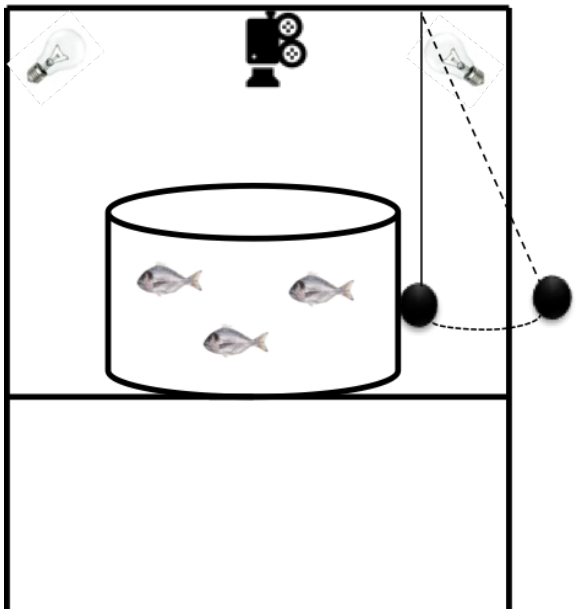

Figure 2.5. Experimental set-up of behaviour test with a mechano-sensory stimulus (pendulum).

2.8 BIOCHEMICAL ANALYSIS

All samples for proximate composition analysis were collected in a pool of 5-6 fish per tank, homogenized, conserved at -80ºC until analysis and analysed in three technical replicates. Target tissues are specified in each chapter.

2.8.1 CRUDE PROTEIN CONTENT

Protein content was determined from the total nitrogen content of each sample, according to the Kjeldahl method (AOAC, 2000). Briefly, approximately 200 mg samples were digested with 10 ml sulphuric acid at 400ºC for 60 min in a presence of catalytic tablet with 1.5 % CuSO4· 5H2O and 2 % of selenium (Panreac, Barcelona, Spain), where nitrogen in the sample is converted to ammonia. Follows a distillation with 20 ml of distilled water and 50 ml of sodium hydroxide (40% w/v) to separate ammonia from the digestate and collection for analysis that is finally quantified by titration with hydrochloric acid (HCl) 0.1 M. Protein content determination is calculated according to the following equation:

$$\%\ \text{Protein} = 100 \times ((\text{volume HCl}_{\text{samples}} - \text{volume HCl}_{\text{blank}}) \times 0.1 \times 14.007 \times 6.25) / \text{weight sample}$$

2.8.2 ASH CONTENT

Determination of ash content of each sample was carried out by incineration in a muffle furnace at 600ºC overnight and until constant weight (AOAC, 2000) and calculated following the equation:

$$\%\ \text{Ash} = (\text{weight sample before incineration} - \text{weight sample after incineration}) \times 100$$

2.8.3 MOISTURE CONTENT

The determination of moisture content was carried out by drying each sample in a temperature-controlled oven at 110ºC for 24h and until constant weight (AOAC, 2000). Moisture content was calculated as follows:

$$\%\ \text{Moisture} = (\text{wet weight sample} - \text{dried weight sample}) \times 100$$

2.8.4 TOTAL, NEUTRAL AND POLAR LIPID CONTENTS

Total lipid content of each sample was extracted by adding to 0.2 g of sample a mixture of chloroform/methanol (2:1 v/v) with 0.01% butylated hydroxytoluene (BHT), homogenising it with an ultraturrax (T25 Digital Ultra-turrax, IKA®, Germany) and lipid quantity determined by a gravimetric method, followed by filtration using anhydrous sodium sulphate, and evaporation to dryness under a nitrogen atmosphere (Folch et al., 1957). Total lipids were weighed and calculated according to the following equation:

$$\%\ \text{Lipids} = (\text{weight lipids} / \text{weight sample}) \times 100$$

Neutral and polar lipid fractions of samples were separated from total lipids. For by elution and filtration of polar lipids with methanol, and neutral lipids with chloroform and chloroform/methanol (49:1 v/v) (Juaneda and Rocquelin, 1985). After separation, both polar and neutral lipids were evaporated in a rotary evaporator and under nitrogen atmosphere and were weighed. Polar and neutral fractions were calculated as follows:

Neutral lipid fraction (LN) + Polar lipid fraction (LP) = Total lipids (TL)

2.8.5 FATTY ACID COMPOSITION

Fatty acid methyl esters (FAMES) of each sample were obtained by acid media transmethylation by adding toluene with BHT 0.1% and methanol: sulphuric (1%) to total lipids and incubating them at 50 °C during 16 h under nitrogen atmosphere and darkness (Christie, 1989). Then, FAMES were recovered by adding a mixture of hexane: diethyl ether (1:1) and centrifuging at 2500 rpm during 5 minutes at room temperature. Fatty acids were then filtered, diluted in hexane, evaporated until dryness under nitrogen atmosphere and weighed. After dilution to 40 mg/ml with hexane, FAMES were stored in micro-vials for separation by gas-liquid chromatography (GLC), under the conditions previously described by Izquierdo et al. (1990) and quantification with a flame ionization detector (Finnigan Focus SG, Thermo Electron Corporation, Milan, Italy). All fatty acids were then identified by comparison with previously characterized standards and fatty acid levels presented as % total fatty acids (Nippai, Tokyo, Japan).

2.9 MORPHOLOGICAL AND HISTOLOGICAL STUDIES

2.9.1 SAMPLES PROCESSING

For histological examinations of liver morphology, 30 whole-larvae (Chapter 3) or livers from 5 juveniles (Chapter 6) per tank were collected and preserved in formalin 4%. Subsequently, samples were dehydrated in a graded ethanol series using a Histokinette 2000 (Leica, Nussloch, Germanyand) and then embedded in paraffin wax. Paraffin blocks were made and cut with a Leica microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) in 4 µm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Panreac) (Martoja and Martoja-Pierson, 1970). Five larvae and one liver were kept per paraffin block and were visualized and photographed under a light microscope (BX51TF, Olympus, Tokyo, Japan).

2.9.2 SEMI-QUANTITATIVE HISTOLOGICAL EXAMINATION

All slides were firstly blinded evaluated by three different researchers for describing general histomorphology of the hepatic tissue. A semi-quantitative score was used to evaluate hepatocyte lipid vacuolization: score 0–1 was considered as normal morphology of liver tissue, with none or very small lipid vacuoles within the hepatocytes; score 1-2 was considered as moderate hepatic steatotic alterations associated to a moderate lipid infiltration; and score 2–3 as severe steatotic alterations referring to a high lipid infiltration and large vacuoles within the hepatocytes.

2.9.3 HISTOMETRIC ANALYSIS

Micrographs from each slide were taken using a Nikon Microphot-FXA microscope (Nikon Instruments Inc., Melville, NY, USA) incorporated with an Olympus DP50 camera (Olympus Optical Co. LTD, Shinjuku-ku, Tokyo, Japan). The total area of 25 hepatocytes per specimen (375 hepatocytes per treatment) were measured, as well as the maximum and minimum length of these hepatocytes considering the hepatocyte nucleus as reference point and using arbitrary units. All the measurements were done with Image Pro-Plus 6.0 (Media Cybenetics, Rockville, USA) software for Windows.

2.10 MOLECULAR ANALYSIS

All samples for molecular studies were collected in a pool of 5-9 fish per tank (specified in the respective chapter; Chapters 3, 5, 6 and 7). Target tissues are specified in each chapter. Samples were conserved in RNA later (Sigma-Aldrich, Madrid, Spain) at -80ºC until analysis and analysed in technical duplicates in each polymerase chain reaction (PCR).

2.10.1 RNA EXTRACTION, QUANTIFICATION AND COMPLEMENTARY DNA SYNTHESIS

Total RNA was extracted from, approximately, 200 mg of samples, using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Samples were homogenized with 1 ml of TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA) at 30 Hz for 30 s cycles using TissueLyser-II (Qiagen) until complete homogenization. Then, 200 µl of chloroform were added to the samples and then centrifuged for phase separation (12,000 g, 15 min, 4 °C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane and RW1 and RPE buffers (Qiagen) were used to wash away contaminants. Purified RNA was eluted with 25-50 µl of RNase-free water. The quality of RNA was checked by 1.4% agarose gel electrophoresis and the quantity was analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Hundred ng of total RNA were reverse transcribed to complementary DNA (cDNA) using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA).

2.10.2 REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (RT-QPCR)

RT-qPCR was used to determine relative gene expression in the samples collected by the Δ–Δ method (Livak and Schmittgen, 2001). The PCR reactions were carried out in 96-well microplates using a an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) or a CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). PCR conditions for every primer were as follows: a first step of 3 min 30 s at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at annealing temperature (specified in each chapter), 30 s at 72 °C, 1 min at 95 °C, and a 95 °C for 1 min, and a final denaturing step from 58 °C to 95 °C for 10 s. All PCR reactions were carried out in in a final volume of 15 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each

primer (10 mM), 5 µl of cDNA (1:10 dilution) and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. In each run, an analysis of the melting curve leading to a melting peak specific for the amplified target DNA was carried out. Primer efficiencies were tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:500). Primer nucleotide sequences, and GenBank access numbers are described in Tables 2.1 and 2.2 for the genes measured in gilthead sea bream and meagre, respectively.

Beta actin (*β-actin), ribosomal protein L27* (*rpl-27*) and *elongation factor 1 alpha* (*ef1*□) were used as house-keeping genes; *5-lipoxygenase* (*5-lox*) and *cyclooxygenase-2 (cox-2*), *heat shock proteins 70* and *90* (*hsp70* and *90*) were used as markers of immune function and eicosanoids production; *glutathione peroxidase* (*gpx*) and *superoxide dismutase* (*sod*) were used as markers of fish antioxidant system; *fatty acyl desaturase 2* (*fads2*) and *fatty acyl elongase 5* (*elovl5*) were used as indicators of PUFA biosynthesis; *glucose-6-phosphatase* (*g6p*) was used as marker of glucose metabolism; *peroxisome proliferator-activated receptor alpha* (*ppar-α*) and *carnitine palmitoyl transferase I* (*cpt1*) were used as markers of lipid catabolism; *stearoyl-CoA desaturase-1 a* (*scd-1a*), *lipoprotein lipase* (*lpl*), *fatty acid synthase* (*fas*) and *sterol regulatory element-binding protein 1* and *2* (*srebp1, 2*) were used as markers of lipogenesis; *neurogenic differentiation factor 6* (*neurod6*); and *early growth response gene 1* (*egr1*) were used as markers of neurogenesis; *nitric oxide synthase 1* (*nos1*), *brain-derived neurotrophic factor* (*bdnf*) and *c-fos* were used as markers of neural activity. Sequences from *neurod6*, *egr1*, *nos*1, *c-fos* and *bdnf* were designed from gilthead sea bream genome using Primer3 software, whereas other primer sequences were obtained from the literature specified in the respective chapter.

Table 2.1 Primer sequences used for molecular studies with gilthead sea bream

	Sequence (5'-3')		
Genes	Forward	Reverse	GenBank Access nº
5-lox	CCTGGCAGATGTGAACTTGA	CGTTCTCCTGATACTGGCTGA	FP334124
cox2	GAG TAC TGG AAG CCG AGC AC	GAT ATC ACT GCC TGA GT	AM296029
fads2	GCAGAGCCACAGCAGCAGGGA	CGG CCT GCG CCT GAG CAG TT	AY055749
g6p	CGCTGGAGTCATTACAGGCGT	CAGGTCCACGCCCAGAACTC	AF151718.1
ppar-α	TCT CTT CAG CCC ACC ATC CC	ATC CCA GCG TGT CGT CTC C	AY590299
fads2	GCAGGCGGAGAGCGACGGTCTGTTCC	AGCAGGATGTGACCCAGGTGGAGGCAGAAG	AY055749
elovl5	CCTCCTGGTGCTCTACAAT	GTGAGTGTCCTGGCAGTA	AY660879
scd-1a	GGAGGCGGAGGCGTTGGAGAAGAAG	AGGGAGACGGCGTACAGGGCACCTATATG	JQ277703
lpl	CGTTGCCAAGTTTGTGACCTG	AGGGTGTTCTGGTTGTCTGC	AY495672.2
fas	TGCCATTGCCATAGCACTCA	ACCTTTGCCCTTTGTGTGGA	JQ277708.1
srebp-1	AGGGCTGACCACAACGTCTCCTCTCC	GCTGTACGTGGGATGTGATGGTTTGGG	JQ277709
srebp-2	GCTCACAAGCAAAATGGCCT	CAAAACTGCTCCCTTCCCCA	AM970922.1
cpt-1	GTGCCTTCGTTCGTTCCATGATC	TGATGCTTATCTGCTGCCTGTTTG	JQ308822
neurod6	TCGGCAGGAAAAGAAAAAG	CACAATATCGGCTCCATGTG	XM_030401584.1
egr1	GACGAGAGGAAGAGGCACAC	ACGGGAGAGGGGTAAGAAGA	XM_030396900.1
nos1	GGTCAACAAAGAGCCTCAGC	ATTCCTCTGGCCTTCTCCAT	XM_030416914.1
c-fos	TGACCTGTCCAACTCCCTCT	GTTGCTGTTGCTTCCTCTCC	XM_030405977.1
bdnf	ATCAGCAACCAAGTGCCTTT	GCCGTCTTTTTATCCACAGC	XM_030413189.1
ß-actin	GACCAACTGGGATGACATGG	GCATACAGGGACAGCACAGC	X89920.1
rpl-27	ACAACTCACTGCCCCACCAT	CTTGCCTTTGCCCAGAACTT	AY188520

Complete genes name: ß-actin, beta-actin; rpl-27, ribosomal protein L27; ef1α, elongation factor 1 alpha; 5-lox, 5-lipoxygenase; cox-2, cyclooxygenase-2; fads2, fatty acyl desaturase 2; elovl5, fatty acyl elongase 5; g6p, glucose-6-phosphatase; ppar-α, peroxisome proliferator-activated receptor alpha; cpt-1, carnitine palmitoyltransferase I; scd-1a, stearoyl-CoA desaturase-1 a; lpl, lipoprotein lipase; fas, fatty acid synthase; srebp1, 2, sterol regulatory element-binding protein 1 and 2; neurod6, neurogenic differentiation factor 6; egr1, early growth response gene 1; nos1, nitric oxide synthase 1 (neuronal); bdnf, brain-derived neurotrophic factor.

Table 2.2. Primer sequences used for molecular studies with meagre

	Sequence (5'-3')		
Genes	Forward	Reverse	GenBank Access nº
hsp70	AACGTTCAGGACTTGCTGCT	CCCTTCGTAGACCTGGATGA	n.a.
hsp90	AAAAGGCCGAGAAGGAAGAG	GGCTTGGTCTTGTTCAGCTC	n.a.
gpx	AAGCAGTTTGCCGAGTCCTA	GCTGGTCTTTCAGCCACTTC	n.a.
sod	GGCCCTCACTTCAATCCCTA	TCCTTTTCCCAGATCGTCGG	n.a.
fads2	TGACTGGGTGACAATGCAGT	TGGTGCTAACTTTGTGCCCT	n.a.
elovl5	CATCACACAGTTACAGCTGGTC	GAATTGTGTGCACGGTTTCT	n.a.
ef1α	GGTGCTGGACAAACTGAAGG	GAACTCACCAACACCAGCAG	n.a.

Complete genes name: fads2, fatty acyl desaturase 2; elovl5, *fatty acyl elongase 5*; hsp70, *heat shock proteins 70; hsp90, heat shock protein 90*; gpx, *glutathione peroxidase*; sod, *super oxide dismutase*; *n. a= not available*

2.11 STATISTICAL ANALYSIS

For all statistical analysis, each tank was considered as one replicate (n=4 and n=3 for each treatment in larval and juveniles' trials, respectively). All data were tested for normal distribution and homogeneity of variances using Shapiro–Wilk and Levene's tests, respectively. Confidence levels were established at 95 % ($P < 0.05$) for the analysis and the results were presented as mean with the standard error (SE) unless otherwise mentioned.

To independently compare the different experimental diets in the different trials, a one-way analysis of variance (ANOVA) was applied to data and means were compared with Tukey's multiple range test (Tukey, 1949). When necessary, transformations to normalize data were applied (Sokal and Rohlf, 1981). If normalization was not possible, the data were analysed using the nonparametric Kruskall-Wallis test.

To determine the specific effect of the dietary lipid source, the fish meal dietary level and its potential interaction with the lipid sources, a two-way ANOVA was also applied to the data (Chapter 4 and 6).

Behaviour response such as responsiveness and type of escape response were analysed applying a Chi-2 square test (critical CHI values =5.99 for two degrees of freedom), while numeric variables including activity levels, latency, distance, and turning rates were analysed using a two-way ANOVA, where diet was used as fixed factor and group within each diet was used as random factor. Total length of each fish (cm) was considered as co-variable for all numeric behavioural variables (Chapter 7).

To obtain a more integrated interpretation of the main effects produced in the fish by the different diets, multivariate analysis of principal components (PCAs) were applied (Chapters 3, 4 and 5). The first two components that explained more than 50% of total variance among the different diets were selected and graphed.

When appropriate, data were subjected to the best-fit regressions (linear, exponential, or logarithmic), which were also checked for significance ($P < 0.05$). Significant correlations between the dietary or tissue components and productive parameters or gene expressions were checked with Spearman or Pearson coefficients.

Statistical treatment of the data was carried out using SPSS 21.0 or Prism7 for macOS 10.15 and Windows.

2.12 GRAPHICAL SCHEMES OF EACH CHAPTER

2.12.1 GENERAL OVERVIEW

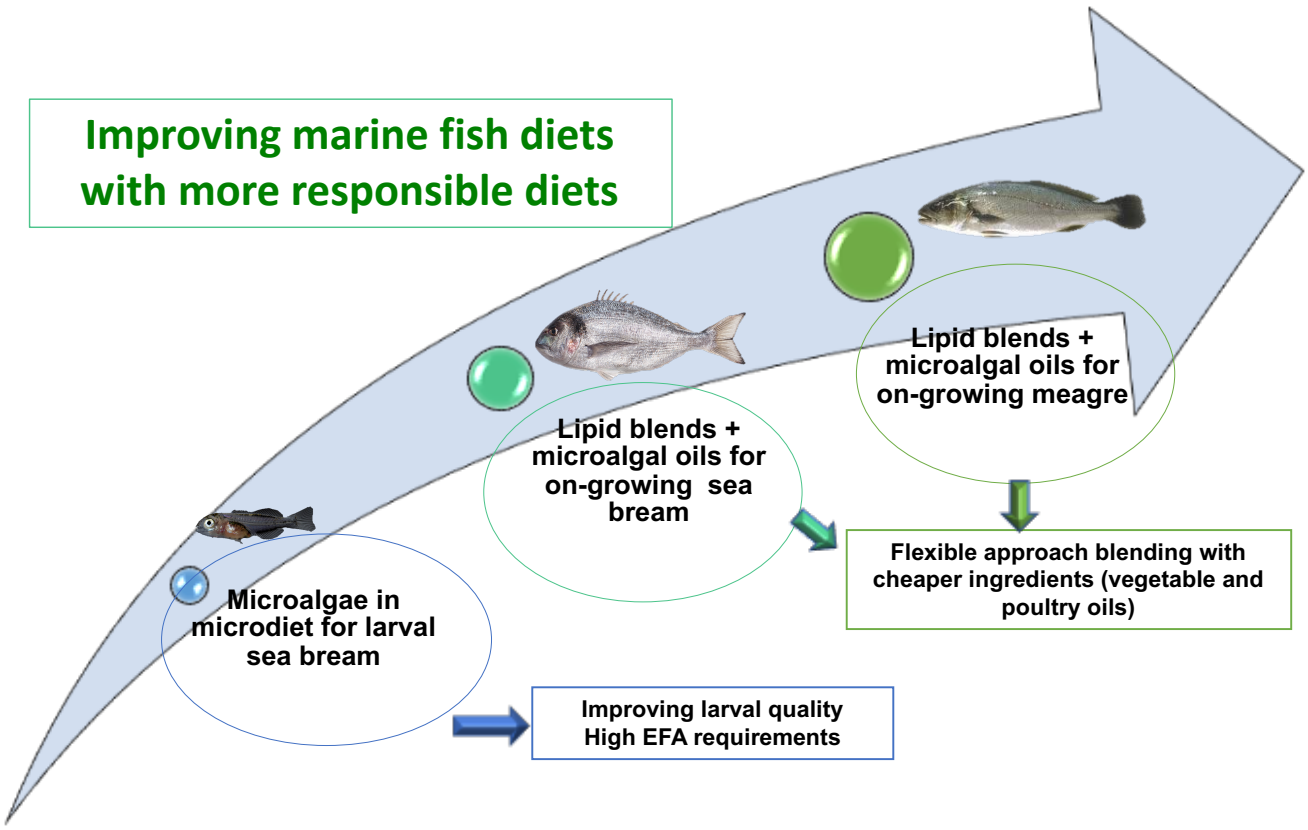

Figure 2.6. Graphical scheme of the general overview of the PhD thesis

2.12.2 Chapter 3- Complete replacement of fish oil by three microalgal products rich in n-3 polyunsaturated fatty acids in early weaning microdiets for gilthead sea bream (Sparus aurata)

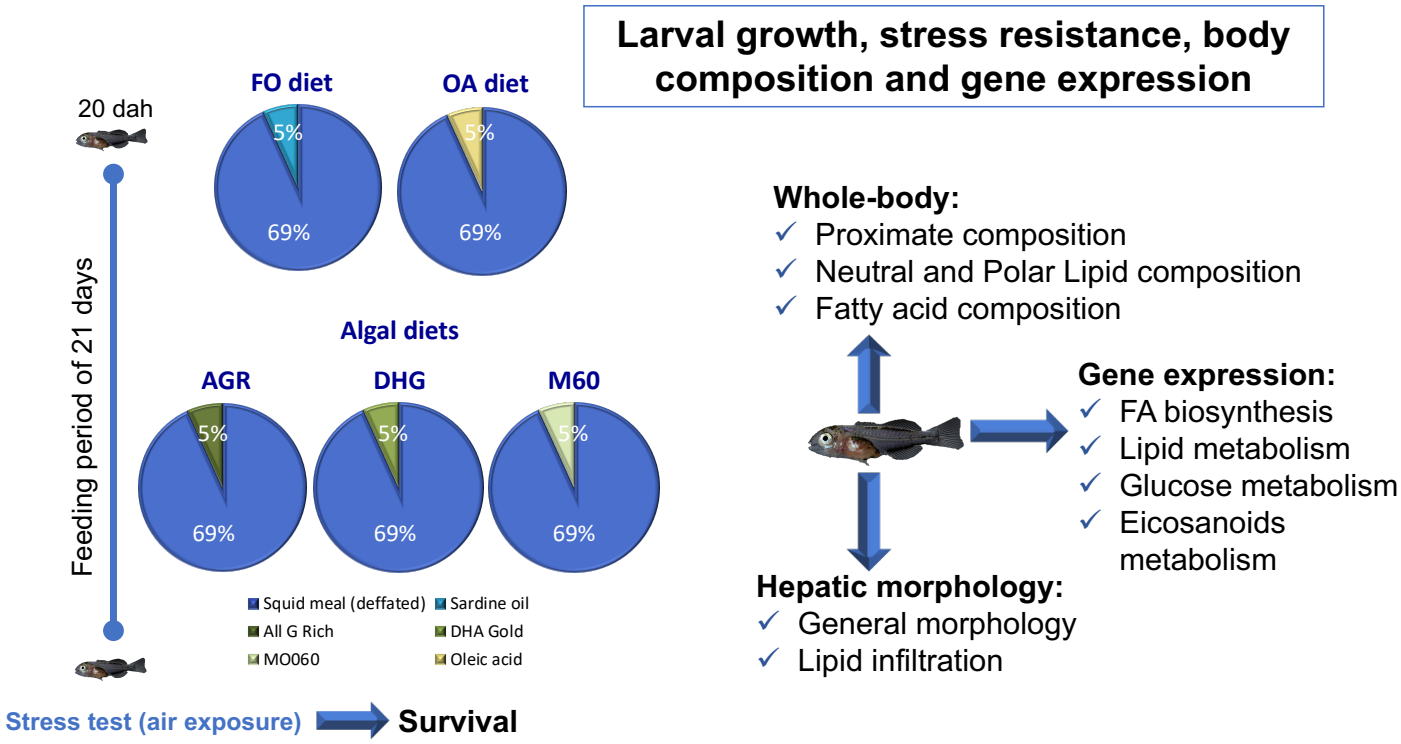

Figure 2.7. Graphical scheme of Chapter 3.

2.12.3 CHAPTER 4- EFFECTIVE COMPLETE REPLACEMENT OF FISH OIL BY COMBINING POULTRY AND MICROALGAE OILS IN PRACTICAL DIETS FOR GILTHEAD SEA BREAM (SPARUS AURATA) FINGERLINGS

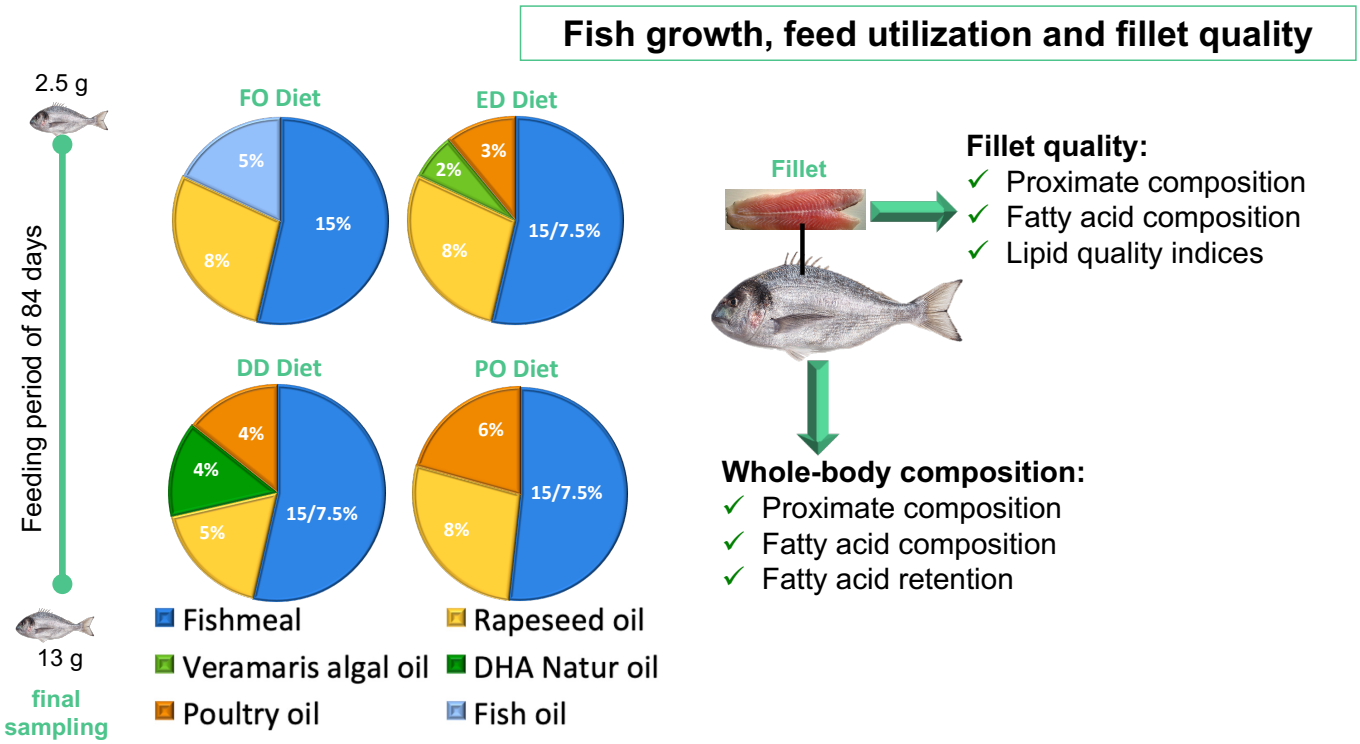

Figure 2.8. Graphical scheme of Chapter 4.

2.12.4 Chapter 5- Oils combination with microalgal products as a strategy for increasing the n-3 long-chain polyunsaturated fatty acids content in fish oil-free diets for meagre (Argyrosomus regius)

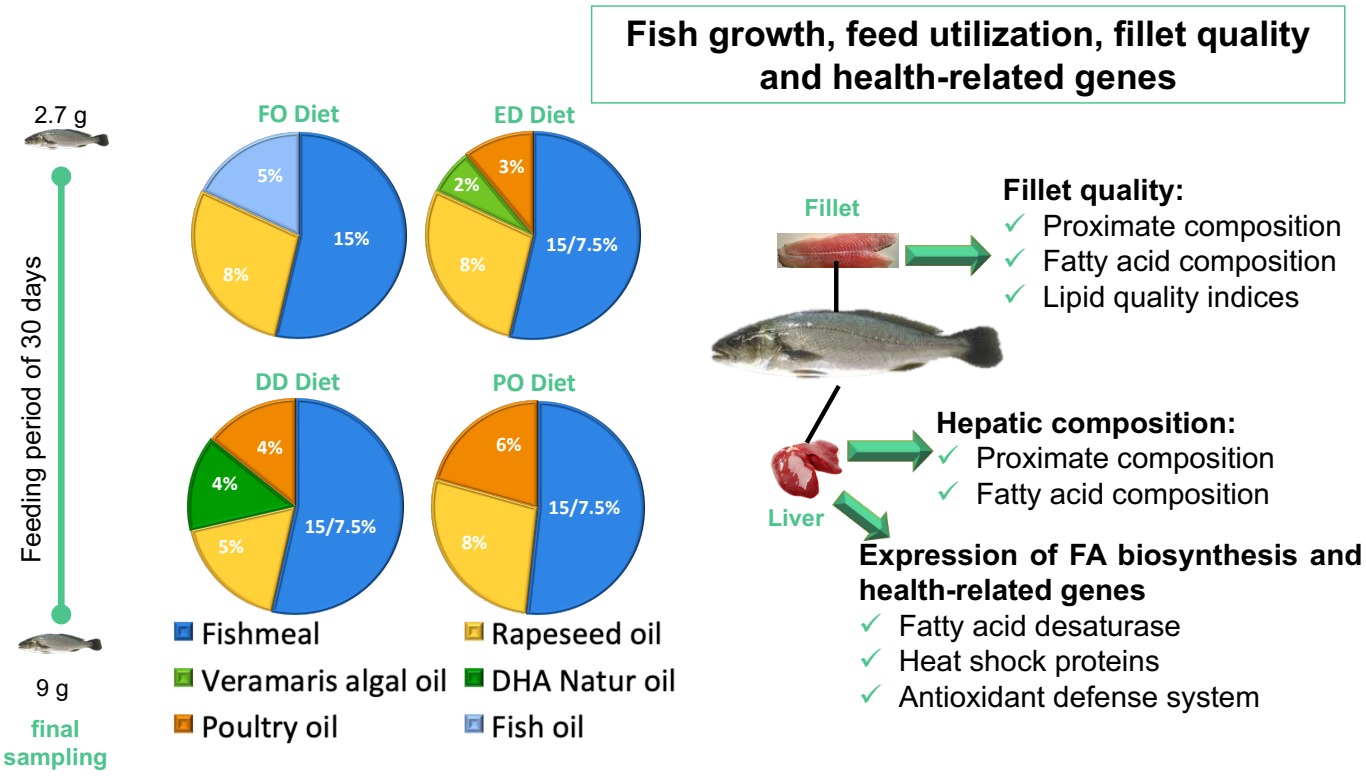

Figure 2.9. Graphical scheme of Chapter 5.

2.12.5 CHAPTER 6- HEPATIC BIOCHEMICAL, MORPHOLOGICAL AND MOLECULAR EFFECTS OF FEEDING MICROALGAE AND POULTRY OILS TO GILTHEAD SEA BREAM (SPARUS AURATA)

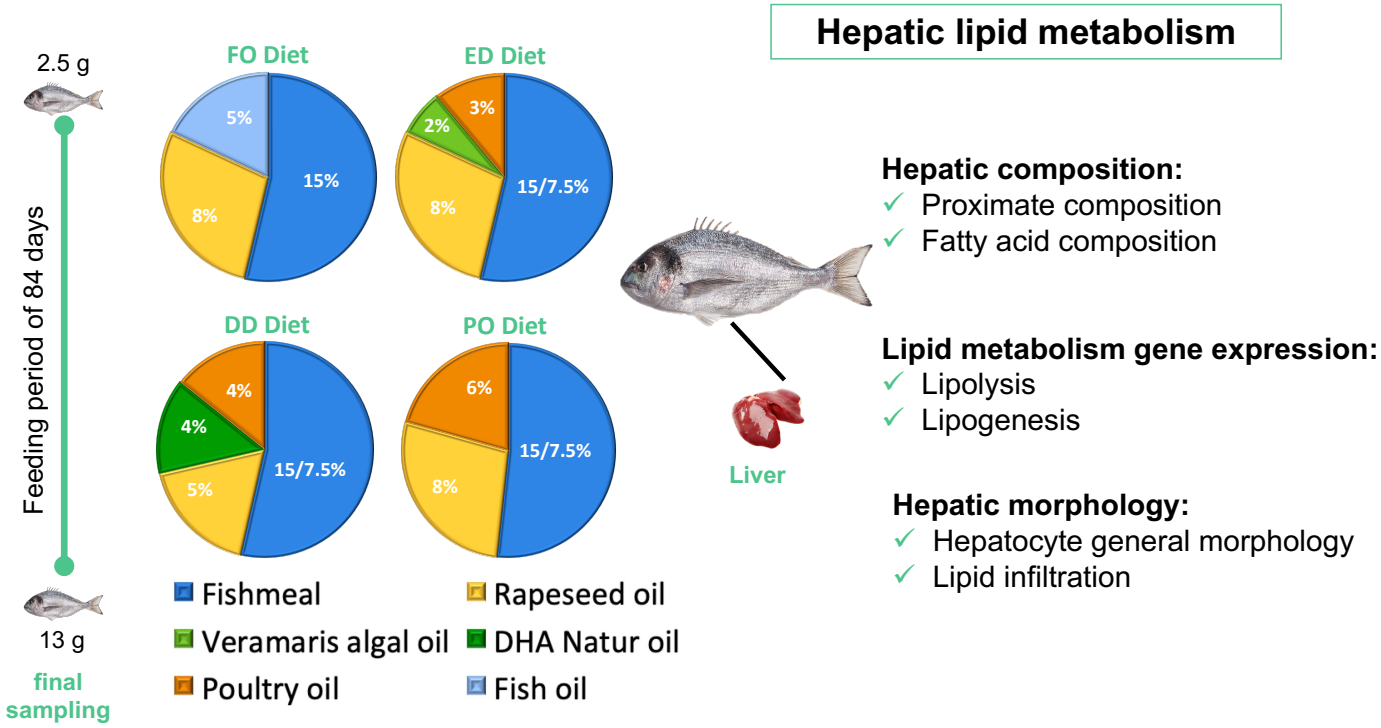

Figure 2.10. Graphical scheme of Chapter 6.

2.12.6 Chapter 7- Dietary novel oils modulate neural function and preserve locomotor response in gilthead sea bream (Sparus aurata) juveniles by regulating synthesis and contents of fatty acids in brain

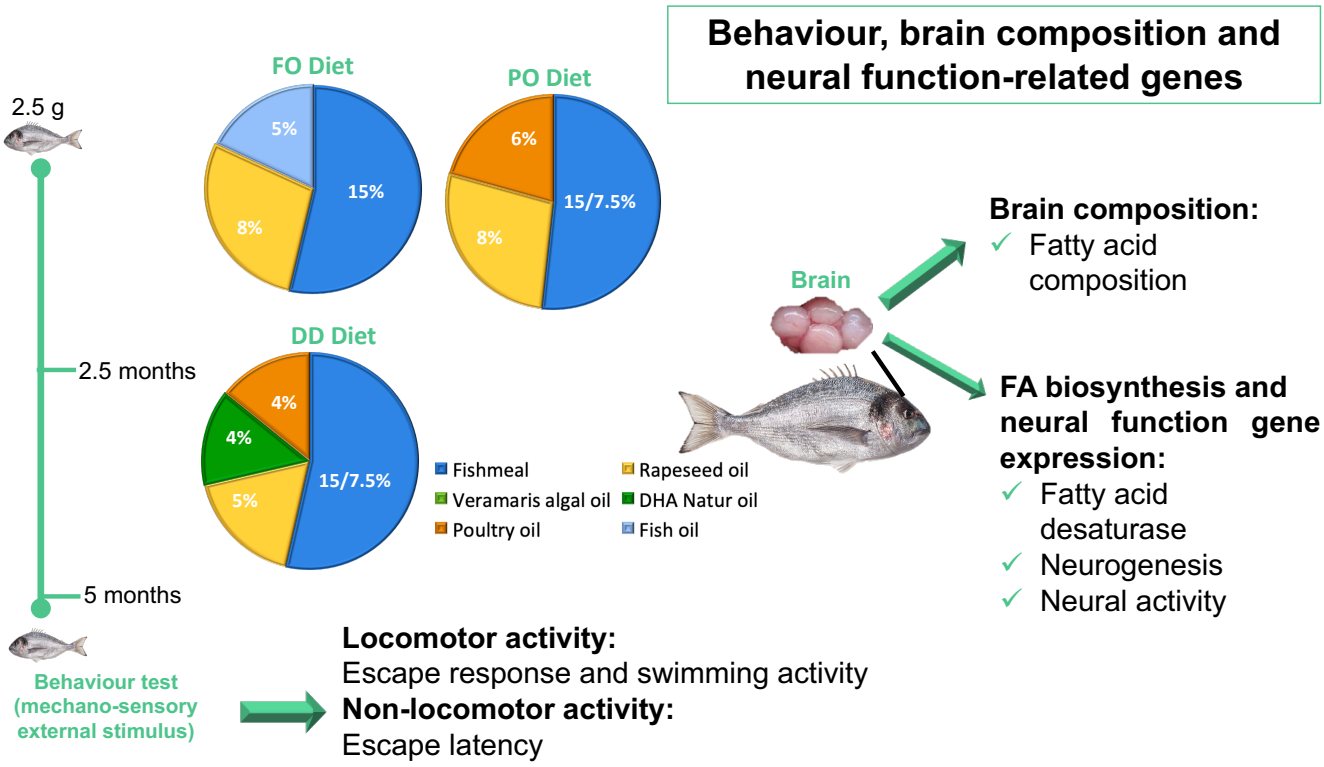

Figure 2.11. Graphical scheme of Chapter 7.

CHAPTER 3. COMPLETE REPLACEMENT OF FISH OIL BY THREE MICROALGAL PRODUCTS RICH IN N-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS IN EARLY WEANING MICRODIETS FOR GILTHEAD SEA BREAM (*SPARUS AURATA*)

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3.1 ABSTRACT

The aim of the present study was to evaluate the effect of different n-3 LC-PUFA-rich microalgae replacing FO in larval diets, on performance, biochemical composition, and health of gilthead sea bream (*Sparus aurata*). Dietary fish oil (FO) was replaced with oleic acid (OA) or three n-3 LC-PUFA-rich algae products (2 commercial products: All G Rich and DHA Gold, and a development product: MO060). The five early weaning microdiets were fed to 20-dah gilthead sea bream larvae for 21 days. Proximate composition and fatty acid profiles of total, neutral and polar lipids of seabream larvae were analysed, as well as hepatocyte morphology and the expression of selected genes related to lipid metabolism. OA diet significantly inhibited larval growth and survival after a stress challenge, whereas FO replacement by DHA Gold and MO60 led to a higher body weight, total length, and stress resistance. Larvae fed the diets containing microalgae had increased DHA content in whole body lipids and showed a down-regulation of expression of *fads2*, indicating an inhibition of LC-PUFA biosynthesis. Relative expression of genes related to lipid (*ppar-α*), or eicosanoids (*cox-2* and *5-lox*) metabolism were unaffected by the replacement of FO by any of the three microalgal products or OA. In conclusion, all the microalgae products tested were effective total replacers of FO in weaning microdiets for gilthead sea bream larvae, providing the necessary dietary n-3 LC-PUFA for their optimal growth and survival.

3.2 INTRODUCTION

Fish oil (FO) is rich in long-chain polyunsaturated fatty acids (LC-PUFA), particularly in docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), which have critical functional and physiological roles in fish larval growth, survival, and health (Tocher, 2010). Indeed, n-3 LC-PUFA participate in the normal immune function of fish and are particularly important in stress response. Concretely, these FA are substrates for lipoxygenases (LOX) and cyclooxygenases (COX), enzymes that modulate the production of eicosanoids, hormone-like metabolites involved, among others, in inflammatory processes in vertebrates (Rowley et al., 1995). Additionally, n-3 LC-PUFA regulates lipid metabolism by controlling fatty acid synthesis and catabolism to maintain lipid homeostasis. Several enzymes are involved in both metabolic pathways, but peroxisome proliferator-activated receptor α (PPAR-α) is a critical enzyme for fatty acid β-oxidation in peroxisomes (Tocher, 2003). Furthermore, PPAR is involved not only in energy balance and lipid metabolism, but also in glucose metabolism (Lee et al., 2003). Gluconeogenesis is promoted during starvation, energy-restricted periods, or different dietary lipid sources, by increasing gluconeogenic enzymes activities, including that of glucose-6-phosphatase (G6P), converting glucose-6-phosphate to glucose and, consequently, increasing blood glucose levels (Enes et al., 2008; Wang et al., 2019). Therefore, changes in fatty acid composition of tissues are also related to affect PPAR and, directly or indirectly glucose metabolism to maintain homeostasis, through glycolysis/gluconeogenesis pathways (Caseras et al., 2002; Wang et al., 2019) because FA oxidation leads to acetyl-CoA that is used in gluconeogenesis (Enes et al., 2008).

Supply of n-3 LC-PUFA through the diet is particularly important in marine teleosts, since they lack the ability to synthesize these FA from their 18-carbon precursors, such as alpha-linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6). This is due to a reduced activity in Δ4, Δ5 and/or Δ6 desaturase steps, regulated at a molecular and enzymatic level by fatty acyl desaturase 2 (FADS2), an enzyme that in most marine fish has been shown to have a Δ6 activity (Tocher, 2003; Tocher, et al., 2006; Izquierdo et al., 2008). Therefore, n-3 LC-PUFA are dietary essentials for marine fish such as the gilthead sea bream (*Sparus aurata*). Furthermore, fish larvae have usually a much higher requirement for n-3 LC-PUFA than later life stages due to the pivotal important plastic purposes of these FA, particularly of DHA, for tissue and organs development (Izquierdo, 2005; Izquierdo and Koven, 2011).

Given the global limited availability of fish oils for aquaculture feeds and the successive increase in the use of alternative lipid sources, mostly vegetable oils (VO), meeting adequately the dietary n-3 LC-PUFA requirements of fish becomes difficult to achieve. Ensuring increased production in aquaculture is dependent on sustainable feeding strategies and the use of new sources of n-3 LC-PUFA as alternatives to FO and VO. Microalgae appear to be the most

promising alternative to the traditional marine derived ingredients, since they are the primary producers of EPA and DHA in the marine ecosystems, and can become a cost competitive product alternative to FO over the next years (Ganuza and Izquierdo, 2007; Chauton et al., 2015; Sprague et al., 2017). The field of microalgae intended for aquaculture is now attracting considerable attention and a few commercial products are available, particularly as a source of n-3 LC-PUFA for larval nutrition (Han et al., 2019; Glencross et al., 2020; Pratiwy et al., 2020). Algal products have been successfully used to replace FO in farmed fish diets. Microalgal meals such as ALL-G RICH™ (Alltech Inc., Nicholasville, KY, USA), obtained from the marine heterotroph *Schizochytrium limacinum*, have been tested as dietary lipid sources and proven effective in longfin yellowtail (*Seriola rivoliana*)(Kissinger et al., 2016), giant grouper (*Epinephelus lanceolatus*)(García-Ortega et al., 2016), rainbow trout (*Oncorhynchus mykiss*) (Lyons et al., 2017), tilapia (Fernandes et al. 2018) and red drum (*Sciaenops ocellatus*) (Perez-Velazquez et al., 2018). Another commercial algal product, DHA GOLD®, produced from (*Crypthecodinium cohnii*) and based on powder algal fermentation has demonstrated its potential as a supplementary lipid source resulting in similar growth performance to that of a FO-based diet (Betiku, et al., 2016). Furthermore, the development microalgae product based on a new fermentation process of *Schizochytrium limacinum* to further enrich in EPA and DHA, (MO060, Adisseo France SAS) has also been tested. However, despite the great potential of microalgal products as a rich source of n-3 LC-PUFA, especially DHA, they generally contain lower levels of EPA than FO, besides having relatively higher contents of other FA that are not commonly found on FO, for instance the n-6 docosapentaenoic acid (DPA, 22:5n-6) (Atalah et al., 2007; Ganuza et al., 2008).

Thus, the purpose of this study was to examine the effects of different commercially available microalgal products (ALL-G RICH™ and DHA GOLD®) or in development (MO060), as complete replacers of dietary FO in early weaning diets for gilthead sea bream. The three products selected were high in n-3 LC-PUFA but differed particularly in their specific fatty acid composition, particularly in DHA, EPA, ARA, or n-6 DPA contents. Besides their effects on larval survival, growth performance and body composition, metabolism and health-related genes expression were also assessed.

3.3 MATERIALS AND METHODS

3.3.1 DIETS AND FEEDING TRIAL

Five practical microdiets (pellet size <250 µm), based on defatted squid meal (defatted 3 consecutive times in our laboratories with a chloroform:methanol ratio of 3:1) as protein source, were formulated and tested in quadruplicate. A positive control diet was based on fish oil (FO diet), and a negative control diet, replaced FO by oleic acid (OA diet). Additionally, three experimental diets were formulated to test three commercial or on-development n-3 LC-PUFA-rich algal products: the AGR diet that included ALL G RICH™ (with 70% fat content, of which 19% of total FA is DHA; Alltech Inc., Nicholasville, KY, USA), the DHG diet that included DHA GOLD (56% of fat of which 39.5% of total FA is DHA; DSM, Basel, Switzerland) and the M60 diet that included MO060 (73 % fat, of which 49% of total FA is DHA; Adisseo, France SAS). For diets preparation, squid powder and water-soluble components were firstly mixed, followed by mixing the fat-soluble vitamins and, finally, gelatine, which was dissolved in warm water. The microdiets paste was pelleted and dried in an oven at 38°C for 24 h. The following day, pellets were grounded and sieved to particle sizes below <250 µm. Diet formulation, proximate composition, and fatty acid contents of microdiets are described in Tables 3.1-3.3.

Fish larvae were manually fed with the experimental diets (each 45 min from 8:00-20:00), for 21 days by a trained researcher. After 5 days of the beginning of the feeding trial, larvae were observed under the binocular microscope to determine feed acceptance by removing (after feeding) 5 larvae per tank, which were kept in distilled water on a slide microscope to observe the presence of the feed inside the digestive tube.

Table 3.1. Ingredients and proximate composition (% dry weight) of the experimental microdiets with different meals fed to gilthead sea bream larvae for 21 days

	Diets				
Ingredients (%)	OA	FO	AGR	DHG	M60
Squid meal[1] (defatted)	69.2	69.2	68.2	68.2	68.2
Gelatin[2]	3.0	3.0	3.0	3.0	3.0
Soy lecithin[3]	8.0	8.0	8.0	8.0	8.0
Sardine oil[4]	0	5.0	0	0	0
Oleic acid[5]	5.0	0	0	0	0
All G Rich[6]	0	0	6.0	0	0
DHA Gold[7]	0	0	0	6.0	0
MO060[8]	0	0	0	0	6.0
Mineral premix[9]	4.5	4.5	4.5	4.5	4.5
Vitamin premix[10]	5.8	5.8	5.8	5.8	5.8
Taurine	1.5	1.5	1.5	1.5	1.5
Attractants[11]	3.0	3.0	3.0	3.0	3.0
Analysed proximate composition (% dw)					
Moisture	6.9	8.4	7.2	6.6	7.8
Proteins (N x 6.25)	67.5	68.5	69.3	69.0	69.5
Total lipids	12.6	13.3	11.6	11.4	11.6
Neutral lipids (% total lipids)	82.8	50.0	60.8	53.3	42.6
Polar lipids (% total lipids)	17.2	50.0	39.3	46.7	57.4
Ash	8.0	8.0	7.8	8.5	7.7

[1] Rieber & Son, Bergen, Norway
[2] Sigma-Aldrich, St. Louis, USA
[3] Novadiet, S.A., Burgos, Spain
[4] Agramar S.A., Spain
[5] Merck KGaA, Darmstadi, Germany
[6] Alltech Inc., Nicholasville, KY, USA
[7] DSM Nutritional Products, Basel, Switzerland
[8] Adisseo, France SAS
[9] Mineral premixes supplied (in mg per 100 g diet): NaCl 215.13, $MgSO_47H_2O$ 677.55, $NaH_2PO_4H_2O$ 381.45, K_2HPO_4 758.95, $Ca(H_2PO_4)2H_2O$ 671.61, $FeC_6H_5O_7$ 146.88, $C_3H_5O_31/2Ca$ 1617.21, $Al_2(SO_4)_36H_2O$ 0.69, $ZnSO_47H_2O$ 14.84, $CuSO_45H_2O$ 1.25, $MnSO_4H_2O$ 2.99, KI 0.74, $CoSO_47H_2O$ 10.71; Sigma-Aldrich, Madrid, Spain
[10] Vitamin premix supplied (in mg per 100 g diet): cyanocobalamine 0.030, astaxanthin 5.00, folic acid 5.44, pyridoxine-HCl 17.28, thiamine 21.77, riboflavin 72.53, ca-pantothenate 101.59, p-aminobenzoic acid145.00, nicotinic acid 290.16, inositol 1450.90, retinol acetate 0.24, ergocalcipherol 3.65, menadione 17.28, alpha-tocopherol acetate 150.00, ascorbyl poliphosphate 180.00, choline chloride 2965.8; Sigma-Aldrich, Madrid, Spain
[11] Attractants premix supplied (in mg per 100 g diet): inosine-5-monophos-phate 500.0, betaine 660.0, L-serine 170.0, L-phenylala-nine 250.0, DL-alanine 500.0, L-sodium aspartate 330.0, L-valine 250.0, glycine 170.0; Sigma-Aldrich, Madrid, Spain

Table 3.2. EPA and DHA contents (% total identified fatty acids) of the microalgae meals used for formulating the experimental microdiets

	Microalgae meals			
	AGR	DHG	M60	Fish oil
Protein	15.63	15.23	10.37	-
Lipid	70	56	73	-
Ash	2.63	11.17	3.26	-
DHA	18.74	39.5	49	4.19
EPA	0.43	0.96	1.73	2.16

Table 3.3. Fatty acid composition (% total identified fatty acids) of the experimental microdiets with different microalgae meals fed to gilthead sea bream larvae for 21 days

	Diets				
Fatty acid	OA	FO	AGR	DHG	M60
14:0	0.09	0.63	1.18	1.63	1.17
14:1n-7	0.01	0.03	0.02	0.01	0.02
14:1n-5	0.01	0.07	0.02	0.01	0.01
15:0	0.04	0.11	0.65	0.15	0.21
15:1n-5	0.01	0.03	0.03	0.02	0.01
16:0ISO	0.01	0.07	0.04	0.01	0.01
16:0	9.94	12.26	29.51	16.26	33.66
16:1n-7	0.24	1.50	0.26	0.44	0.19
16:1n-5	0.02	0.16	0.04	0.03	0.03
16:2n-4	0.01	0.17	0.03	0.02	0.01
17:0	0.01	0.09	0.02	0.01	0.02
16:3n-4	0.06	0.19	0.05	0.06	0.06
16:3n-3	0.03	0.13	0.04	0.04	0.04
16:3n-1	0.08	0.12	0.11	0.10	0.09
16:4n-3	0.03	0.08	0.02	0.02	0.05
16:4n-1	0.01	ND	0.02	0.01	0.01
18:0	4.23	3.76	3.49	3.07	3.37
18:1n-9	45.25	27.06	7.30	10.90	6.43
18:1n-7	1.29	2.52	1.04	1.24	0.88
18:1n-5	0.05	0.10	0.04	0.05	0.04
18:2n-9	0.02	0.10	0.02	0.05	0.00
18:2n-6	31.99	26.96	22.77	23.62	20.94
18:2n-4	0.01	0.10	0.03	0.02	0.02
18:3n-6	0.05	0.24	0.08	0.15	0.10
18:3n-4	0.03	0.17	0.04	0.03	0.03
18:3n-3	1.94	4.22	2.02	2.73	1.80
18:3n-1	0.02	ND	ND	0.01	0.01
18:4n-3	0.03	0.52	0.08	0.18	0.10
18:4n-1	0.02	0.18	0.06	0.03	0.02
20:0	0.33	0.30	0.31	0.24	0.24
20:1n-9	0.09	0.43	0.09	0.12	0.08
20:1n-7	1.31	3.43	1.42	1.46	1.37
20:1n-5	0.05	0.17	0.05	0.06	0.04
20:2n-9	0.03	0.12	0.05	0.09	0.02
20:2n-6	0.09	0.67	0.09	0.17	0.08
20:3n-9	0.03	0.07	0.08	0.22	0.11
20:3n-6	0.05	0.27	0.06	0.03	0.04
20:4n-6	0.11	0.36	0.76	0.96	0.27
20:3n-3	0.07	0.30	0.20	0.21	0.10
20:4n-3	0.03	0.51	0.31	0.50	0.27
20:5n-3	0.51	2.67	0.84	1.24	0.88
22:1n-1	0.04	1.79	0.08	0.23	0.28
22:1n-9	0.24	0.52	0.25	0.30	0.04
22:4n-6	0.03	0.14	0.07	0.08	0.04
22:5n-6	0.05	0.20	3.48	8.27	5.39
22:5n-3	0.14	0.98	0.18	0.43	0.12
22:6n-3	1.30	5.49	22.63	24.50	21.26
∑ SFA	14.65	17.22	35.21	21.37	38.68
∑ MUFA	48.60	37.81	10.64	14.87	9.43
∑ n-9	45.64	28.30	7.80	11.67	6.69
∑ n-6	32.36	28.84	27.32	33.28	26.85
∑ n-3	4.08	14.91	26.33	29.85	24.63
n-3/ n-6	0.13	0.52	0.96	0.90	0.92
∑ n-6 LC-PUFA	0.33	1.64	4.47	9.51	5.82
∑ n-3 LC-PUFA	2.06	9.95	24.16	26.88	22.65
EPA/ARA	4.8	7.4	1.1	1.3	3.2
EPA/DHA	0.4	0.5	0.1	0.1	0.1

ND, not detected

3.3.2 LARVAL PERFORMANCE

Quadruplicate groups of 20 days after hatching (dah) gilthead sea bream larvae, with an initial total length of 8.04 ± 0.1 mm and initial dry body weight of 0.48 ± 0.1 mg, were allocated in a total of 24 tanks of 200 L with a water flow of 150L hour-1, at a density of 600 larvae tank-1. Temperature along the trial was 22.6 ± 0.12ºC, dissolved oxygen 5.6 mg L^{-1}, pH 8.2 ± 0.02 and salinity 37 mg L^{-1}. After the feeding period, larval growth was determined by measuring body weight of 30 larvae per tank, that were dried at 105°C until constant weight (~24 h). Additionally, 30 larvae per tank were removed, placed on ice, and transferred to a microscope slide with distiller water to measure individual total length (Profile Projector V-12A; Nikon, Tokyo, Japan). Final survival was calculated by individually counting all the alive larvae that survived to the whole feeding period.

To test larval stress resistance, at the end of the feeding period, an air-exposure stress test was conducted with 20 larvae per tank that were placed in a scoop net out of the water for 2 min. Subsequently, larvae were allocated in a new tank supplied with clean seawater and aeration, and survival was determined after 24 h.

Whole-larvae samples were also, washed with distilled water after starvation of 16h and sampled for analysis (biochemical, histological, and molecular analysis).

3.3.3 BIOCHEMICAL ANALYSIS

Moisture (AOAC, 1995), protein by nitrogen determination N x 6.25 (Kjeldahl, 1883) and crude lipid (Folch et al., 1957) contents of diets and larvae were analysed. Neutral (NL) and polar lipid (PL) fractions of larvae were separated for characterising FA profile of each fraction, eluting PL with methanol and NL with chloroform/methanol (49:1 v/v) (Juaneda and Rocquelin, 1985). For that, total lipids were filtered with a Sep-Pack NH2 cartridge, firstly eluting NL with 30 ml of chloroform and 20 ml of chloroform/methanol (49:1 v/v) and secondly eluting PL with 30 ml of methanol (Juaneda and Rocquelin, 1985). Eluents were added to sample in series of 10 ml. Then, each lipid fraction was evaporated in rotary evaporators and finalizing under N2 atmosphere for subsequent transmethylation. Fatty acid methyl esters were obtained by transmethylation of lipids (Christie, 1982), separated by gas-liquid chromatography (GLC), quantified by FID (GC-14A; Shimadzu, Tokyo, Japan) (Izquierdo et al., 1992), and identified by comparison with previously characterized standards.

3.3.4 LARVAL MORPHOLOGY

At the beginning and at the end of the feeding trial, 30 larvae per tank were sampled and preserved in 4% buffered formaldehyde, dehydrated through graded alcohol and xylene, and finally embedded in paraffin wax for histological study. Paraffin blocks were made and cut with a Leica microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) in 4 µm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) to visualization under a light microscope (BX51TF, Olympus, Tokyo, Japan). Five larvae were kept per paraffin block. Livers were blinded assessed by three different researchers for cytoplasmic lipid vacuolization infiltration, using a semi-quantitative four graded examination scheme as follows: 0, no lipid infiltration; 1, few lipid vacuolization; 2, medium lipid vacuolization; 3, severe lipid vacuolization.

3.3.5 GENE EXPRESSION ANALYSES

Total RNA from whole larvae samples was extracted using TRI Reagen (Merck KGaA, Darmstadt, Germany) and the RNeasy Mini Kit (Qiagen). Total body tissue was homogenized using the TissueLyzer-II (Qiagen, Hilden, Germany) at 30Hz for 2 min. 200 µl of chloroform were added to the samples and then centrifuged for phase separation (12,000 g, 15 min, 4 °C). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into a RNeasy spin column where total RNA bonded to a membrane and RW1 and RPE buffers (Qiagen) were used to wash away contaminants. Purified RNA was eluted with 50 µl of RNase-free water. The quality of RNA was check by 1.4% agarose gel electrophoresis and the quantity were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Hundred ng of total RNA were reverse transcribed to complementary cDNA using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1:5, 1:10, 1:20, 1:50, 1:100, 1:200 and 1:500). Real-time quantitative PCR was performed in an CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using β-actin and ribosomal protein L27 (rpl-27) as housekeeping genes in a final volume of 15 µl per reaction well. The PCR conditions were as follows: 95 °C for 3 min 30 s followed by 40 cycles of 95 °C for 15 s, Tm °C for 30 s, and 72 °C for 30 s, and then 95 °C for 1 min, final denaturing step from 58 °C to 95 °C for 10 s. All PCR reactions were carried out in in a final volume of 15 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA (1:10 dilution) and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. In each run, an analysis of melting curve leading to a melting peak specific for the

amplified target DNA was carried out. *5-lipoxygenase* (*5-lox*), *cyclooxygenase-2* (*cox-2*), *fatty acyl desaturase 2* (*fads2*), *glucose-6-phosphatase* (*g6p*) and *peroxisome proliferator-activated receptor α* (*ppar-α*) and expressions were determined. Relative gene expression was estimated by the Δ–Δ method (Livak and Schmittgen, 2001). Sequences of primers are shown in Table 3.4.

Table 3.4. Sequences of primers used to determine gene expression in 20 dah-gilthead sea bream larvae

	Sequence (5'-3')				
Genes	Forward	Reverse	Tm	GenBank Access nº	Reference
5-lox	CCTGGCAGATGTGAACTTGA	CGTTCTCCTGATACTGGCTGA	61	FP334124	Alves Martins et al., 2012
cox2	GAG TAC TGG AAG CCG AGC AC	GAT ATC ACT GCC TGA GT	61	AM296029	Chaves-Pozo et al., 2008
fads2	GCAGAGCCACAGCAGCAGGGA	CGG CCT GCG CCT GAG CAG TT	61	AY055749	Izquierdo et al., 2015
g6p	CGCTGGAGTCATTACAGGCGT	CAGGTCCACGCCCAGAACTC	61	AF151718.1	Enes et al., 2008
ppar-α	TCT CTT CAG CCC ACC ATC CC	ATC CCA GCG TGT CGT CTC C	58.1	AY590299	Benedito-Palos et al., 2014
ß-actin	GACCAACTGGGATGACATGG	GCATACAGGGACAGCACAGC	58.1	X89920.1	Minghetti et al. 2010
rpl-27	ACAACTCACTGCCCCACCAT	CTTGCCTTTGCCCAGAACTT	58.1	AY188520	Laizé et al., 2005

3.3.6 STATISTICAL ANALYSES

All data are presented as mean ± SE and were tested for normality and homogeneity of variances with Shapiro-Wilk and Levene′s test, respectively. Each tank was treated as a replicate (n=4 for each treatment). A one-way ANOVA was applied to data and, when applicable, means comparisons among different experimental groups were compared by Tukey's test (when homogeneity was assumed) or Games-Howell test (when homogeneity was not assumed). Statistical differences were considered when $P<0.05$. Histological evaluation was statistically treated with a Mann-Whitney U test. All statistical analyses were done using the SPSS 21.0 software package. In addition, principal component analysis (PCAs) were carried out in overall larvae whole-body fatty acid composition for each lipid fraction, using Prism9 software for Windows.

3.3.7 ETHICAL STATEMENT

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (reference OEBA-ULPGC-21/ 2018).

3.4 RESULTS

3.4.1 LARVAL PERFORMANCE

All experimental microdiets were well accepted by larvae denoted by the presence of feed inside digestive tube and the good feed intake. After three weeks of feeding the experimental diets, larval dry body weight had increased over threefold, and total length had increased over 44% percent (Table 3.5). Survival during the feeding period was 28% lower in larvae fed OA diet, but not significantly different ($P>0.05$), than that of larvae fed the other diets (Table 3.5). Larvae fed OA diet also showed the lowest growth ($P<0.05$), as well as the lowest survival after the air exposure (Table 3.5). In contrast, FO, AGR, DHG and M60 diets led to the highest larval growth and survival after the air exposure test ($P<0.0$; Table 3.5).

Table 3.5. Survival and growth performance of 20 dah-gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days

	Diets				
	OA	FO	AGR	DHG	M60
Survival (%)	38 ± 3.2	53 ± 1.76	46 ± 3.55	52 ± 2.02	46 ± 6.35
Body weight (mg dry weight)	1.61 ± 0.11^{b}	2.12 ± 0.33^{ab}	2.03 ± 0.21^{ab}	2.76 ± 0.17^{a}	2.82 ± 0.29^{a}
Total length (mm)	10.54 ± 0.21^{b}	11.31 ± 0.43^{ab}	11.27 ± 0.23^{ab}	12.47 ± 0.27^{a}	12.17 ± 0.38^{a}
Survival after air exposure test (%)	28.33 ± 2.89^{b}	90.00 ± 8.66^{a}	78.33 ± 16.65^{a}	91.25± 3.15^{a}	95.00 ± 5.00^{a}

Values (mean ± SE; n=4) with the different superscripts are significantly different.

3.4.2 DIETS AND LARVAE BIOCHEMICAL AND FATTY ACID COMPOSITION

Regarding the fatty acid composition of the microalgae meals used, MO060 was the highest in DHA and EPA contents, followed by DHA GOLD and then ALL-G RICH (Table 3.2). Fatty acid analysis of the diets showed that the diet containing oleic acid oil (OA Diet) was characterized by the highest content in monounsaturated fatty acids (MUFA), particularly, oleic acid (OA, 18:1n-9), and LA (18:2n-6) (Table 3.3) and the lowest n-3 LC-PUFA content. In contrast, the diet containing FO was characterized by the highest content in EPA (20:5n-3), and consequently the highest levels of EPA/ARA and EPA/DHA, as well as the highest LNA (18:3n-3) content (Table 3.3). The diets containing algal products were higher in n-3 LC-PUFA, particularly DHA (22:6n-3) and n-6 DPA (22:5n-6), as well as a higher n-3/n-6, than FO and OA diet. Among them, diet DHG showed the highest n-6 DPA and n-3 LC-PUFA levels. AGR diet was slightly high in n-3/n-6 fatty acids and M60 was highest in saturated fatty acids (SFA), particularly palmitic acid (16:0) (Table 3.3). Thus, compared to FO diet, the inclusion of M60 or AGR doubled the contents in SFA and all the microalgal products increased the DHA content by almost four-fold (Table 3.3).

Regarding fatty acid profiles of larval total lipids, they generally reflected the fatty acid composition of the diets, with significantly higher (P>0.05) n-3 LC-PUFA, DHA, and n-6 DPA contents in fish fed the algal diets (Table 3.6). Besides, EPA/ARA and EPA/DHA followed a similar pattern to the diet. However, although 18:1n-9 was 7 times higher in OA diet with respect to M60 diet, the contents of this fatty acid in larvae fed these two diets were not significantly (P>0.05) different (Table 3.6). Similar results were also found for 18:2n-6 and 18:3n-3 (Table 3.6). On the contrary, the contents in 18:3n-6, 18:2n-9 or 20:2n-9, products of desaturation and elongation from 18:2n-6 and 18:1n-9, respectively, were significantly (P<0.05) increased in OA larvae, followed by FO larvae, in comparison to those fed the algal products (Table 3.6). The PCA results showed that PC1 explained 40.85 % of the total variation in fatty acid composition of total lipids in sea bream larvae, whereas PC2 explained 20.70% (Figure 3.1). PC1 was positively correlated with 20:4n-6, 17:0, 16:0 ISO and 16:3n-3 contents, while negatively correlated with n-6, 16:0 and 18:0, tending to separate M60 from DHG larvae. In contrast, PC2 was positively correlated with 18:3n-6, n-9, MUFA, 18:2n-9, 20:2n-9, 16:1n-7 and 18:2n-6, while negatively correlated with 22:5n-6, n-3/n-6, n-3, n-3 LC-PUFA and 22:6n-3, clearly separating FO and OA from AGR and DHG.

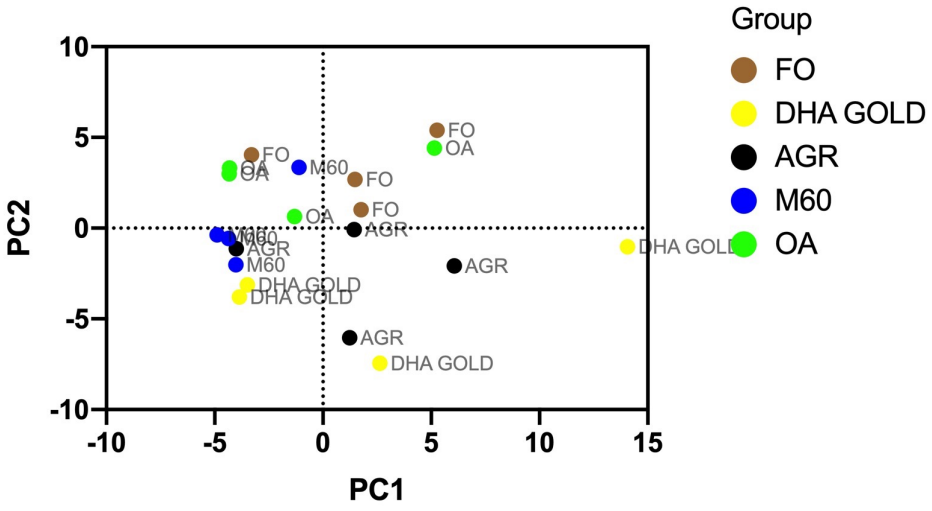

Figure 3.1. Principal components of total lipids fatty acid composition of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days.

PC1: 40.85%; PC2: 20.70%.

In neutral lipids, most fatty acids also followed the dietary profile (Table 3.7). However, the levels of 18:3n-6, 18:2n-9 or 20:2n-9, products of desaturation and elongation from 18:2n-6 and 18:1n-9, were significantly ($P<0.05$) increased in OA, FO and M60 larvae, in comparison to those fed the algal products (Table 3.7), regardless the dietary levels. The lowest levels of these fatty acids were found in DHG larvae (Table 3.7). Besides, both ARA (20:4n-6) and DHA contents in OA larvae were high and similar to those of FO larvae, despite the lower contents of those fatty acids in the OA diet (Table 3.7). The PCA results showed that PC1 explained 36.31 % of the total variation in fatty acid composition of neutral lipids in sea bream larvae, whereas PC2 explained 17.80% (Figure 3.2). PC1 was positively correlated with MUFA, n-9 and, particularly, 20:2n-6, 18:2n-9, 20:2n-9, as well as 18:4n-3 contents, while negatively correlated with SFA, 16:0, 18:0, n-3, n-3 LC-PUFA, 22:5n-6 and 22:6n-3, clearly differentiating FO and OA diets from those containing algal products. PC2 was positively correlated with 20:5n-3 and n-3/n-6 while negatively correlated with n-6 and 18:2n-6, separating FO from OA and DHG from M60.

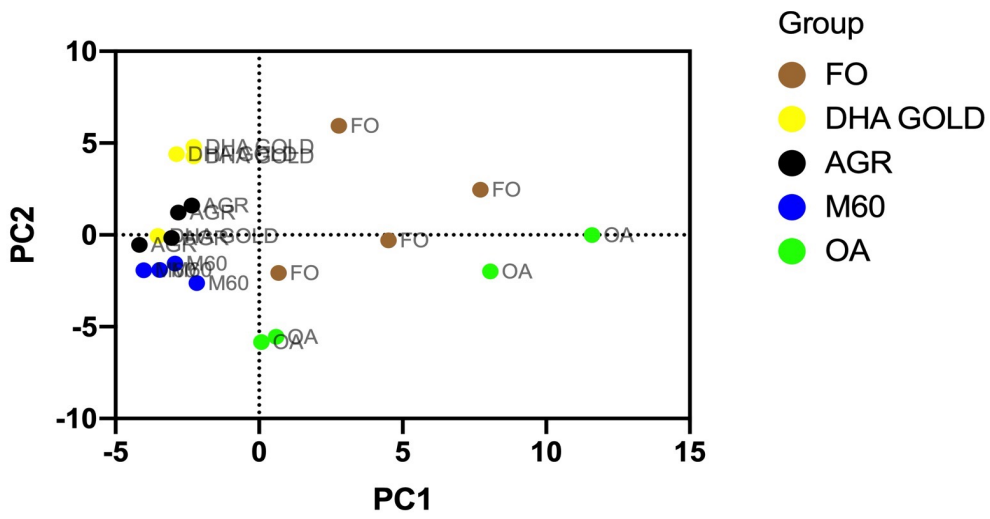

Figure 3.2. Principal components of neutral lipids fatty acid composition of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days.

PC1: 36.31%; PC2: 17.80%.

Fatty acid profiles of polar lipids from larvae (Table 3.8) fed the different diets was more homogeneous than that of neutral lipids and reflected the dietary profiles in a lesser extent than neutral lipids. For instance, the contents of 16:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and, particularly, 22:6n-3 in larvae fed the different diets were more similar among them (Table 3.8), than among the contents of these fatty acids in the different diets (Table 3.3). SFA, as well as 16:0 and 18:0, were higher in PL than in NL, and were also higher in

larvae fed the algal products than in OA larvae (Table 3.8). MUFA contents in PL, including 18:1n-9 (Table 3.8), were lower than in diet (Table 3.3) and in NL (Table 3.7), and were higher in OA and FO larvae than in the larvae fed the algal products (Table 3.8). However, the contents in 18:2n-9, 18:3n-6, 20:2n-9, 20:2n-6, 20:3n-9 or 20:3n-6 in PL (Table 3.8), were not related with the dietary levels of these fatty acids and were higher than in the diet but lower than in NL. Besides, all these fatty acids, products of elongation and desaturation from 18:1n-9 or 18:6n-6, were higher in OA larvae, followed by FO larvae (Table 3.8). The contents on 18:2n-6 in PL were lower than in diet and similar that their respective values in NL, and despite the values of this fatty acid for OA larvae were the highest in agreement with the dietary levels, they were also high in PL of M60 larvae, regardless the low content in M60 larvae. Finally, ARA, EPA, and DHA contents in PL (Table 3.8) were higher than their respective values in the diet. The PCA results showed that PC1 explained 36.24 % of the total variation in fatty acid composition of PL in sea bream larvae, whereas PC2 explained 18.96% (Figure 3.3). PC1 was positively correlated with MUFA, n-9 and, particularly, with 20:2n-6, 18:2n-9, 20:2n-9, 22:1n-9, as well as 18:4n-3 contents and negatively correlated with SFA, 16:0, n-3, n-3 LC-PUFA, 22:5n-6 and 22:6n-3, clearly differentiating FO and OA diets from those containing algal products. PC2 was positively correlated with 18:0 and negatively correlated with n-3 LC-PUFA.

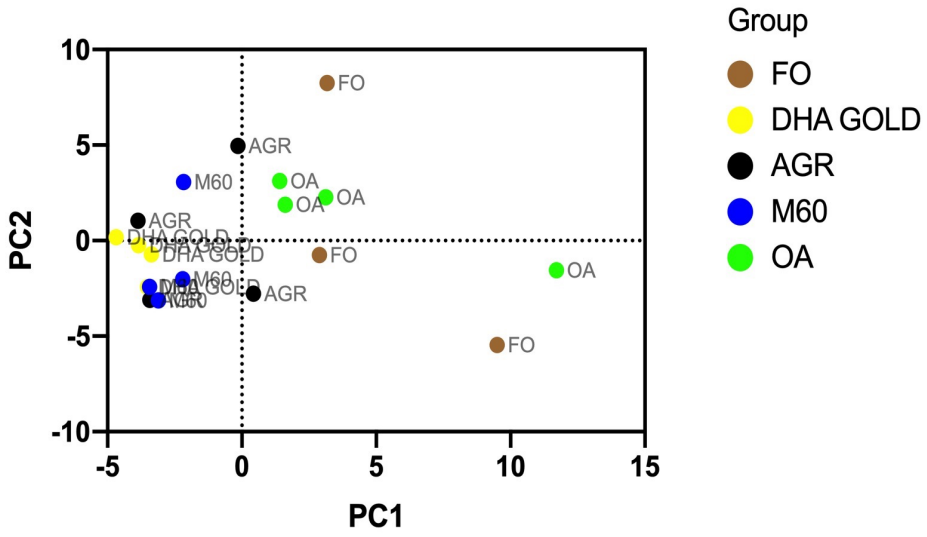

Figure 3.3. Principal components of polar lipids fatty acid composition of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days.

PC1: 36.24%; PC2: 18.96%.

Table 3.6. Total lipids (% dry weight) and respective fatty acid composition (% total fatty acids) of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days

	Diets				
	OA	FO	AGR	DHG	M60
Total lipids	14.13 ± 0.19	12.91 ± 0.64	14.43 ± 0.33	13.72 ± 0.4	13.14 ± 0.42
14:0	0.49 ± 0.11	0.51 ± 0.22	0.51 ± 0.20	0.48 ± 0.16	0.40 ± 0.15
14:1n-7	0.12 ± 0.16	0.19 ± 0.17	0.16 ± 0.14	0.20 ± 0.23	0.01 ± 0.01
14:1n-5	0.15 ± 0.20	0.25 ± 0.21	0.21 ± 0.19	0.24 ± 0.29	0.02 ± 0.01
15:0	0.24 ± 0.08	0.40 ± 0.18	0.32 ± 0.06	0.33 ± 0.30	0.17 ± 0.04
15:1n-5	0.03 ± 0.02	0.19 ± 0.15	0.25 ± 0.14	0.28 ± 0.35	0.03 ± 0.00
16:0ISO	0.12 ± 0.14	0.20 ± 0.16	0.23 ± 0.15	0.19 ± 0.22	0.03 ± 0.01
16:0	13.32 ± 3.66	14.89 ± 4.12	16.52 ±6.40	12.00 ±5.20	19.82 ± 3.96
16:1n-7	0.72 ± 0.08	0.95 ± 0.26	0.77 ± 0.28	0.64 ± 0.19	0.85 ± 0.13
16:1n-5	0.23 ± 0.10	0.28 ± 0.13	0.30 ± 0.14	0.26 ± 0.20	0.10 ± 0.01
16:2n-4	0.29 ± 0.05	0.31 ± 0.19	0.37 ± 0.06	0.35 ± 0.17	0.27 ± 0.05
17:0	0.25 ± 0.17	0.28 ± 0.13	0.24 ± 0.10	0.26 ± 0.22	0.08 ± 0.01
16:3n-4	0.26 ± 0.15	0.29 ± 0.11	0.28 ± 0.07	0.31 ± 0.19	0.16 ± 0.02
16:3n-3	0.18 ± 0.24	0.21 ± 0.16	0.25 ± 0.13	0.31 ± 0.28	0.05 ± 0.02
16:3n-1	0.76 ± 0.18	0.66 ± 0.07	0.80 ± 0.06	0.73 ± 0.10	0.77 ± 0.06
16:4n-3	0.64 ± 0.12^{ab}	0.48 ± 0.04^{a}	0.35 ± 0.07^{ab}	0.40 ± 0.23^{ab}	0.30 ± 0.01^{b}
16:4n-1	0.15 ± 0.15	0.22 ± 0.13	0.25 ± 0.11	0.08 ± 0.07	0.08 ± 0.01
18:0	8.01 ± 0.56	7.49 ± 0.75	7.99 ± 1.18	6.95 ± 1.73	9.00 ± 0.15
18:1n-9	18.56 ± 8.86^{ab}	16.95 ± 1.99^{a}	10.26 ± 1.66^{b}	9.74 ± 2.84^{ab}	12.85 ± 2.12^{ab}
18:1n-7	1.53 ± 0.08^{b}	2.34 ± 0.34^{a}	1.54 ± 0.19^{b}	1.49 ± 0.23^{b}	1.82 ± 0.17^{ab}
18:1n-5	0.14 ± 0.11	0.24 ± 0.14	0.25 ± 0.12	0.23 ± 0.24	0.06 ± 0.03
18:2n-9	3.92 ± 0.43^{a}	2.43 ± 0.27^{b}	0.84 ± 0.23^{cd}	0.42 ± 0.08^{d}	1.37 ± 0.11^{c}
18:2n-6	15.39 ± 4.78	14.90 ± 1.54	12.02 ±2.13	10.88±2.71	16.89 ± 0.32
18:2n-4	0.14 ± 0.15	0.22 ± 0.13	0.21 ± 0.11	0.28 ± 0.23	0.03 ± 0.02
18:3n-6	2.54 ± 0.21^{a}	2.25 ± 0.27^{a}	1.30 ± 0.27^{b}	0.93 ± 0.10^{b}	2.25 ± 0.16^{a}
18:3n-4	0.14 ± 0.13	0.21 ± 0.14	0.24 ± 0.14	0.30 ± 0.30	0.08 ± 0.01
18:3n-3	0.56 ± 0.12^{b}	1.22 ± 0.04^{a}	0.68 ± 0.15^{b}	0.78 ± 0.25^{ab}	1.33 ± 1.05^{ab}
18:4n-3	0.30 ± 0.23	0.43 ± 0.07	0.37 ± 0.16	0.32 ± 0.18	0.26 ± 0.16
18:4n-1	0.16 ± 0.15	0.18 ± 0.16	0.23 ± 0.12	0.23 ± 0.23	0.04 ± 0.02
20:0	0.47 ± 0.18	0.48 ± 0.13	0.38 ± 0.04	0.43 ± 0.15	0.36 ± 0.15
20:1n-9	0.15 ± 0.12	0.34 ± 0.10	0.27 ± .15	0.28 ± 0.19	0.13 ± 0.06
20:1n-7	1.04 ± 0.25	1.55 ± 0.31	1.16 ± 0.13	1.20 ± 0.09	1.22 ± 0.58
20:1n-5	0.21 ± 0.21	0.30 ± 0.16	0.26 ± 0.07	0.30 ± 0.20	0.16 ± 0.07
20:2n-9	0.83 ± 0.09^{a}	0.69 ± 0.12^{ab}	0.43 ± 0.15^{bc}	0.28 ± 0.23^{c}	0.47 ± 0.08^{abc}
20:2n-6	0.66 ± 0.21	0.79 ± 0.22	0.61 ± 0.18	0.53 ± 0.10	0.46 ± 0.20
20:3n-9	0.16 ± 0.14	0.21 ± 0.17	0.40 ± 0.39	0.29 ± 0.31	0.03 ± 0.03
20:3n-6	1.10 ± 0.21	1.23 ± 0.26	0.88 ± 0.28	0.90 ± 0.45	0.99 ± 0.18
20:4n-6	1.60 ± 0.08^{b}	1.65 ± 0.25^{ab}	1.74 ± 0.15^{ab}	2.11 ± 0.06^{a}	1.64 ± 0.72^{ab}
20:3n-3	0.27 ± 0.31	0.41 ± 0.16	0.56 ± 0.49	0.43 ± 0.28	0.24 ± 0.15
20:4n-3	0.26 ± 0.27	0.59 ± 0.13	0.26 ± 0.18	0.47 ± 0.32	0.38 ± 0.29
20:5n-3	1.08 ± 0.04^{b}	3.01 ± 0.64^{a}	1.37 ± 0.23^{ab}	1.35 ± 0.15^{ab}	1.81 ± 1.20^{ab}
22:1n-1	0.45 ± 0.25	0.67 ± 0.30	0.51 ± 0.34	1.77 ± 2.51	0.23 ± 0.19
22:1n-9	0.87 ± 0.49	0.97 ± 0.44	0.84 ± 0.32	1.56 ± 1.71	0.38 ± 0.22
22:4n-6	0.31 ± 0.15	0.50 ± 0.30	0.50 ± 0.30	0.52 ± 0.55	0.21 ± 0.17
22:5n-6	1.61 ± 0.22^{b}	1.24 ± 0.44^{b}	4.44 ± 1.30^{ab}	7.21 ± 4.12^{a}	3.62 ± 1.80^{ab}
22:5n-3	0.49 ± 0.16	1.74 ± 0.76	0.79 ± 0.33	0.60 ± 0.30	1.07 ± 1.24
22:6n-3	9.08 ± 0.38^{c}	14.38 ± 5.71^{bc}	26.78 ± 8.82^{ab}	29.96 ± 6.17^{a}	17.43 ± 4.74^{abc}
∑ SFA	22.90 ± 3.52	24.25 ± 4.84	26.17 ± 7.60	20.64 ± 6.32	29.86 ± 4.09
∑ MUFA	24.22 ± 8.73	25.23 ± 2.03	16.77 ± 2.58	18.19 ± 4.85	17.87 ± 3.14
∑ n-9	24.50 ± 8.41^{ab}	21.60 ± 1.81^{a}	13.06 ± 1.90^{b}	12.57 ± 2.43^{b}	15.23 ± 2.28^{ab}
∑ n-6	23.20 ± 4.85^{ab}	22.62 ± 0.81^{b}	21.59 ± 0.72^{b}	23.14 ± 4.50^{ab}	26.09 ± 0.51^{a}
∑ n-3	12.59 ± 0.80^{b}	22.07 ± 7.11^{ab}	30.84 ± 9.05^{ab}	34.19 ± 6.52^{a}	22.63 ± 2.66^{a}
n-3/ n-6	0.57 ± 0.15	0.98 ± 0.33	1.43 ± 0.42	1.56 ± 0.51	0.87 ± 0.09
∑ n-6 LC-PUFA	5.27 ± 0.81	5.42 ± 1.18	8.16 ± 1.94	11.27 ± 3.47	6.92 ± 0.88
∑ n-3 LC-PUFA	10.91 ± 0.24^{b}	$19.72 \pm 7.^{17ab}$	29.20 ± 9.02^{ab}	32.37 ± 6.29^{a}	20.69 ± 2.96^{a}
EPA/ARA	0.68 ± 0.06^{c}	1.82 ± 0.16^{a}	0.79 ± 0.12^{bc}	0.64 ± 0.09^{c}	1.02 ± 0.19^{b}
EPA/DHA	0.12 ± 0.01^{a}	0.23 ± 0.05^{a}	0.06 ± 0.02^{b}	0.05 ± 0.01^{b}	0.14 ± 0.14^{ab}

Values (mean ± SE; n=4) with the different superscripts are significantly different

Table 3.7. Neutral lipids fatty acid composition (% total fatty acids) of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days

	Diets				
	OA	FO	AGR	DHG	M60
Fatty acids					
14:0	0.35 ± 0.08^{b}	0.91 ± 0.25^{ab}	0.77 ± 0.07^{a}	2.66 ± 1.17^{ab}	0.68 ± 0.20^{ab}
14:1n-7	0.21 ± 0.20	0.08 ± 0.08	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.00
14:1n-5	0.29 ± 0.28	0.13± 0.09	0.03 ± 0.00	0.08 ± 0.04	0.02 ± 0.00
15:0	0.24± 0.11	0.43 ± 0.06	0.50 ± 0.02	0.70 ± 0.22	0.33 ± 0.09
15:1n-5	0.13 ± 0.09	0.09 ± 0.06	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01
16:0ISO	0.21 ± 0.18	0.12 ± 0.08	0.05 ± 0.04	0.08 ± 0.03	0.02 ± 0.01
16:0	7.04 ± 1.71^{c}	12.29 ± 2.67^{bc}	16.82 ± 1.09^{ab}	22.85 ± 3.30^{a}	11.58 ± 6.76^{bc}
16:1n-7	0.79 ± 0.19^{b}	1.47 ± 0.12^{a}	0.87 ± 0.16^{b}	2.62 ±1.44^{ab}	0.75 ± 0.15^{b}
16:1n-5	0.27 ± 0.16	0.30 ± 0.11	0.10 ± 0.03	0.20 ± 0.10	0.09 ± 0.02
16:2n-4	0.27 ± 0.11^{ab}	0.30 ± 0.09^{a}	0.29 ± 0.07^{a}	0.08 ± 0.03^{b}	0.23 ± 0.08^{ab}
17:0	0.33 ± 0.23	0.19 ± 0.08	0.10 ± 0.01	0.06 ± 0.04	0.08 ± 0.02
16:3n-4	0.29 ± 0.16	0.29 ± 0.07	0.22 ± 0.07	0.20 ± 0.14	0.14 ± 0.04
16:3n-3	0.18 ± 0.15	0.19 ± 0.12	0.06 ± 0.02	0.17 ± 0.05	0.05 ± 0.01
16:3n-1	0.25 ± 0.13	0.17 ± 0.05	0.17 ± 0.06	0.13 ± 0.02	0.12 ± 0.01
16:4n-3	0.34 ± 0.15	0.19 ± 0.06	0.15 ± 0.06	0.09 ± 0.01	0.09 ± 0.03
18:0	6.10 ± 0.82^{b}	6.72 ± 0.53^{ab}	8.07 ± 0.25^{a}	8.35 ± 0.56^{a}	8.51 ± 1.28^{a}
18:1n-9	24.42 ± 3.89^{a}	18.55 ± 2.69^{ab}	10.29 ± 0.31^{c}	13.65 ± 1.22^{abc}	12.36 ± 1.32^{bc}
18:1n-7	1.52 ± 0.05^{b}	2.48 ± 0.06^{a}	1.76 ± 0.12^{b}	2.48 ± 0.42^{ab}	1.78 ± 0.22^{b}
18.1n-5	0.16 ± 0.20	0.17 ± 0.06	0.09 ± 0.02	0.09 ± 0.02	0.07 ± 0.02
18:2n-9	5.00 ± 0.72^{a}	3.42 ± 0.35^{b}	1.11 ± 0.32^{cd}	0.43 ± 0.10^{d}	1.90 ± 0.18^{c}
18:2n-6	16.93 ± 3.18	11.92 ± 4.03	14.80 ± 1.09	11.01 ± 3.11	18.18 ± 2.15
18:2n-4	0.18 ± 0.14	0.18 ± 0.10	0.04 ± 0.01	0.09 ± 0.04	0.02 ± 0.01
18:3n-6	3.24 ± 0.35^{a}	4.97 ± 3.93^{ab}	2.43 ± 0.41^{ab}	1.67 ± 0.32^{b}	3.40 ± 0.42^{a}
18:3n-4	0.19 ± 0.15	4.17 ± 6.84	0.09 ± 0.04	0.09 ± 0.04	0.05 ± 0.01
18:3n-3	0.73 ± 0.07^{b}	1.34 ± 0.32^{a}	0.87 ± 0.07^{b}	0.91 ± 0.13^{ab}	1.04 ± 0.16^{ab}
18:4n-3	0.38 ± 0.26^{ab}	0.95 ± 0.56^{ab}	0.24 ± 0.03^{b}	0.42 ± 0.04^{a}	0.31 ± 0.05^{ab}
18:4n-1	0.26 ± 0.20	0.20 ± 0.13	0.04 ± 0.01	0.07 ± 0.03	0.04 ± 0.02
20:0	0.48 ± 0.11	0.57 ± 0.19	0.49 ± 0.09	0.43 ± 0.07	0.46 ± 0.07
20:1n-9	0.24 ± 0.12	0.60 ± 0.30	0.16 ± 0.04	0.19 ± 0.03	0.13 ± 0.02
20:1n-7	1.24 ± 0.15	1.83 ± 0.31	1.40 ± 0.12	1.24 ± 0.12	1.51 ± 0.17
20:1n-5	0.29 ± 0.24	0.30 ± 0.14	0.24 ± 0.16	0.17 ± 0.03	0.16 ± 0.06
20:2n-9	1.12 ± 0.10^{a}	0.60 ± 0.19^{b}	0.32 ± 0.08^{c}	0.12 ± 0.04^{c}	0.65 ± 0.05^{b}
20:2n-6	0.65 ± 0.14^{ab}	0.66 ± 0.16^{a}	0.48 ± 0.13^{ab}	0.36 ±0.06^{b}	0.41 ± 0.07^{ab}
20:3n-9	1.26 ± 0.68^{abc}	0.43 ± 0.48^{abc}	0.76 ± 0.07^{b}	0.04 ± 0.02^{c}	1.07 ± 0.03^{a}
20:3n-6	0.76 ± 0.50	0.59 ± 0.41	0.23 ± 0.06	0.38 ± 0.10	0.23 ± 0.07
20:4n-6	2.05 ± 0.15^{a}	2.34 ± 1.76^{ab}	2.18 ± 0.14^{a}	2.02 ± 0.38^{ab}	1.41 ± 0.19^{b}
20:3n-3	0.43 ± 0.32	0.31 ± 0.09	0.22 ± 0.04	0.20 ± 0.04	0.14 ± 0.03
20:4n-3	0.42 ± 0.32	0.56 ± 0.23	0.35 ± 0.07	0.38 ± 0.06	0.26 ± 0.03
20:5n-3	1.63 ± 0.37	2.81 ± 1.10	1.76 ± 0.41	2.83 ± 1.38	1.25 ± 0.08
22:1n-1	0.42 ± 0.30	0.85 ± 0.33	0.12 ± 0.02	0.13 ± 0.06	0.08 ± 0.03
22:1n-9	3.15 ± 1.10	1.26 ± 0.39	1.03 ± 0.33	0.57 ± 0.15	1.37 ± 0.48
22:4n-6	0.78 ± 0.65	0.96 ± 0.79	0.15 ± 0.03	0.23 ± 0.09	0.15 ± 0.04
22:5n-6	1.76 ± 0.47^{b}	0.86 ± 0.53^{b}	3.70 ± 0.08^{a}	4.13 ± 1.32^{ab}	5.24 ± 0.15^{a}
22:5n-3	1.00 ± 0.63	1.75 ± 1.02	0.49 ± 0.11	0.90 ± 0.42	0.30 ± 0.05
22:6n-3	12.03 ± 2.13^{bc}	10.42 ± 2.67^{c}	25.86 ± 0.80^{a}	16.39 ± 2.38^{b}	23.25 ± 0.80^{a}
∑ SFA	14.74 ± 1.92^{c}	21.23 ± 3.44^{bc}	26.79 ± 1.32^{ab}	35.13 ± 4.27^{a}	21.67 ± 5.16^{bc}
∑ MUFA	33.12 ± 1.11^{a}	28.12 ± 2.83^{a}	16.15 ± 0.60^{c}	21.48 ± 2.71^{b}	18.37 ± 1.82^{bc}
∑ n-9	35.19 ± 2.82^{a}	24.87 ± 2.88^{b}	13.66 ± 0.66^{c}	15.01 ± 0.98^{c}	17.47 ± 1.47^{bc}
∑ n-3	17.14 ± 4.34^{b}	18.52 ± 4.55^{ab}	30.00 ± 1.24^{a}	22.30 ± 3.25^{ab}	26.69 ± 1.00^{ab}
∑ n-6	26.17 ± 2.29^{ab}	22.30 ± 4.39^{ab}	23.96 ± 1.13^{ab}	19.81 ± 4.88^{b}	29.02 ± 2.67^{a}
n-3/ n-6	0.67 ± 0.22^{b}	0.90 ± 0.37^{ab}	1.26 ± 0.10^{a}	1.17 ± 0.23^{ab}	0.93 ± 0.08^{ab}
∑ n-6 LC-PUFA	6 ± 0.86	5.41 ± 1.63	6.73 ± 0.13	7.12 ± 0.89	7.44 ± 0.24
∑ n-3 LC-PUFA	15.51 ± 3.74^{b}	15.85 ± 4.54^{ab}	28. 68 ± 1.22^{a}	20.71 ± 3.18^{ab}	25.19 ± 0.92^{a}
EPA/ARA	0.79 ± 0.13	1.47 ± 0.36	0.80 ± 0.14	1.36 ± 0.51	0.90 ± 0.09
EPA/DHA	0.13 ± 0.01^{a}	0.27 ± 0.07^{ab}	0.07 ± 0.02^{b}	0.17 ± 0.08^{ab}	0.05 ± 0.00^{b}

Values (mean ± SE; n=4) with the different superscripts are significantly different.

Table 3.8. Polar lipids fatty acid composition (% total fatty acids) of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days

	Diets				
Fatty acids	OA	FO	AGR	DHG	M60
14:0	0.19 ± 0.06^{b}	0.31 ± 0.13^{b}	0.39 ± 0.10^{ab}	0.69 ± 0.13^{a}	0.30 ± 0.17^{b}
14:1n-7	0.06 ± 0.05	0.06 ± 0.04	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
14:1n-5	0.13 ± 0.15	0.06 ± 0.03	0.03 ± 0.02	0.03 ± 0.00	0.01 ± 0.01
15:0	0.20 ± 0.08	0.22 ± 0.05	0.35 ± 0.08	0.27 ± 0.03	0.18 ± 0.07
15:1n-5	0.08 ± 0.07	0.06 ± 0.06	0.03 ± 0.03	0.02 ± 0.00	0.04 ± 0.04
16:0ISO	0.08 ± 0.08	0.07 ± 0.05	0.04 ± 0.02	0.03 ± 0.01	0.02 ± 0.01
16:0	14.11 ± 2.56^{b}	19.28 ± 7.23^{ab}	22.58 ± 3.83^{ab}	24.69 ± 2.69^{a}	21.29 ± 3.53^{ab}
16:1n-7	0.56 ± 0.05^{b}	0.75 ± 0.18^{ab}	1.02 ± 0.22^{a}	1.10 ± 0.16^{a}	0.83 ± 0.18^{ab}
16:1n-5	0.19 ± 0.07	0.20 ± 0.05	0.13 ± 0.06	0.10 ± 0.01	0.09 ± 0.01
16:2n-4	0.33 ± 0.08^{a}	0.40 ± 0.06^{a}	0.42 ± 0.08^{a}	0.05 ± 0.01^{b}	0.30 ± 0.07^{a}
17:0	0.15 ± 0.11	0.16 ± 0.04	0.12 ± 0.07	0.06 ± 0.00	0.11 ± 0.06
16:3n-4	0.24 ± 0.06	0.21 ± 0.01	0.30 ± 0.09	0.21 ± 0.00	0.17 ± 0.02
16:3n-3	0.12 ± 0.12	0.18 ± 0.06	0.07 ± 0.03	0.10 ± 0.03	0.04 ± 0.01
16:3n-1	1.04 ± 0.11	1.03 ± 0.03	1.37 ± 0.14	0.99 ± 0.11	1.27 ± 0.14
16:4n-3	0.86 ± 0.05^{a}	0.59 ± 0.12^{b}	0.38 ± 0.05^{c}	0.35 ± 0.03^{c}	0.46 ± 0.07^{bc}
16:4n-1	0.15 ± 0.06	0.17 ± 0.08	0.10 ± 0.07	0.09 ± 0.01	0.11 ± 0.01
18:0	9.14 ± 0.45	11.42 ± 2.94	11.24 ± 2.01	10.00 ± 0.22	10.45 ± 1.79
18:1n-9	21.17 ± 1.45^{a}	17.20 ± 2.95^{ab}	11.99 ± 2.18^{bc}	10.94 ± 0.30^{bc}	8.46 ± 5.03^{c}
18:1n-7	1.66 ± 0.01	2.42 ± 0.38	1.83 ± 0.24	1.73 ± 0.04	1.72 ± 0.18
18:1n-5	0.11 ± 0.06	0.12 ± 0.01	0.09 ± 0.02	0.10 ± 0.04	0.06 ± 0.01
18:2n-9	2.26 ± 0.11^{a}	1.49 ± 0.11^{b}	0.62 ± 0.20^{cd}	0.33 ± 0.01^{d}	0.95 ± 0.13^{c}
18:2n-6	17.32 ± 1.18^{a}	13.81 ± 0.10^{ab}	12.01 ± 2.83^{b}	11.53 ± 0.73^{b}	15.72 ± 1.41^{ab}
18:3n-6	1.49 ± 0.07^{a}	1.23 ± 0.20^{ab}	0.89 ± 0.06^{b}	2.18 ± 1.11^{ab}	1.15 ± 0.15^{ab}
18:3n-4	0.08 ± 0.07	0.11 ± 0.07	0.05 ± 0.01	0.06 ± 0.02	0.07 ± 0.03
18:3n-3	0.44 ± 0.08	0.69 ± 0.16	0.43 ± 0.05	0.45 ± 0.02	0.58 ± 0.14
18:4n-3	0.17 ± 0.10	0.18 ± 0.07	0.08 ± 0.01	0.11 ± 0.04	0.09 ± 0.02
18:4n-1	0.16 ± 0.12	0.08 ± 0.02	0.04 ± 0.02	0.06 ± 0.03	0.03 ± 0.01
20:0	0.53 ± 0.07^{a}	0.46 ± 0.05^{ab}	0.40 ± 0.16^{ab}	0.29 ± 0.05^{b}	0.34 ± 0.06^{ab}
20:1n-9	0.18 ± 0.12^{ab}	0.26 ± 0.00^{a}	0.17 ± 0.16^{ab}	0.06 ± 0.01^{b}	0.07 ± 0.01^{b}
20:1n-7	1.32 ± 0.07^{a}	1.48 ± 0.14^{a}	0.85 ± 0.22^{b}	0.67 ± 0.04^{b}	0.87 ± 0.14^{b}
20:1n-5	0.17 ± 0.09	0.18 ± 0.05	0.15 ± 0.09	0.07 ± 0.02	0.11 ± 0.04
20:2n-9	0.78 ± 0.10^{a}	0.56 ± 0.07^{b}	0.19 ± 0.08^{cd}	0.10 ± 0.02^{d}	0.35 ± 0.04^{c}
20:2n-6	0.84 ± 0.13^{a}	0.85 ± 0.04^{a}	0.52 ± 0.12^{b}	0.42 ± 0.03^{b}	0.53 ± 0.06^{b}
20:3n-9	1.22 ± 0.16^{a}	0.91 ± 0.66^{abc}	0.63 ± 0.09^{b}	0.02 ± 0.01^{c}	0.95 ± 0.04^{a}
20:3n-6	0.18 ± 0.09^{b}	0.31 ± 0.28^{ab}	0.05 ± 0.02^{b}	0.49 ± 0.01^{a}	0.04 ± 0.01^{b}
20:4n-6	1.87 ± 0.21^{ab}	1.71 ± 0.87^{ab}	1.98 ± 0.43^{ab}	1.97 ± 0.09^{a}	1.34 ± 0.09^{b}
20:3n-3	0.10 ± 0.06	0.27 ± 0.06	0.12 ± 0.01	0.14 ± 0.02	0.10 ± 0.01
20:4n-3	0.20 ± 0.17	0.40 ± 0.15	0.20 ± 0.05	0.21 ± 0.02	0.18 ± 0.03
20:5n-3	1.29 ± 0.05	2.26 ± 1.31	1.55 ± 0.94	1.23 ± 0.18	1.14 ± 0.26
22:1n-1	0.23 ± 0.19^{ab}	0.41 ± 0.19^{a}	0.12 ± 0.06^{ab}	0.15 ± 0.10^{ab}	0.07 ± 0.04^{b}
22:1n-9	0.44 ± 0.17^{a}	0.35 ± 0.09^{ab}	0.20 ± 0.08^{ab}	0.18 ± 0.12^{ab}	0.16 ± 0.02^{b}
22:4n-6	0.36 ± 0.12	0.62 ± 0.42	0.56 ± 0.66	0.17 ± 0.04	0.11 ± 0.02
22:5n-6	2.39 ± 0.38^{bc}	1.51 ± 0.85^{c}	3.60 ± 0.84^{b}	6.28 ± 0.57^{a}	5.58 ± 0.80^{a}
22:5n-3	0.78 ± 0.20	1.57 ± 0.93	0.71 ± 0.66	0.38 ± 0.08	0.27 ± 0.06
22:6n-3	14.57 ± 2.00	13.25 ± 8.15	19.56 ± 7.30	20.89 ± 2.27	23.20 ± 6.31
∑ SFA	24.40 ± 2.64^{b}	31.92 ± 10.32^{ab}	35.11 ± 5.70^{ab}	36.03 ± 2.94^{a}	32.69 ± 5.32^{ab}
∑ MUFA	26.28 ± 0.49^{a}	23.55 ± 3.41^{ab}	16.61 ± 2.77^{bc}	15.16 ± 0.46^{c}	12.52 ± 4.85^{c}
∑ n-9	26.04 ± 0.96^{a}	20.75 ± 2.14^{ab}	13.80 ± 2.45^{bc}	11.62 ± 0.33^{c}	10.93 ± 4.82^{c}
∑ n-3	18.53 ± 2.48	19.39 ± 10.89	23.09 ± 7.75	23.86 ± 2.49	26.07 ± 6.64
∑ n-6	24.45 ± 0.85^{a}	20.04 ± 1.89^{b}	21.42 ± 1.55^{ab}	23.03 ± 0.97^{ab}	24.50 ± 1.24^{a}
n-3/ n-6	0.76 ± 0.11	0.92 ± 0.49	1.07 ± 0.35	1.03 ± 0.07	1.07 ± 0.28
∑ n-6 LC-PUFA	5.64 ± 0.88^{b}	5.00 ± 1.74^{ab}	8.51 ± 4.36^{ab}	9.32 ± 0.74^{a}	7.60 ± 0.83^{ab}
∑ n-3 LC-PUFA	16.93 ± 2.27	17.75 ± 10.58	22.13 ± 7.78	22.84 ± 2.52	24.90 ± 6.63
EPA/ARA	0.70 ± 0.08^{ab}	1.22 ± 0.46^{a}	0.50 ± 0.13^{b}	0.62 ± 0.06^{b}	0.84 ± 0.15^{ab}
EPA/DHA	0.09 ± 0.01^{b}	0.19 ± 0.02^{a}	0.08 ± 0.05^{b}	0.06 ± 0.01^{b}	0.05 ± 0.01^{b}

Values (mean ± SE; n=4) with the different superscripts are significantly different.

3.4.3 LARVAL HEPATIC HISTOMORPHOLOGY

In general, larvae were well developed irrespectively of the diets. Morphological studies focused on liver as a main organ for lipid metabolism. Hepatocytes presented a round and prominent nucleus, sometimes displaced from the central position of the cell by lipid vacuoles. Irrespective of the experimental group, a moderate lipid infiltration within the hepatocytes was observed (Table 3.9; Figure 3.4).

Table 3.9. Hepatic histological evaluation of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days

	Diets				
	OA	FO	AGR	DHG	M60
Lipid infiltration*	2.00 ± 0.76	2.83 ± 0.79	2.83 ± 0.62	2.50 ± 0.83	2.23 ± 0.42

Values are presented as mean ± SE.
*0, no lipid infiltration; 1, few lipid vacuolization; 2, medium lipid vacuolization; 3, severe lipid vacuolization.

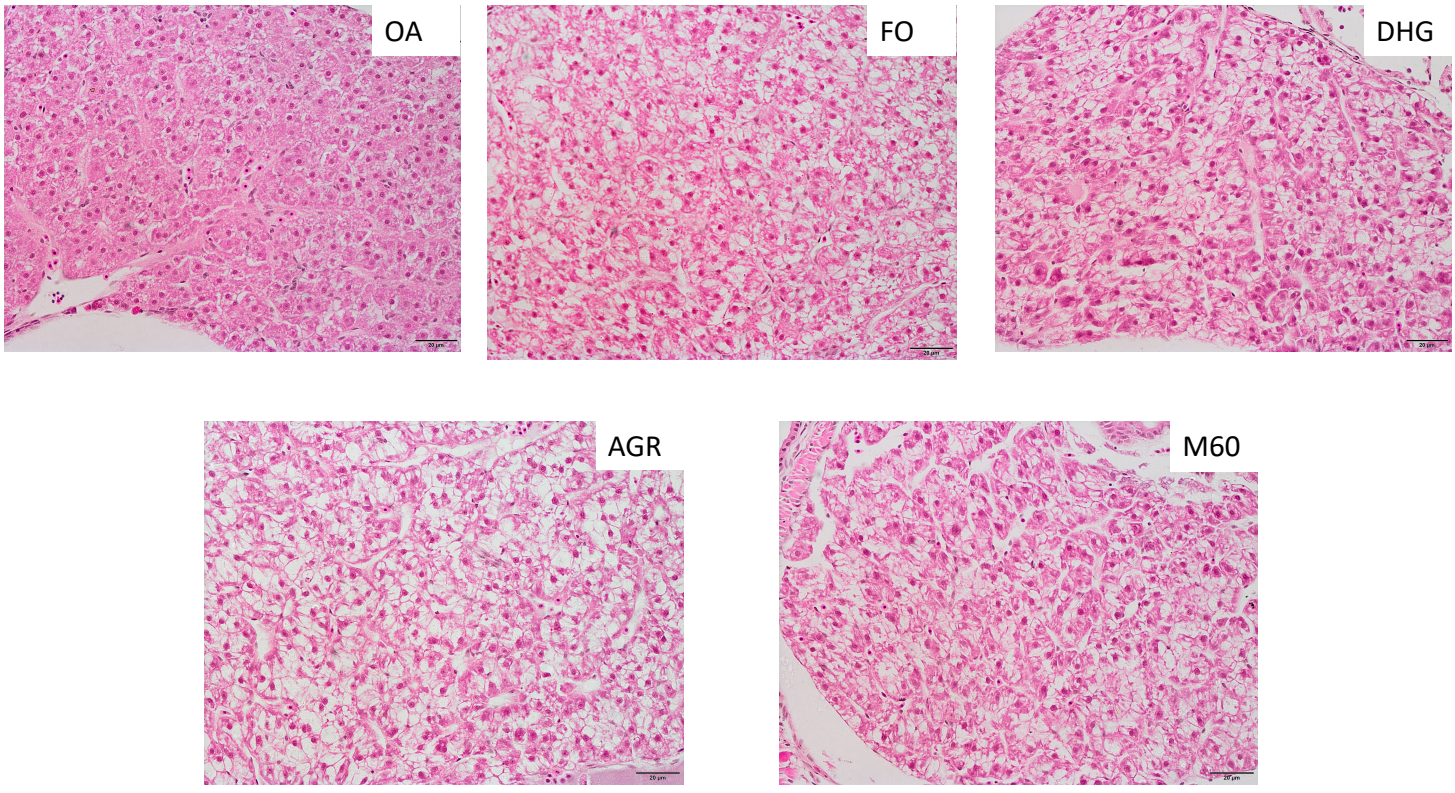

Figure 3.4. Histological aspect of livers of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days (stained with H&E; scale 20 μm).

3.4.4 GENE EXPRESSION

The expression of *fads2* and *g6p* was the highest in larvae fed the OA diet, followed by those fed FO and was downregulated in sea bream larvae fed the diets containing microalgae (P<0.05; Figure 3.5). Indeed, expressions of both genes were negatively correlated with the dietary levels of DHA (r^2=0.99, P=0.001 and r^2=0.87, P=0.02, respectively) and total n-3 LC-PUFA (r^2=0.98, P=0.001 and r^2=0.90 P=0.01, respectively). Furthermore, *fads2* expression was also correlated with DHA content in larval phospholipids (r^2=0.83, P=0.04). In contrast, no significant differences were found in the relative expression of *5-lox*, *cox-2* and *ppar-α* among larvae fed the different microdiets. However, there was a moderate negative correlation (R^2=0.67; P=0.03) between the EPA/ARA ratio in larval PL and *cox-2* expression, as well as a positive, although not significant, relation between DHA contents in PL and *5-lox* expression (R^2=0.59).

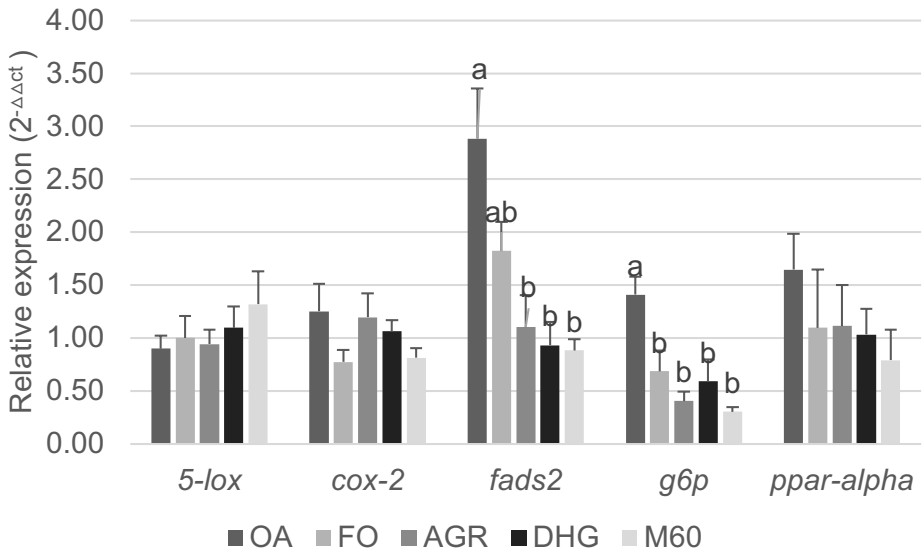

Figure 3.5. Relative expression of fatty acid metabolism-related genes of gilthead sea bream larvae fed the experimental microdiets with different microalgae meals for 21 days.

Values (mean ± SE; n=4) with the different letters above SE bars are significantly (P<0.05) different.

3.5. DISCUSSION

In marine fish, an insufficient supply of n-3 LC-PUFA, especially DHA and EPA, can lead to low survival, reduced stress resistance, growth depletion, altered feeding and swimming activities, and immune-suppression (Izquierdo, 2005; Hamre et al. 2013). This is particularly important in larvae, which have higher essential fatty acids (EFA) requirements than juveniles (Izquierdo and Koven, 2010). In the present study, FO replacement by OA in microdiets for seabream, reduced final larval survival by 30% and survival after stress exposure by 70%, in agreement with the increase demand in EFA to deal with stress and recover homeostasis (Montero et al., 2003). Moreover, FO replacement by OA lead to the lowest growth in terms of body weight and total length, confirming the symptoms of EFA deficiency (Tocher, 2010; Hamre et al., 2013). In fact, the OA diet contained only 0.3% n-3 LC-PUFA on a dry matter basis (dw), well below the minimum requirement estimated for gilthead sea bream (1.5% dw; Izquierdo and Koven, 2010), in agreement with the poor survival and growth of the larvae fed this diet.

Larvae fed OA diet also showed the lowest n-3 LC-PUFA, particularly DHA, levels in total and neutral lipids, denoting the marked influence of dietary fatty acids on body composition (Cowey and Sargent, 1972). Particularly, the n-3 LC-PUFA contents in fish are markedly dependent on dietary levels, since marine fish larvae have a very limited capacity for their synthesis (Izquierdo et al., 2001). Interestingly, OA larvae also showed the highest contents on 18:2n-9, 20:2n-9, 18:3n-6, 20:3n-6 and ARA, products from the elongation and desaturation pathways of 18:1n-9 and 18:2n-6, respectively, in the total and, particularly, the neutral lipids, despite the lowest dietary contents in these fatty acids. These results suggested the activation of the biosynthetic pathway of PUFA trough FADS2 in OA larvae, in agreement with the highest mRNA relative levels for *fads2*. This gene was first isolated in a marine fish in gilthead seabream (Seiliez et al., 2003) and its expression is modulated by dietary lipids (Izquierdo et al., 2008), although the ability to synthesize DHA was insufficient to satisfy its requirement (Hamre et al., 2013). Posteriorly, other studies have reported the up-regulation of f*ads2* in tissues of fish fed low n-3 LC-PUFA levels (Carvalho et al., 2018, 2020). This up-regulation could be modulated by deficient contents of the products (DHA, EPA or ARA, essential fatty acids for marine species) or/and by high fatty acids substrates (often LA or LNA). In the present study, desaturation products in OA larvae appeared in n-6 and n-9 FA rather than in n-3 series, according to the higher levels of 18:1n-9 and 18:2n-6 in this diet. Both FA are substrates for FADS2 which have typically demonstrated a Δ6 activity in gilthead sea bream (Houston et al., 2017). Thus, the poor survival and growth performance of OA larvae confirms the low efficiency of seabream to synthesize more elongated and desaturated FA, such as DHA, possibly in relation to the low activity of Δ4 desaturase, which may also

produce this EFA. In addition, an activation of the elongation pathways through ELOVL5 and/or 6 is also often observed in marine fish fed OA-based diets (Houston et al., 2017; Carvalho et al., 2018).

In contrast, despite the lowest DHA contents in OA diet, as well as in total and neutral lipids of OA larvae, the levels of DHA in phospholipids of these larvae were similar to those of fish fed the FO diet. The relatively high content of DHA in polar lipids of OA larvae in comparison to FO larvae, could be related to the selective incorporation of DHA by some of the enzymes involved in phospholipid synthesis and its low catabolic rate. Thus, this fatty acid is preferentially retained in PL, even when they are deficient in diet, to preserve cell membrane fluidity and functionality (Izquierdo, 2005). This selective retention was reported in previous studies in gilthead sea bream (Rodriguez et al., 1994; Izquierdo et al., 2005; Fountoulaki et al., 2009), as well in other fish species (Bell et al., 2001; Borges et al., 2014; Carvalho et al., 2018), in concordance with the important biological functions of DHA in the early stages of development for plastic purposes (Izquierdo, 2005).

Gluconeogenesis is also an important energy supply metabolic pathway during starvation, nutrient, or energy-restricted periods, which leads to an endogenous glucose production (Enes et al., 2008; Wang et al., 2019). Studies in mammals have reported the inducible effect of the dietary lipids in gluconeogenesis (Massillon et al., 1997) and this effect have been described in fish as well (Panserat et al., 2002). Indeed, a previous study with rainbow trout reported a higher expression and activity of G6P with the increase of the dietary lipids and free fatty acids. For instance, the activation of PPAR, involved in free FA oxidation, stimulates the expression of G6P and other gluconeogenic enzymes (Caseras et al., 2002). In agreement, in the present study sea bream larvae fed the OA diet also showed the highest expression in *g6p*, which is consistent with the highest absolute numeric value in *ppar* expression. The expression of *g6p* alone is not sufficient to estimate an effect in glucose metabolism and further analysis of glucose or other related parameters would be necessary. Furthermore, whether this possible effect is associated to an energy restriction in these EFA-deficient fish or due to a possible higher FA oxidation needs to be further studied. For instance, in mammals, high levels of free FA impaired glucose metabolism, leading to impaired glucose tolerance (Randle, 1998). A similar interaction was described in rainbow trout, which led to high fat-induced persistent hyperglycaemia and reduced insulin sensitivity (Figueiredo-Silva et al., 2012). Furthermore, linoleic acid and oleic acid increase glucose production in chicken hepatocytes (Su et al., 2008), which is agreement with the highest levels of these FA in OA diet. Further studies would be needed to confirm if this condition is impairing fish health and metabolism with metabolic consequences for fish production. On the contrary, sea bream fed microalgae diets presented similar *g6p* expression levels than those fed FO diet, indicating

that the replacement of FO by these microalgal products is possible without adverse effects on fish metabolism.

Indeed, replacement of dietary FO by any of the microalgal products did not negatively affected survival, stress resistance or growth of larval gilthead seabream. For instance, survival of gilthead sea bream larvae ranged around 46-53%, in agreement with the survival rates observed in previous studies with this species reared with microalgae supplemented-microdiets during a similar period (Eryalcyn et al., 2015). Furthermore, survival after air exposure of larvae fed the microalgae-based diets, was very high and similar to that of larvae fed the FO-based diet. This, along with the highest growth performance found in these fish, indicates that these microalgae products were very efficient in meeting the n-3 LC-PUFA requirements for gilthead sea bream larvae. The three microdiets tested containing microalgal products, AGR, DHG and M60, had very high dietary n-3 LC-PUFA levels (2.8, 3.1 and 2.6% dw, respectively). However, a slight improvement in growth performance and survival after stress of larvae fed DHG and M60 diets compared to those fed FO or AGR was observed and might be related with the higher n-6 DPA (22:5n-6) in the former diets, in agreement with recent studies in fish larvae (Garcia et al., 2008; Basford et al., 2020). This result suggests the need to further study the role of n-6 DPA, not commonly found on the traditional lipid sources used in aquafeeds such as FO or VO but present in high quantities in some microalgal products. Thus, feeding AGR, DHG or M60 diets led to the highest contents of n-3 LC-PUFA, particularly DHA, as well as n-6 DPA in the larval lipid fractions. Whereas FA profiles of neutral lipids clearly reflect dietary profiles, phospholipid composition is more stable, in relation to a substrate specificity for certain fatty acids during phospholipids synthesis and a limited catabolism, supporting cell membrane structure and fluidity functions (Sargent et al., 1993).

Interestingly, despite the high DHA content in AGR diet, reflected in the high DHA contents in larval total and neutral lipids, the larval polar lipids did not show a so high DHA content. Moreover, the DHA content in larval neutral lipids fed this diet was the highest among all larvae, suggesting a good absorption and incorporation into the neutral lipids, but a poorer incorporation of DHA into polar lipids and, hence, into biomembranes. These results suggested the lower bioavailability of DHA in All G Rich than in DHA Gold or M060. Indeed, although not significant, feeding the AGR diet led to a slight lower growth or survival after activity test than DHG and M60 diets. The incorporation of dietary DHA into larval polar lipids may be related to the total PL content of the diet, which was higher in DHG and M60 diets compared to AGR, or the DHA content in the PL. Thus, enzymatic, histological, and biochemical evidence demonstrate that feeding with PL, particularly if they are rich in n-3 LC-PUFA, enhances lipid transport and the incorporation n-3 LC-PUFA into larval membrane lipids promoting fish growth (Izquierdo et al., 2000). In agreement, in the present study, there

was a correlation between final total length and n-3 LC-PUFA contents in larval PL (R^2=0.63), whereas such relation was not found for larval NL (R^2=0.17). All G Rich as well as M60 are obtained from *Schizochytrium limacinum,* whose lipids contain mainly triacylglycerol, sterol esters, and phosphatidylcholine (Morita et al., 2006). However, PL content and the DHA content in the PL fraction may decrease along the culture in *Schizochytrium limacinum* (Morita et al., 2006), what could explain the lower incorporation into larval polar lipids according to previous studies in this species (Izquierdo et al., 2000). DHA Gold is obtained from an improved strain of *Schizochytrium sp.* with a higher content of EPA, as reflected in the DHG diet.

Alternative lipid sources can affect fish production by altering EPA/ARA ratios in fish, closely associated with an alteration in fish health metabolic functions, namely eicosanoids production. Indeed, it is recognized that ARA-derived eicosanoids (pro-inflammatory) are more active than EPA-derived eicosanoids (anti-inflammatory). In mammals, LOX and COX have a higher affinity for ARA than for EPA (Calder, 2012), although in marine fish the production of EPA-derived eicosanoids has an important role in metabolism (Ganga et al., 2005). Consequently, the imbalance in EPA/ARA ratios may lead to an imbalance in eicosanoids metabolism, resulting in excessive production of pro-inflammatory eicosanoids and affecting fish health and growth. Indeed, EPA has a down-regulating effect on the production of prostaglandin 2 (PGE2), an ARA-derived eicosanoid in fish (Ghioni et al., 2002). Moreover, *cox-2* and *5-lox* gene expressions are significantly down-regulated in sea bream larvae with EPA/ARA ratios in whole-body lower than 1 (Martins et al., 2012). In agreement, in the present study there was a moderate negative relation (R^2=0.67) between the EPA/ARA ratio in larval PL and *cox-2* expression, suggesting a down-regulation of *cox-2* by the increase in EPA/ARA ratios in fish membranes. On the contrary, no relation was found in the present study between EPA/ARA ratios in diets or larval lipids and *5-lox* expression, but a positive relation was found between DHA contents in PL and *5-lox* expression (R^2=0.59). DHA has been found to be a good substrate for a few lipoxygenases (LOX). For instance, in sea bream, DHA regulates cortisol production through LOX-derived products in adrenocorticotropic hormone stimulated interrenal cells (Ganga et al., 2005, Ganga et al., 2006). Nevertheless, FO replacement by the microalgal products tested in the present study did not significantly affect the expression of these genes, suggesting that the eicosanoid cascade metabolism was not compromised. Further studies on the expression of these genes, under stressed conditions for instance, would be of interest.

Overall, the results of the present study agree with the successful replacement of fish oil by other microalgae in larvae as well as in juveniles of the same species (Eryalçyn et al., 2015; Carvalho et al., 2020), corroborating the potential of microalgae as DHA sources for fish at

different developmental stages.

3.6 CONCLUSIONS

In conclusion, all microalgae products used in the present study (All G RICH, DHA Gold and MO060) were effective total replacers of FO in the diets of gilthead sea bream larvae, providing the necessary dietary n-3 LC-PUFA for optimal growth and survival. Even though, DHA GOLD and MO060 further improved growth and survival under stress conditions, possibly related with a higher dietary n-6 DPA or PL contents. Worth noting is that microalgae diets raised the DHA content in the whole body of larvae compared to OA or FO. The relative gene expression of lipid catabolism (*ppar-α*), eicosanoid metabolism (*cox-2* and *5-lox*) were unaffected, but FA synthesis though *fads2* was down-regulated by microalgae when compared to a diet based in oleic acid as lipid source, possibly due to the high DHA level in microalgae diets.

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CHAPTER 4. EFFECTIVE COMPLETE REPLACEMENT OF FISH OIL BY COMBINING POULTRY AND MICROALGAE OILS IN PRACTICAL DIETS FOR GILTHEAD SEA BREAM (*SPARUS AURATA*) FINGERLINGS

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4.1 ABSTRACT

Few ingredients allow the complete replacement of fish meals (FM) and fish oils (FO) in aquaculture feeds without affecting fish performance or fillet nutritive value. This is due to the adequate content of essential nutrients, including the n-3 long-chain polyunsaturated fatty acids (n-3 LC PUFA), and the unique high palatability of FM and fish oil. Some microalgae present abundant amounts of these fatty acids, for instance docosahexaenoic acid (DHA). Therefore, the aim of the present study was to evaluate the effect of two different microalgae products, one providing DHA and eicosapentaenoic acid (EPA, 20:5n-3; ED diet) and the other one DHA and n-6 docosapentaenoic acid (DPA, 22:5n-6; DD diet), in combination with poultry oil and rapeseed oil, as total replacers of fish oil, at two different dietary fish meal contents (15 and 7.5%). The effects of these dietary oil combinations on performance, composition, and nutritive quality indices of gilthead sea bream (*Sparus aurata*) juveniles were studied and compared against a positive control diet (FO) and two negative (PO diets) controls, one for each dietary fish meal content tested, giving in total 7 experimental diets. Both microalgae products in combination with poultry and rapeseed oils were able to completely replace fish oil in practical diets with 15% FM without affecting growth performance, utilization of dietary fatty acids or the nutritional quality of fish fillet for the consumer. On the contrary, PO alone was not able to completely replace fish oil and negatively affected fish performance, in relation to an insufficient dietary n-3 LC-PUFA content. A similar decrease in growth performance was also observed with the reduction of the dietary FM content to 7.5%. In conclusion, both oils from microalgae, providing either DHA and EPA or DHA and n-6 DPA, were effective n-3 LC-PUFA sources for sea bream juveniles and allowed the complete replacement of fish oil in combination with more cheaper lipid sources, such as poultry and rapeseed oils.

4.2 INTRODUCTION

The stagnation of fish wild stocks and fisheries, along with high global fish consumption, has increased the demand for aquaculture products over the last decades (FAO, 2016). Fish meal (FM) and fish oil have been major dietary nutrient sources in feeds for farmed fish. However, their limited availability, high cost and potential bioaccumulation of toxic pollutants, have promoted the search for more sustainable alternative ingredients to reduce the inclusion of FM and fish oil in aquafeeds (FSAI, 2002; Lundebye et al., 2004). The main bulk of research has focused on the replacement of FM and fish oil by terrestrial vegetable meals (VM) and oils (VO), respectively (Montero and Izquierdo, 2010; Turchini et al., 2010; Nasopoulou and Zabetakis, 2012; Torrecillas et al., 2017a; Benedito-Palos et al., 2007). These terrestrial ingredients have been considered good alternatives in fish diets, for their competitive price and availability and good catabolic potential to obtain dietary energy (Kerdawy and Salama, 1997). Although many studies have successfully incorporated VO as lipid sources in fish feeds, some others have reported reduced performance at high dietary inclusions, and most of them stated a decreased n-3 LC-PUFA content in fish tissues, including fillets (reviewed in Nasopoulpou and Zabetakis, 2012). Therefore, high fish oil replacement levels by VO may constrain production efficiency while also decreasing the benefits of eating fish for the consumers (Simopoulos, 2012). Indeed, intake of n-3 LC PUFA, particularly docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), is important for human health (Gill et al., 2012; Simopoulos, 2012). These fatty acids are well recognized for their benefits in prevention of diseases, particularly metabolic, cardiovascular, and coronary disorders, or cancers (Connor, 2000; Simopoulos, 2012). DHA and EPA also play important roles in the development and growth of marine fish (Izquierdo and Koven, 2011). Since fish have a limited capacity to biosynthesize LC-PUFA through endogenous pathways, these fatty acids must be supplied through the diet (Tocher, 2015). As the lack of LC-PUFA, particularly from the n-3 series, is responsible for the lower performance of marine fish when VO almost completely replace dietary fish oil (Montero and Izquierdo, 2010; Montero et al., 2015; Torrecillas et al., 2017a, b), important efforts are made to develop novel cost-effective lipid sources containing n-3 LC-PUFA to meet fish requirements, aquaculture sustainability and fillet nutritional quality for the consumers.

Several lipid sources have been tested in recent years, including microorganisms, krill, and genetically modified plants (Naylor et al., 2009; Adarme-Vega et al., 2014; Betancor et al., 2016a,b; Tocher et al., 2019). Among them, microorganisms have several advantages as ingredients for aquaculture feeds due to their simple nutritional composition, being particularly rich in EPA and/or DHA (Shah et al., 2018). Being autotrophs or very simple heterotrophs, these microorganisms are considered more sustainable than other lipid sources for their low

footprint (Tibbetts, 2018; Shah et al., 2018). Several microalgae species autotrophically or heterotrophically produced have been tested in marine fish diets for their high EPA and/or DHA contents including those from the genus *Crypthecodinium* (Eryalcyn et al., 2015; Schafberg et al., 2018, 2020), *Nannochloropsis* (Eryalcyn et al., 2015; Metsoviti et al., 2018), *Phaeodactylum* (Ruyter et al., 2016; Sørensen et al., 2016) or *Schizochytrium* (Metsoviti et al., 2018; Perez-Velazquez et al., 2018; Kosoulaki et al., 2020). The lipid contents and dietary fatty acid profiles of these microorganisms depend on the species and growth stage, but also on different aspects of the production and the processing technology employed. Therefore, dietary fatty acid profiles of these lipid sources or their combinations can be tailored to match the essential fatty acid requirements of the different fish and crustacean species and life stages. For instance, products derived from the genus *Crypthecodinium, Thraustochytrium* or *Schizochytrium* are very high in DHA (Qiu et al., 2001; Ganuza and Izquierdo, 2007), whereas those from *Phaeodactylum* or *Nannochloropsis* are rich in EPA (Atalah et al., 2007; Eryalcyn et al., 2015). Thus, complete replacement of fish oil by a *Crypthecodinium* product in early weaning diets for gilthead seabream (*Sparus aurata*) allowed to cover the DHA requirements of this species (Eryalcyn et al., 2015). However, seabream requirements for EPA were not covered by this microalga, and EPA sources, such as the algae *Nannochloropsis gaditana,* had to be supplemented to sustain maximum growth (Eryalcyn et al., 2015).

The use of microalgae as lipid sources in fish feeds has been constricted by their high production costs and their low availability (Tibbetts, 2018; Sarker et al., 2016). For this reason, their inclusion in fish diets has been mostly limited to highly valuable fish species or during early developmental stages, when DHA and EPA requirements are higher. However, the successful use of lipid rich biomass from microalgae to replace fish oil in diets for salmonids, has resulted in an increased demand for such products. As a result, global production and commercial availability of microalgae biomass obtained by fermentation technology have increased. Recently, algal oils extracted from such biomasses have also become commercially available. Compared to the biomass, the oils can more easily be combined with other oils to optimize the dietary fatty acid profile, thus providing higher flexibility in formulation and production of fish feeds. In general, algal oils can be considered as LC-PUFA concentrates containing 40-60 % LC-PUFA in addition to saturated fatty acids (SFA). The LC-PUFA fraction in an oil from fermentation of *Aurantiochytrium sp*. contains mainly DHA and n-6 docosapentaenoic acid (n-6 DPA, 22:5n-6) and minor amounts of EPA, whereas an oil from *Schizochytrium sp.* can contain both EPA and DHA and less n-6 DPA.

In addition to VO, oils obtained from terrestrial animal by-products could be considered as sustainable alternatives to complement algal oils rich in LC-PUFA. Poultry oil is a readily available ingredient that constitutes a good energy source for fish for its adequate content in

SFA and monounsaturated fatty acids (MUFA) (Rosenlund et al. 2001; Higgs et al. 2006; Hatlen et al., 2013). Nonetheless, n-3 LC-PUFA content of poultry oil is very limited (Turchini et al., 2009). Poultry oil has been investigated as supplemental lipid source or partial replacer of fish oil in diets for many farmed species (Higgs et al., 2006; Hatlen et al., 2013; Turchini et al., 2013; Liland et al., 2015; Salini et al., 2015; Campos et al., 2019), without negative effects on growth performance (Hatlen et al., 2015). However, the potential use of poultry oil or the combination of this ingredient with microalgae and VO in diets for gilthead sea bream has not yet been investigated.

Therefore, the aim of the present study was to evaluate the effect of two algal oils, one containing mainly EPA and DHA and the other DHA and n-6 DPA, in combination with poultry and rapeseed oils, as total replacers of fish oil. Moreover, since there is an increased interest in total replacement of fish oil and FM, but few studies have focused on the concomitant replacement of FM and fish oil (Dias et al., 2005; 2009; Torrecillas et al., 2017a), these lipid sources combinations were tested at two FM dietary levels: 15 and 7.5%. Furthermore, diets where fish oil was replaced with poultry oil alone providing insignificant amounts of LC-PUFA, were included to investigate if the requirement for these fatty acids could be covered through the dietary FM inclusion. The effect of these formulations on gilthead sea bream growth performance, nutrient deposition and fillet nutritional quality were assessed and discussed.

4.3 MATERIALS AND METHODS

4.3.1 FEEDING TRIAL AND FISH PERFORMANCE

Seven experimental diets were formulated to contain similar amounts of protein, lipid, and energy. The control diet (FO diet) contained 15% FM and 5% fish oil. In the experimental diets, the fish oil was replaced by either poultry oil (PO diets) or by a blend of poultry and one of two algal oils to approximately reach the EPA+DHA level of the control diet. One algal oil (Veramaris algal oil, Veramaris, The Netherlands) contained both EPA and DHA (ED diets), whereas the other algal oil (DHA Natur Oil, Archer Daniels Midland, USA) contained mainly DHA and n-6 DPA (DD diets). The major fatty acid composition of the oils used are described in Table 4.1.

Table 4.1. Major fatty acids (% of total FA) of oils used in the experimental diets

Oil	Veramaris	DHA Natur	Poultry	Rapeseed	Fish
Fatty acid					
12:0	0.08	0.51	1.1		
14:0	1.47	24.53	1.19	0.1	7.3
16:0	23.14	10.13	20.99	4.6	18.7
16:1n-7	0.06	0.18	4.79	0.3	
18:0	1.53	0.38	5.67	1.6	3.5
18:1n-9		0.59	35.83	56.6	
18:1n-7		0.17	1.99	3.1	
18:2n-6		1.23	21.79		
18:3n-3	0.03	0.29	2.12	7.8	
20:0	0.57	0.09	0.09		
∑ 20:1 isomers			0.48	1.1	
20:4n-6	0.66	0.37	0.49		
20:4n-3	1.66	0.46			
20:5n-3	21.42	0.25	0.27	0.2	15.7
∑ 22:1 isomers				1.1	
22:5n-3	5.07		0.07		
22:5n-6	1.53	9.13			
22:6n-3	39.98	48.82			11.7
∑ SFA	27.34	35.89	29.24	7.2	30.6
∑ MUFA	0.06	0.94	43.39	63.3	22.9
∑ n-6	3.24	10.89	22.71	19.9	4.4
∑ n-3	67.41	50.05	2.46	8.1	33.6

The experimental oils were analysed by Near Infrared Refraction (NIR) to give FA used to formulate (Aziziam and Kramer, 2005). The experimental diets were produced with two different FM levels: 15 and 7.5% FM (Table 4.2). Except the PO diets, all diets covered the n-3 LC-PUFA requirements for gilthead sea bream juveniles (Izquierdo, 2005). Feeds were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) at a pellet size of 1 mm and shipped to the ECOAQUA Institute laboratories (Canary Islands, Spain), where they were analyzed for proximate (Table 4.2) and fatty acid composition (Table 4.3) following

the methods described in section 4.3.2 All diets were kept at 10º C before and during the feeding trial.

Table 4.2. Formulation and proximate composition of the experimental diets

	Diets						
		15% FM			7.5% FM		
Ingredients (%)	FO	ED	DD	PO	ED	DD	PO
Fish meal[a]	15.00	15.00	15.00	15.00	7.50	7.50	7.50
Wheat [a]	12.30	12.43	12.13	11.43	9.90	9.52	10.88
Corn gluten [a]	6.58	6.12	6.20	10.00	10.00	10.00	10.00
Hi-pro soya [a]	5.00	5.00	5.00	5.00	8.00	8.00	5.00
Wheat gluten [a]	17.71	18.02	17.92	15.38	18.25	17.00	17.72
Soya protein concentrate [a]	25.00	25.00	25.00	25.00	27.50	29.00	30.00
Faba beans [a]	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil [a]	5.28	0.00	0.00	0.00	0.00	0.00	0.00
Rapseed oil [a]	7.92	7.60	5.69	7.29	7.52	5.58	7.57
Veramaris algal oil[b]	0.00	2.46	0.00	0.00	2.76	0.00	0.00
DHA Natur oil [c]	0.00	0.00	3.67	0.00	0.00	4.11	0.00
Poultry oil [f]	0.00	3.16	3.92	5.70	3.38	4.09	6.12
Vitamin premix[d]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[e]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Microalgae:PO	-	1:1.3	1:1.1	-	1:1.2	1:1	-
Proximate analysis (%)							
Crude protein	49.31	48.35	48.06	50.18	49.31	49.14	50.38
Crude lipids	18.19	17.53	18.23	17.50	17.92	17.32	17.58
Neutral lipids	89.32	89.91	90.15	91.96	90.37	90.00	94.17
Polar lipids	10.68	10.09	9.85	8.04	9.63	10.00	5.83
Moisture	6.60	8.23	7.85	8.28	8.05	7.09	7.67
Ash	4.66	4.51	4.70	4.64	4.60	4.19	3.94
Starch (theorical value)	10.03	10.04	10.18	9.87	8.64	8.52	9.05
Fiber (theorical value)	2.53	2.53	2.53	2.54	2.78	2.85	2.81

[a] Skretting AS (Norway)

[b] Veramaris algal oil (Veramaris, The Netherlands)

[c] DHA Natur oil (ADM Animal Nutrition, USA)

[d, e] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC,

[f] Poultry oil: Sonac. B.V. The Netherlands

Table 4.3. Fatty acid composition of the experimental diets (% total identified FA)

	Diets						
		15% FM			7.5% FM		
Fatty acid	FO	ED	DD	PO	ED	DD	PO
14:0	2.05	0.73	1.33	0.72	0.39	1.30	0.82
14:1n-7	0.40	0.59	0.34	0.08	0.16	0.05	0.08
14:1n-5	0.37	0.52	0.35	0.03	0.19	0.06	0.03
15:0	0.45	0.41	0.35	0.10	0.16	0.15	0.08
15:1n-5	0.29	0.42	0.36	0.03	0.16	0.02	0.02
16:0ISO	0.37	0.49	0.40	0.03	0.19	0.07	0.03
16:0	9.72	8.80	10.51	11.59	9.41	12.09	13.08
16:1n-7	2.51	1.24	1.42	1.90	0.87	1.43	2.13
16:1n-5	0.31	0.64	0.38	0.06	0.15	0.02	0.03
16:2n-4	0.44	0.59	0.52	0.07	0.17	0.10	0.11
17:0	0.49	0.00	0.41	0.06	0.17	0.05	0.05
16:3n-4	0.28	0.68	0.35	0.11	0.24	0.09	0.10
16:3n-3	0.31	0.65	0.49	0.04	0.26	0.07	0.02
16:3n-1	0.28	0.42	0.46	0.05	0.32	0.05	0.01
16:4n-3	0.54	0.55	0.44	0.13	0.22	0.06	0.07
18:0	2.72	2.48	2.70	3.40	2.92	2.95	3.50
18:1n-9	30.01	28.16	29.26	39.78	33.58	34.87	40.78
18:1n-7	2.68	1.94	1.73	2.38	2.09	1.88	2.38
18:1n-5	0.37	0.47	0.47	0.07	0.15	0.08	0.05
18:2n-9	0.41	0.49	0.39	0.00	0.18	0.02	0.02
18:2n-6	15.74	15.52	16.36	23.16	19.44	19.69	24.58
18:2n-4	0.32	0.62	0.41	0.03	0.20	0.03	0.00
18:3n-6	0.00	0.94	0.52	0.08	0.19	0.15	0.11
18:3n-4	0.34	0.53	0.49	0.10	0.23	0.06	0.06
18:3n-3	5.02	4.30	3.61	5.29	4.63	4.04	5.27
18:3n-1	0.29	0.47	0.46	0.00	0.26	0.03	0.03
18:4n-3	0.94	0.57	0.53	0.39	0.29	0.23	0.20
18:4n-1	0.27	0.42	0.47	0.03	0.21	0.03	0.03
20:0	0.68	0.73	0.50	0.52	0.85	0.41	0.42
20:1n-9	0.37	0.41	0.43	0.18	0.21	0.14	0.11
20:1n-7	2.33	2.20	1.91	1.96	1.77	1.29	1.36
20:1n-5	0.47	0.47	0.41	0.17	0.22	0.10	0.07
20:2n-9	0.30	0.56	0.35	0.05	0.23	0.04	0.06
20:2n-6	0.32	1.10	0.56	0.27	0.32	0.13	0.16
20:3n-6	0.32	0.66	0.58	0.11	0.24	0.14	0.07
20:4n-6	0.59	0.80	0.48	0.32	0.50	0.40	0.21
20:3n-3	0.29	0.60	0.46	0.10	0.40	0.11	0.04
20:4n-3	0.44	0.72	0.64	0.11	0.38	0.22	0.07
20:5n-3	5.30	3.33	1.54	1.24	3.65	1.01	0.69
22:1n-11	2.37	2.26	1.56	1.94	1.45	1.02	0.93
22:1n-9	0.56	0.56	0.58	0.39	0.37	0.27	0.26
22:4n-6	0.36	1.08	1.32	0.15	0.25	0.11	0.06
22:5n-6	0.47	1.11	3.05	0.13	0.46	3.97	0.22
22:5n-3	1.31	1.04	1.09	0.31	0.68	0.31	0.13
22:6n-3	5.36	8.72	9.04	2.11	10.62	10.67	1.46
∑ SFA	16.11	13.15	15.80	16.39	13.90	16.96	17.95
∑ MUFA	43.02	39.89	39.20	48.96	41.35	41.21	48.23
∑ n-6	17.79	21.21	22.88	24.29	21.40	24.59	25.41
∑ n-6 LC-PUFA	2.06	4.75	5.99	0.98	1.77	4.75	0.72
∑ n-3	19.51	20.49	17.84	9.72	21.12	16.73	7.96
∑ n-3 LC-PUFA	12.70	14.41	12.77	3.87	15.73	12.32	2.39
EPA+DHA	10.66	12.05	10.58	3.35	14.27	11.68	2.15
EPA/ARA	8.91	4.18	3.22	3.88	7.35	2.51	3.28
DHA/EPA	1.0	2.6	5.9	1.7	2.9	10.6	2.1
n-6/n-3	0.91	1.04	1.28	2.50	1.01	1.47	3.19

A feeding trial was conducted with gilthead sea bream (*Sparus aurata*) fingerlings with an initial body weight of 2.50 ± 0.01 g (mean ± SE) and an initial fork length of 5.33 ± 0.01 cm. Fish, which were produced at the ECOAQUA Institute facilities, were randomly distributed in groups of 55 fish in 21x250 L fiberglass cylinder tanks with conical bottom and painted with light grey colour (stocking density of 0.55 kg m^{-3}). Tanks were continuously supplied with filtered sea water (35 mg L^{-1} salinity) and aeration. Water temperature (23.1 ± 0.3 ºC) and dissolved oxygen (6.4 ± 0.2 mg L^{-1}) were recorded daily. Tanks were illuminated by fluorescent lights placed above the tank at an intensity of 100 lx and programmed for 12h light photoperiod (from 8 a.m. to 8 p.m.). Each experimental diet was randomly assigned to an experimental tank and tested in triplicate (n=3). Fish were manually fed until visual apparent satiety by a trained researcher 3 times a day, 6 days per week for 74 days. Feed delivery was calculated daily, and uneaten pellets were collected in a net by opening the water outlet 30 min after each meal, dried in an oven for 24 h and weighed to estimate feed intake (FI). Mortalities were removed daily, and their biomass accounted to more accurately estimate FI expressed per average body weight (g feed g $fish^{-1}$).

Individual weight and length (fork length) were assessed after 74 days of feeding. Prior to sampling, fish were fasted for 24 h. Fish were anesthetized with clove oil (6 mL 100 L^{-1}) and, after handling, fish that were not used for sample collection, were allowed to recover in a tank with aerated seawater before returning them to their original tank.

Growth performance and feed utilization were estimated using the following formulas:

Mortality (%) = 100* (nº dead fish/ nº total fish)

Specific growth rate (SGR, % day^{-1}) = 100* (ln BW_1−ln BW_0) / nº days of the trial

Feed intake (FI, g feed g $fish^{-1}$) = Feed delivered (g feed $fish^{-1}$) / fish body weight (g)

Biological feed conversion ratio (FCR) = Feed delivered ($t_1 - t_0$)/ (Biomass t_1 – Biomass t_0 + $Biomass_{harvested}$ + $Biomass_{lost}$)

Condition factor (CF, %) = 100* Bw_0 / fork $length^3$

At the beginning of the trial, 12 fish were sampled from the starting stock, whereas at the end of the trial 12 fish were sampled from each experimental tank. Six of those fish were pooled per tank (n=3) and used for determining whole-body composition, while the other six fish were pooled for determining fillet composition. Fish were painlessly killed by immersion in iced water.

4.3.2 BIOCHEMICAL ANALYSIS

Feeds and fish samples were frozen at -80ºC until analysis. All samples were homogenized prior to analysis. Moisture, ash, and protein contents were determined according to A.O.A.C. (2000). Lipids were extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1989). FAMES were then separated by gas liquid chromatography (Izquierdo et al., 1990), quantified by a flame ionization detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified comparing with previously characterized standards.

Nutrient utilization and deposition were estimated based on the following equations:

Protein efficiency ratio (PER) = (BW_1 - BW_0) / protein intake (g $fish^{-1}$)

Lipid efficiency ratio (LER) = (BW_1 - BW_0) / lipid intake (g $fish^{-1}$)

Relative fatty acid retention efficiency (% FA intake) = 100* (final weight (g) * FA in final whole-body (g 100 g FA^{-1}) * final whole-body lipids (%)– initial weight (g) * FA in initial whole-body (g 100 g FA^{-1})* initial whole-body lipids (%) / FI (g) * dietary lipids (%) * dietary FA (g 100 g FA^{-1}) (according to Carvalho et al., 2018).

Additionally, indexes of lipid quality for human nutrition were calculated from fish fillet composition, according to the equations:

Hypercholesterolemic fatty acids (H) = 12:0 + 14:0 + 16:0

Hypocholesterolemic fatty acids (h) = 18:0 + Σ MUFA + Σ PUFA

Atherogenic index (AI) = (12:0 + 4 * 14:0 + 16:0) / (Σ MUFA + Σ n-3 PUFA + Σ n-6 PUFA)

Thrombogenic index (TI) = (14:0 + 16:0 + 18:0) / (0.5* Σ 18:1) + (0.5* Σ MUFA) + (0.5* Σ n-6 PUFA) + (3* Σ n-3 PUFA) + (Σ n-3 PUFA / Σ n-6 PUFA)

Peroxidation index (PI) = 0.025 × (Σ monoenoic fatty acids) + 1 × (Σ dienoic fatty acids) + 2 × (Σ trienoic fatty acids) + 4 × (Σ tetraenoic fatty acids) + 6 × (Σ pentaenoic fatty acids) + 8 × (Σ hexaenoic fatty acids).

4.3.3 STATISTICAL ANALYSIS

All the resulting data are presented in the corresponding tables as mean ± standard error (SE). For all statistical analysis carried out, each tank was considered as a replicate (n=3 for each treatment). All data were tested for normal distribution and homogeneity of variances using Shapiro–Wilk and Levene's tests, respectively. To independently compare the different experimental diets, a one-way ANOVA was applied to all data and means were compared with Tukey's multiple range test (Tukey, 1949). To determine the specific effect of the alternative dietary source, the FM dietary level and their potential interaction, a two-way ANOVA was applied to the data. To understand the dietary effects on the fish tissues fatty acid profiles a multivariate analysis of principal components (PCA) was applied, followed by a PERMANOVA. Homogeneity of data dispersions was checked with a distance-based test for multivariate dispersions (PERMDISP). The number of permutations was established in 999. Confidence levels were established at 95 % ($P < 0.05$), and statistical analyses were done using the SPSS 21.0 software package and PRIMER 7 for Windows. When appropriate, data were also subjected to the best-fit regressions (linear, exponential, or logarithmic), which were checked for significance using SPSS 21.0 software.

4.3.4 ETHICAL STATEMENT

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were approved by the Canary Island Council of Agriculture, Fishing and Water and the Bioethical Committee of the University of Las Palmas de Gran Canaria (reference OEBA-ULPGC-21/ 2018).

4.4 RESULTS

4.4.1 DIETARY PROXIMATE AND FATTY ACID COMPOSITION

The experimental diets had similar proximate composition (Table 4.2), but their fatty acid composition differed according to the dietary lipid source used (Table 4.3). Therefore, the level of EPA+DHA in the two diets containing algal oils (ED and DD) was comparable to that of the FO diet. However, DHA was higher and EPA lower in the diets with algal oils, particularly in DD diets, reflecting the fatty acid composition of the microalgae oils used (Table 4.1). In accordance to oils fatty acid composition, PO diets contained the lowest levels of these essential fatty acids (EFA), as well as the lowest levels of arachidonic acid (ARA, 20:4n-6) (Table 4.3). As expected, DD contained much more n-6 DPA than the other experimental diets (Table 4.3), in line with the fatty acid profile of this algal oil (Table 4.1). The PO diets presented the highest content in MUFA, particularly in oleic acid (OA, 18:1n-9), as well as in linoleic acid (LA, 18:2n-6) and, to a lesser extent, in linolenic acid (LNA, 18:3n-3) (Table 4.3). Therefore, n-6/n-3 ratio was lower in the FO, ED, and DD diets than in the PO ones (Table 4.3).

4.4.2 FEEDING TRIAL AND FISH PERFORMANCE

Survival was very high in all experimental groups except in fish fed 7.5% FM PO that had lower survival (92.12%) than fish fed FO or 7.5% FM ED diet ($P<0.05$; Table 4.4). Moreover, two-way ANOVA showed that fish fed PO diets had significantly higher mortality than those fed ED or DD (Table 4.4), regardless of dietary FM level. Furthermore, fish fed PO (15 or 7.5% FM) showed the lowest FBW, FBL, and SGR, together with the highest FCR and lowest PER and LER ($P<0.05$; Table 4.4).

Diets ED and DD with 15% FM did not negatively affect fish FBW, FBL, SGR, FCR and PER compared to the FO diet ($P<0.05$; Table 4.4). Nevertheless, fish fed 15% FM DD showed a 7% lower LER than fish fed the FO diet ($P<0.05$; Table 4.4). On the contrary, sea bream fed 7.5% FM ED and DD showed significantly lower growth together with poorer feed utilization parameters than those fed FO or 15% FM ED or DD ($P<0.05$; Table 4.4).

Therefore, a reduction of dietary FM from 15% to 7.5% negatively affected growth parameters as denoted by the two-way ANOVA analysis ($P<0.05$; Table 4.4). No interaction was found between the lipid source and the dietary FM content, except for FCR, PER and LER, where a significant shared effect was observed ($P<0.05$; Table 4.4).

Table 4.4. Growth performance of gilthead sea bream fingerlings fed the experimental diets for 74 days

Diets										
		15% FM			7.5% FM			Two-way ANOVA		
	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
Survival (%)	98.18±1.05^{a}	97.57±0.61^{ab}	97.58±1.60^{ab}	96.97±1.60^{ab}	98.79±0.61^{a}	97.58±1.60^{ab}	92.12±1.19^{b}	PO>ED, DD	NS	NS
FBW (g)	12.42±0.26^{a}	12.37±0.50^{a}	11.78±0.31^{a}	9.41±0.46^{b}	9.93±0.02^{b}	9.48±0.15^{b}	7.76±0.15^{c}	ED, DD>PO	P=0.00	NS
FBL (cm)	8.50±0.05^{a}	8.53±0.10^{a}	8.46±0.05^{a}	7.98±0.01^{b}	8.04±0.05^{b}	7.93±0.05^{b}	7.52±0.07^{c}	ED, DD>PO	P=0.00	NS
SGR (% day^{-1})	2.17±0.03^{a}	2.16±0.05^{a}	2.09±0.03^{a}	1.78±0.08^{b}	1.86±0.01^{b}	1.80±0.02^{b}	1.52±0.03^{c}	ED, DD>PO	P=0.00	NS
FI (g feed^{-1} g fish^{-1})	0.97±0.01	0.97±0.01	0.97±0.01	0.99±0.02	0.96±0.01	0.98±0.02	1.01±0.01	NS	NS	NS
FCR	1.22±0.02^{d}	1.22±0.00^{d}	1.27±0.01^{cd}	1.37±0.02^{b}	1.35±0.01^{bc}	1.35±0.01^{bc}	1.56±0.01^{a}	PO>ED, DD	P=0.00	P=0.01
CF	2.23±0.39^{abc}	2.37±0.38^{a}	2.30±0.38^{ab}	1.87±0.15^{bc}	2.16±0.56^{abc}	2.43±0.57^{a}	1.80±0.10^{c}	ED, DD>PO	NS	NS
PER	1.56±0.02^{a}	1.56±0.01^{a}	1.49±0.01^{a}	1.34±0.02^{b}	1.38±0.01^{b}	1.40±0.01^{b}	1.17±0.02^{c}	ED, DD>PO	P=0.00	P=0.00
LER	4.46±0.06^{a}	4.30±0.02^{ab}	4.15±0.02^{bc}	3.84±0.07^{d}	3.81±0.04^{d}	3.98±0.03^{cd}	3.37±0.05^{e}	ED, DD>PO	P=0.00	P=0.02

Values (mean ± SE, n=3) with different superscript letters in the same row or separated by > are significantly different (P < 0.05).

Survival (r^2=0.74; P<0.05, Figure 4.1a), SGR (r^2=0.64; P<0.05) and PER (r^2=0.66; P<0.05) were directly correlated, while FCR (r^2=0.64; P<0.05) was inversely correlated with dietary n-3 LC-PUFA in % DW, all of them following a logarithmic equation. Besides, survival (r^2=0.78; P<0.05), SGR (r^2=0.73; P<0.05), PER (r^2=0.75; P<0.05, Figure 4.1b) and LER (r^2=0.70; P<0.05) were inversely related to the dietary n-6/n-3 ratio, whereas FCR (r^2=0.76; P<0.05) was linearly correlated to this ratio.

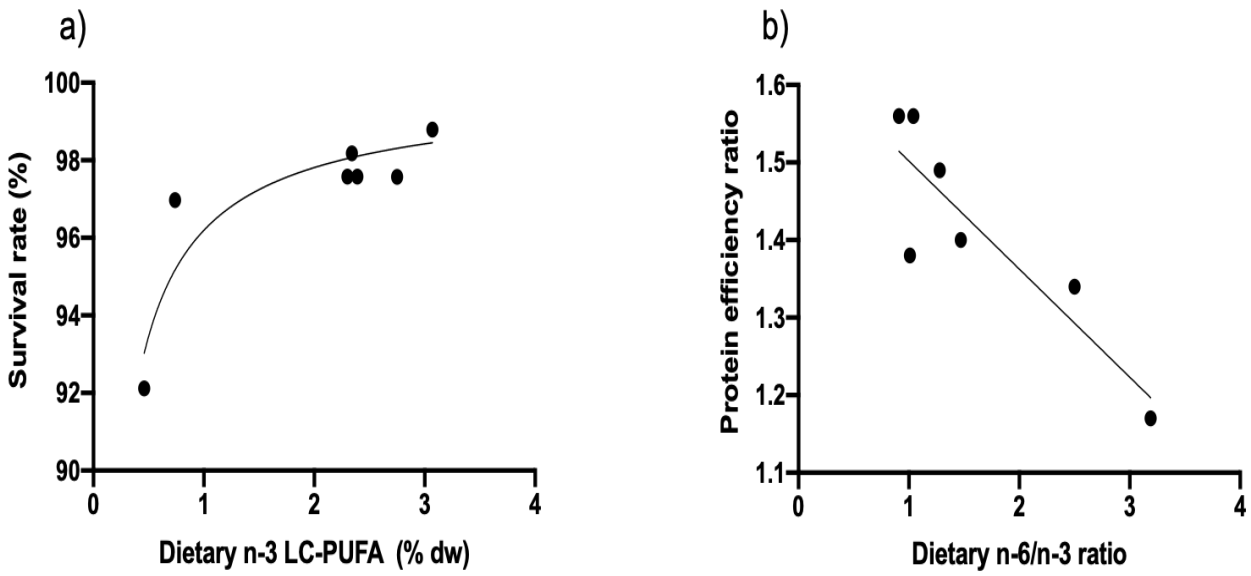

Figure 4.1. Significant correlations between dietary factors and performance parameters: a) dietary n-3 LC-PUFA (% DW) vs survival rate (%); b) dietary n-6/n-3 ratio vs protein efficiency ratio.

4.4.3 BIOCHEMICAL ANALYSIS

4.4.3.1 PROXIMATE COMPOSITION

Whole body and fillet lipid content of fish fed the experimental diets for 74 days were increased in comparison to the initial fish composition. The proximate composition of whole body or fillets did not differ among fish fed the different diets (Table 4.5).

4.4.3.2 WHOLE-BODY FATTY ACID PROFILES

Regardless of dietary lipid source or FM content, the most abundant fatty acids in fish whole-body were oleic acid (OA, 18:1n-9), palmitic acid (16:0), linoleic acid (LA, 18:2n-6) and DHA (Table 4.6). Certain fatty acids such as 14:0, 15:0, 17:0, 16:1n-7, 18:1n-9, 18:1n-7, 22:1n-9, 20:2n-6, 22:5n-6, n-6, 18:3n-3, 20:5n-3, 22:6n-3, monounsaturated fatty acids (MUFA), n-6/n-3, n-9, n-3 or EPA/DHA, reflected their respective dietary levels, whereas sum SFA and 16:0 were similar in all the fish groups, regardless of dietary levels (Table 4.6). Dietary FM reduction significantly reduced 16:4n-3, 20:1n-9, 20:1n-7, 20:2n-9, 20:3n-6, 20:4n-3 and 22:1n-11 and increased 18:2n-6 (Table 4.6). Contrary to the diet, whole-body levels of 18:3n-6, a product of Δ6 desaturase from 18:2n-6, were increased by the reduction of FM. Fish fed PO diets had the lowest contents in sum n-3 FA and n-3 LC-PUFA and the highest contents in MUFA, n-9 fatty acids and 18:2n-6 ($P<0.05$; Table 4.6). Noteworthy, fish fed PO diets had higher contents of 18:3n-6 ($P<0.05$), as well as its elongation product 20:3n-6, regardless of their low dietary levels. In addition, 20:4n-6 levels in fish fed PO diets, were not significantly lower than in control fish, although ARA levels in the PO diets were low ($P<0.05$; Table 4.6). Similarly, DHA contents in fish fed PO diets were not significantly different from fish fed the FO diet. Besides, fish fed PO diets showed the highest contents of 18:2n-9 ($P<0.05$) and 20:2n-9, despite the dietary levels were the lowest (Table 4.6). In fish fed DD, the whole-body contents in 18:2n-6 were lower than in fish fed control or ED diets, whereas 18:3n-6 and, particularly, 20:3n-6 were higher, regardless the dietary levels. Also, DD fish had the highest content in n-6 LC-PUFA, especially due to the highest content in ARA and n-6 DPA. Finally, the whole-body fatty acid profiles of fish fed the ED diets were very similar to those of control fish, except for their higher contents of n-6 and some minor fatty acids ($P<0.05$; Table 4.6).

Table 4.5. Whole-body composition (% wet weight) of gilthead sea bream fingerlings fed the experimental diets for 74 days

			Diets						
				15% FM			7.5% FM		
	(% ww)	Initial	FO	ED	DD	PO	ED	DD	PO
Whole-body	Protein	15.72±0.74	16.34±0.13	16.24±0.30	15.72±0.41	15.62±0.22	15.78±0.13	15.93±0.29	15.59±0.21
	Lipids	4.49±0.06	12.47±0.58	12.33±0.45	12.60±0.10	12.17±0.42	12.05±0.14	12.65±0.42	12.11±0.49
	Ash	3.91±0.51	3.70±0.23	3.88±0.18	3.78±0.14	3.64±0.30	4.30±0.07	3.63±0.17	3.78±0.28
	Moisture	75.49±0.35	67.79±0.44	67.77±0.83	68.07±0.47	69.55±0.73	68.63±0.23	68.09±0.42	68.98±0.06
Fillet	Protein	20.85±0.26	19.51±0.32	19.50±0.36	19.17±0.13	19.65±0.23	19.20±0.16	19.22±0.09	19.54±0.22
	Lipids	3.22±0.23	6.81±0.50	7.70±0.68	7.50±0.69	7.02±0.34	6.96±0.70	8.63±0.55	6.47±0.38
	Moisture	77.85±0.75	72.25±0.79	72.83±0.59	72.99±0.60	72.60±0.26	73.76±0.43	73.17±0.34	72.68±0.56

Values (mean ± SE, n=3) with different superscript letters in the same row are significantly different ($P < 0.05$).

Table 4.6. Whole-body fatty acid composition (% total identified FA) of gilthead sea bream fingerlings fed the experimental diets for 74 days

	Diets									
		15% FM			7.5% FM			Two-way ANOVA		
Fatty acid	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
14:0	1.66±0.30^{a}	0.86±0.20^{b}	1.02±0.03^{ab}	0.96±0.06^{ab}	0.74±0.15^{b}	0.92±0.11^{ab}	0.97±0.06^{ab}	NS	NS	NS
14:1n-7	0.04±0.01	0.03±0.01	0.04±0.00	0.04±0.00	0.03±0.00	0.04±0.00	0.04±0.00	PO>ED	NS	NS
14:1n-5	0.06±0.00^{a}	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}	NS	NS	NS
15:0	0.18±0.02^{a}	0.13±0.01^{ab}	0.12±0.00^{ab}	0.11±0.01^{b}	0.12±0.02^{ab}	0.12±0.01^{ab}	0.12±0.01^{ab}	NS	NS	NS
15:1n-5	0.03±0.01^{a}	0.02±0.01^{ab}	0.01±0.00^{b}	0.02±0.00^{ab}	0.02±0.00^{ab}	0.01±0.00^{ab}	0.02±0.00^{ab}	NS	NS	NS
16:0	11.75±1.01	10.93±0.83	10.82±0.12	10.93±0.37	10.49±0.74	10.34±0.64	11.28±0.48	NS	NS	NS
16:1n-7	3.20±0.21^{a}	2.14±0.28^{b}	2.28±0.05^{b}	2.70±0.03^{ab}	2.02±0.21^{b}	2.09±0.13^{b}	2.79±0.03^{ab}	PO>ED, DD	NS	NS
16:1n-5	0.10±0.01^{a}	0.05±0.00^{b}	0.03±0.00^{b}	0.04±0.00^{b}	0.04±0.01^{b}	0.03±0.00^{b}	0.05±0.00^{b}	PO>DD	NS	NS
16:2n-4	0.23±0.01^{a}	0.08±0.00^{b}	0.06±0.00^{b}	0.07±0.00^{b}	0.06±0.01^{b}	0.05±0.00^{b}	0.07±0.01^{b}	NS	NS	NS
17:0	0.24±0.00^{a}	0.07±0.01^{b}	0.06±0.01^{b}	0.08±0.01^{b}	0.07±0.01^{b}	0.07±0.01^{b}	0.09±0.01^{b}	NS	NS	NS
16:3n-4	0.19±0.01^{a}	0.13±0.01^{b}	0.12±0.00^{b}	0.14±0.00^{b}	0.12±0.01^{b}	0.12±0.01^{b}	0.15±0.01^{b}	PO>ED, DD	NS	NS
16:3n-3	0.07±0.01^{a}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.24^{b}	0.03±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{b}	NS	NS	NS
16:3n-1	0.13±0.03	0.11±0.01	0.10±0.00	0.09±0.00	0.14±0.02	0.11±0.01	0.10±0.01	ED>DD, PO	NS	NS
16:4n-3	0.22±0.03^{a}	0.10±0.02^{b}	0.12±0.01^{b}	0.10±0.08^{b}	0.08±0.01^{b}	0.07±0.01^{b}	0.08±0.01^{b}	NS	P=0.02	NS
18:0	3.84±0.15^{ab}	3.58±0.10^{ab}	3.28±0.12^{b}	3.85±0.14^{ab}	3.44±0.13^{ab}	3.21±0.10^{b}	4.03±0.20^{a}	PO>ED, DD	NS	NS
18:1n-9	36.52±1.24^{abc}	36.44±0.61^{abc}	35.05±0.82^{c}	40.28±0.92^{ab}	36.07±0.93^{bc}	34.95±1.03^{c}	40.85±1.02^{a}	PO>ED, DD	NS	NS
18:1n-7	3.04±0.09^{a}	2.39±0.06^{b}	2.21±0.05^{b}	2.72±0.08^{a}	2.39±0.05^{b}	2.12±0.06^{b}	2.79±0.06^{a}	PO>ED>DD	NS	NS
18:1n-5	0.12±0.01^{a}	0.08±0.01^{b}	0.07±0.00^{b}	0.09±0.00^{ab}	0.08±0.01^{b}	0.06±0.00^{b}	0.08±0.00^{ab}	PO>DD	NS	NS
18:2n-9	0.55±0.03^{b}	0.58±0.06^{b}	0.48±0.01^{b}	1.02±0.07^{a}	0.57±0.01^{b}	0.69±0.02^{b}	1.09±0.09^{a}	PO>ED, DD	NS	NS

Table 4.6. Whole-body fatty acid composition (% total identified FA) of gilthead sea bream fingerlings fed the experimental diets for 74 days (continued)

	Diets									
		15% FM			7.5% FM			Two-way ANOVA		
Fatty acid	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
18:2n-6	15.25±0.12^{d}	17.28±0.03^{c}	16.62±0.11^{c}	19.72±0.31^{ab}	18.88±0.14^{b}	17.55±0.29^{c}	20.12±0.35^{a}	PO>ED>DD	P=0.00	NS
18:2n-4	0.16±0.02^{a}	0.04±0.00^{b}	0.04±0.00^{b}	0.05±0.00^{b}	0.04±0.00^{b}	0.04±0.00^{b}	0.04±0.00^{b}	NS	NS	NS
18:3n-6	0.65±0.03^{b}	0.72±0.03^{b}	0.64±0.02^{b}	1.34±0.14^{a}	0.86±0.03^{b}	0.93±0.01^{b}	1.39±0.12^{a}	PO>ED>DD	P=0.03	NS
18:3n-4	0.16±0.06	0.09±0.01	0.07±0.01	0.09±0.01	0.06±0.00	0.07±0.01	0.07±0.01	NS	NS	NS
18:3n-3	3.65±0.18	3.75±0.14	3.17±0.11	3.75±0.24	3.86±0.13	3.20±0.05	3.55±0.24	ED, PO>DD	NS	NS
18:3n-1	0.02±0.00	0.02±0.02	0.01±0.00	0.01±0.00	0.02±0.01	0.01±0.01	0.01±0.00	NS	NS	NS
18:4n-3	0.69±0.09	0.48±0.04	0.42±0.04	0.54±0.08	0.43±0.04	0.42±0.04	0.47±0.08	NS	NS	NS
18:4n-1	0.11±0.04^{a}	0.03±0.00^{b}	0.02±0.00^{b}	0.03±0.00^{b}	0.03±0.01^{ab}	0.02±0.01^{b}	0.02±0.01^{b}	NS	NS	NS
20:0	0.43±0.03	0.41±0.08	0.30±0.02	0.32±0.02	0.38±0.05	0.31±0.01	0.33±0.01	NS	NS	NS
20:1n-9	0.33±0.01^{a}	0.29±0.04^{ab}	0.27±0.00^{abc}	0.28±0.02^{ab}	0.21±0.02^{bc}	0.18±0.01^{c}	0.23±0.00^{bc}	NS	P=0.01	NS
20:1n-7	1.99±0.02^{a}	1.78±0.23^{ab}	1.59±0.05^{abc}	1.55±0.08^{abc}	1.44±0.16^{bc}	1.20±0.04^{c}	1.36±0.02^{bc}	NS	P=0.01	NS
20:1n-5	0.16±0.01^{a}	0.09±0.02^{b}	0.07±0.01^{b}	0.09±0.00^{b}	0.09±0.01^{b}	0.07±0.01^{b}	0.09±0.00^{b}	NS	NS	NS
20:2n-9	0.27±0.02	0.27±0.04	0.23±0.02	0.29±0.01	0.17±0.02	0.16±0.02	0.25±0.04	NS	P=0.01	NS
20:2n-6	0.34±0.02	0.41±0.08	0.35±0.04	0.38±0.01	0.35±0.05	0.30±0.01	0.37±0.02	NS	NS	NS
20:3n-6	0.22±0.05	0.21±0.03	0.25±0.01	0.27±0.02	0.17±0.01	0.23±0.01	0.22±0.03	PO, DD>ED	P=0.04	NS
20:4n-6	0.41±0.06^{ab}	0.44±0.04^{ab}	0.51±0.01^{a}	0.32±0.02^{b}	0.45±0.00^{ab}	0.55±0.03^{a}	0.33±0.03^{b}	DD>ED>PO	NS	NS
20:3n-3	0.15±0.03	0.15±0.02	0.12±0.00	0.12±0.01	0.14±0.01	0.13±0.02	0.12±0.01	NS	NS	NS
20:4n-3	0.40±0.05^{a}	0.33±0.02^{ab}	0.34±0.02^{ab}	0.23±0.02^{bc}	0.25±0.01^{bc}	0.29±0.01^{abc}	0.18±0.03^{c}	ED, DD>PO	P=0.00	NS
20:5n-3	3.24±0.66^{a}	2.46±0.23^{abc}	1.34±0.14^{bc}	1.26±0.18^{bc}	2.53±0.17^{ab}	1.13±0.10^{bc}	1.03±0.23^{c}	ED> DD, PO	NS	NS
22:1n-11	1.60±0.04^{a}	1.38±0.23^{ab}	1.29±0.09^{abc}	1.10±0.08^{abc}	0.91±0.15^{bc}	0.76±0.03^{c}	0.78±0.00^{c}	NS	P=0.00	NS
22:1n-9	0.55±0.02	0.52±0.10	0.41±0.03	0.48±0.03	0.40±0.06	0.32±0.01	0.41±0.01	NS	NS	NS
22:4n-6	0.15±0.06	0.13±0.03	0.12±0.01	0.10±0.00	0.12±0.02	0.14±0.03	0.10±0.01	NS	NS	NS
22:5n-6	0.19±0.04^{b}	0.33±0.02^{b}	3.30±0.15^{a}	0.13±0.01^{b}	0.40±0.02^{b}	3.88±0.34^{a}	0.22±0.03^{b}	DD> ED, PO	NS	NS
22:5n-3	1.25±0.30^{a}	0.98±0.06^{abc}	0.60±0.15^{bc}	0.48±0.06^{bc}	1.04±0.08^{ab}	0.50±0.05^{bc}	0.39±0.08^{c}	ED> DD, PO	NS	NS
22:6n-3	5.35±1.22^{bc}	9.54±0.09^{ab}	11.93±0.86^{a}	3.74±0.41^{c}	10.17±0.25^{a}	12.50±1.64^{a}	3.20±0.69^{c}	DD>ED>PO	NS	NS
∑ SFA	18.14±1.43	15.99±0.86	15.62±0.24	16.26±0.55	15.26±0.74	14.98±0.84	16.84±0.71	NS	NS	NS
∑ MUFA	47.73±1.41^{ab}	45.23±1.0^{abc}	43.34±0.99^{bc}	49.40±1.20^{a}	43.70±1.17^{bc}	41.85±1.26^{c}	49.52±1.08^{a}	PO>ED, DD	NS	NS
∑ n-9	38.25±1.21^{ab}	38.13±0.87^{ab}	36.44±0.87^{b}	42.35±0.91^{a}	37.44±1.03^{b}	36.31±1.04^{b}	42.84±1.13^{a}	PO>ED, DD	NS	NS
∑ n-6	17.23±0.11^{e}	19.55±0.23^{d}	21.84±0.03^{bc}	22.28±0.47^{bc}	21.24±0.14^{c}	23.60±0.14^{a}	22.76±0.40^{ab}	PO, DD>ED	P=0.00	NS

Table 4.6. Whole-body fatty acid composition (% total identified FA) of gilthead sea bream fingerlings fed the experimental diets for 74 days (continued)

Diets										
		15% FM			7.5% FM			Two-way ANOVA		
Fatty acid	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
∑ n-6 LC-PUFA	1.31±0.23^{b}	1.52±0.18^{b}	4.54±0.13^{a}	1.20±0.05^{b}	1.48±0.09^{b}	5.11±0.39^{a}	1.24±0.09^{b}	DD>ED, PO	NS	NS
∑ n-3	15.03±2.55^{ab}	17.82±0.51^{a}	18.06±1.20^{a}	10.26±0.98^{b}	18.53±0.52^{a}	18.27±1.88^{a}	9.05±1.32^{b}	ED, DD>PO	NS	NS
∑ n-3 LC-PUFA	10.39±2.26^{ab}	13.46±0.33^{a}	14.33±1.06^{a}	5.84±0.67^{b}	14.13±0.37^{a}	14.56±1.80^{a}	4.95±0.35^{b}	ED, DD>PO	NS	NS
EPA+DHA	8.59±1.88^{ab}	11.99±0.32^{a}	13.27±0.99^{a}	5.01±0.58^{b}	12.70±0.42^{a}	13.63±1.73^{a}	4.23±0.91^{b}	ED, DD>PO	NS	NS
EPA/ARA	7.78±0.49^{a}	5.78±0.91^{ab}	2.60±0.22^{c}	3.91±0.32^{bc}	5.70±0.42^{ab}	2.05±0.12^{c}	3.10±0.39^{c}	ED> DD, PO	NS	NS
DHA/EPA	1.65±0.03^{a}	3.88±0.02^{c}	8.90±0.00^{d}	2.97±0.01^{b}	4.02±0.01^{c}	11.06±0.00^{d}	3.11±0.01^{bc}	PO> ED>DD	NS	NS
n-6/n-3	1.20±0.17^{b}	1.10±0.05^{b}	1.22±0.09^{b}	2.20±0.17^{a}	1.15±0.04^{b}	1.32±0.14^{b}	2.62±0.35^{a}	PO>ED, DD	NS	NS
16:0/16:1	3.55±0.09^{c}	5.06±0.29^{a}	4.68±0.16^{ab}	3.98±0.09^{bc}	5.15±0.20^{a}	4.88±0.01^{a}	3.97±0.15^{bc}	ED> DD, PO	NS	NS
18:0/18:1	0.10±0.00^{a}	0.09±0.00^{ab}	0.09±0.00^{b}	0.09±0.00^{ab}	0.09±0.00^{ab}	0.09±0.00^{b}	0.09±0.00^{ab}	NS	NS	NS

Values (mean ± SE, n=3) with different superscript letters in the same row or separated by > are significantly different (P < 0.05).

These differences in whole-body FA composition of gilthead sea bream were clearly reflected in the PCA analysis, where PC1 explained 77.1% of the distribution of whole-body FA composition of fish, while PC2 explained only 12.3% (Figure 4.2a). Sea bream fed PO diets were distinctly separated horizontally from fish fed DD diets, with fish fed ED or FO diet occupying intermediate positions (Figure 4.2a). Vertically, FO fish was separated from fish fed PO and DD, with fish fed ED occupying an intermediate position (Figure 4.2a). As shown in the correlation circle, the horizontal distribution of the fatty acid profiles from the different diets was driven principally by OA, LA, and DHA contents in whole-body, where fish fed PO had the highest OA and LA contents and those fed ED or DD the highest DHA contents (Figure 4.2b). In contrast, EPA was important for the vertical distribution, separating fish fed FO diet with the highest whole-body EPA content (Figure 4.2b). Furthermore, the PERMANOVAs showed that the lipid source significantly affected FA composition ($P<0.05$) whereas FM content of the diets had no significant effect on general whole-body FA composition of fish (data not shown). These results were also corroborated by the two-way ANOVA, where lipid source significantly affected most of the FA contents in whole-body ($P<0.05$), while the dietary content of FM had less impact, and no interaction between the two factors was observed (Table 4.6).

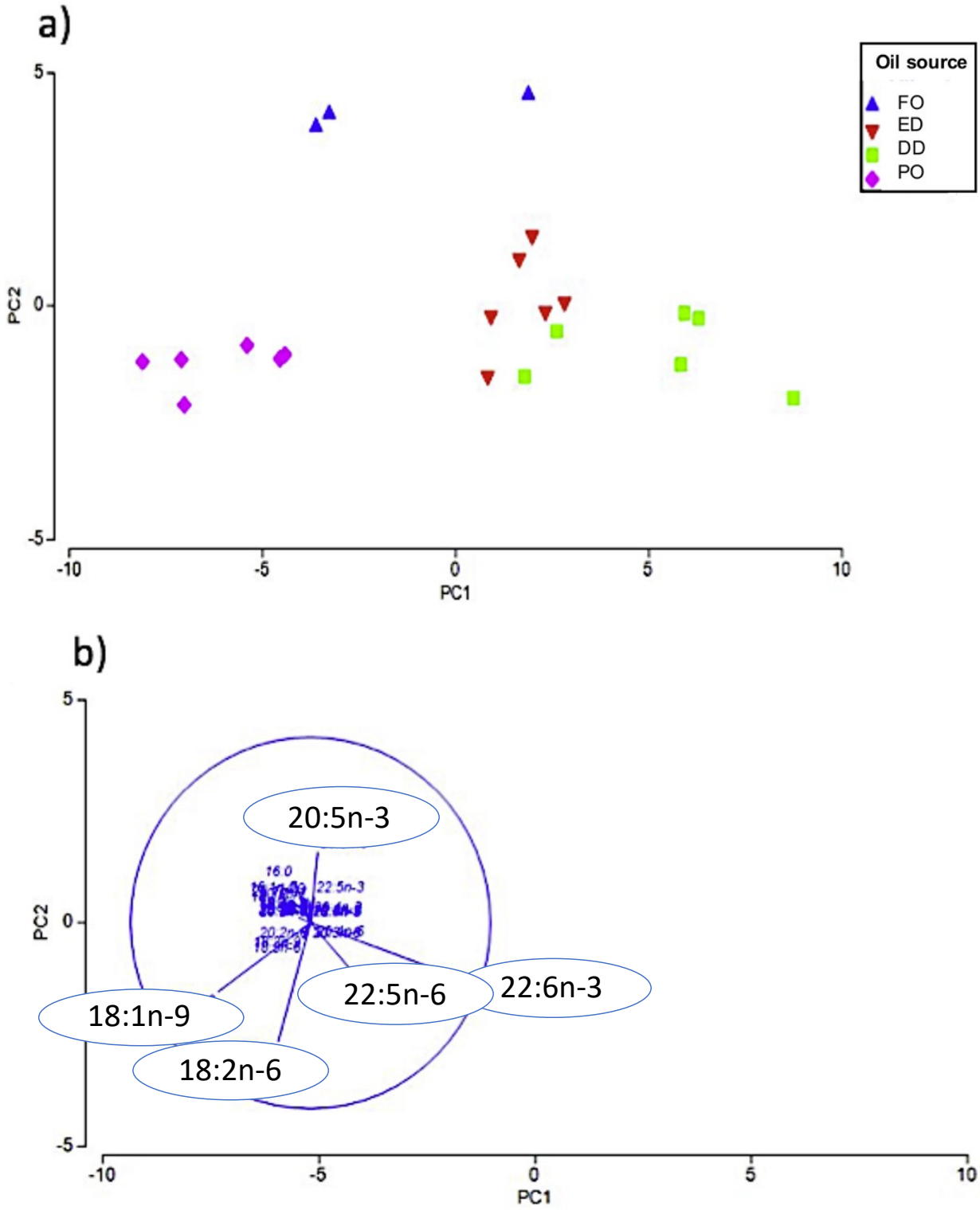

Figure 4.2. Principal components analysis of gilthead sea bream whole-body fatty acid composition: a) Plot of the first two principal components (PC1 and PC2) of gilthead sea bream whole-body fatty acid profiles; (b) Variables used to form the PCA, in which the circle represents the correlation circle; correlations to PC1 or PC2 are stronger as closer are the arrows to the circle.

4.4.3.3 WHOLE-BODY FATTY ACID RELATIVE RETENTIONS

There were no significant differences in the relative retention values for EPA or n-3 LC-PUFA among fish fed the different diets, despite the different dietary levels in these fatty acids (Table 4.7).

Fish fed PO, particularly at 7.5% FM, showed the highest relative retention of 18:2n-9, 18:3n-6, 18:4n-3 and 20:4n-3, products of Δ6 desaturase (fads2), as well as the highest relative retention of total n-6 LC-PUFA ($P<0.05$; Table 4.7). Besides, fish fed 7.5% FM PO showed the highest relative retention of 20:3n-3, and along with fish fed 15% FM PO, the highest 20:3n-6 and 20:2n-9 relative retention, all products of elongase 5 (elovl5) ($P<0.05$; Table 4.7). Finally, fish fed 7.5% FM PO also had the highest relative retention of 22:4n-6, 22:5n-3, 22:6n-3 and, together with 15% FM PO, of 20:3n-6 all of them products of both fads2 and elovl5.

There were no significant differences in the DHA relative retention among fish fed the control diet or any of the algal products used. However, fish fed the DD diet showed a higher ARA relative retention than those fed ED ($P<0.05$; Table 4.7). Besides, fish fed 7.5% FM DD also had a high relative retention of 18:2n-9, 20:3n-6, 20:2n-9, 20:2n-6 and 20:3n-6 ($P<0.05$; Table 4.7). In fish fed ED there was a high utilization of certain fatty acids, such as 22:4n-6, 20:3n-3, 20:2n-9, 20:2n-6 and 20:3n-6 ($P<0.05$; Table 4.7).

Finally, as showed by two-way ANOVA, a reduction in dietary FM content diminished the relative retention of 18:1n-9, 18:2n-6 and 18:3n-3, while increasing the retention on 18:2n-9, 18:4n-3, 20:2n-9, 20:2n-6, 20:4n-6, 20:3n-3, 20:4n-3, 22:1n-9, 22:4n-6, 22:5n-6 and n-6 LC-PUFA ($P<0.05$; Table 4.7). Besides, there was an interaction between the dietary FM levels and lipid sources on the retention of 18:1n-9, 18:2n-9, 18:3n-6, 20:2n-9, 20:2n-6, 20:3n-6, 20:3n-3, 22:4n-6 and 22:5n-6 ($P<0.05$; Table 4.7).

Table 4.7. Fatty acid retention (% FA intake) in whole-body of gilthead sea bream fingerlings fed the experimental diets for 74 days

Diets										
		15% FM			7.5% FM			Two-way ANOVA		
Fatty acid	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
18:1n-9	86.5±4.1^{a}	89.3±2.6^{a}	82.0±2.2^{ab}	66.5±1.4^{c}	67.4±0.6^{c}	69.3±3.6^{bc}	61.3±3.6^{c}	ED, DD>PO	P=0.00	P=0.02
18:2n-9	93.7±2.1^{b}	79.7±7.9^{b}	80.8±1.4^{b}	115.9±16.5^{b}	192.6±3.8^{b}	2890.2±164.7^{a}	3027.3±115.1^{a}	DD, PO>ED	P=0.00	P=0.00
18:2n-6	69.3±3.3^{ab}	77.6±2.9^{a}	70.3±0.6^{ab}	56.8±2.3^{c}	62.0±1.2^{bc}	62.6±2.4^{bc}	50.9±3.4^{c}	ED, DD>PO	P=0.00	NS
18:3n-6	98.1±5.4^{d}	52.3±2.9^{d}	82.8±2.3^{d}	1052.0±140.6^{a}	286.1±16.1^{cd}	436.6±12.1^{c}	760.5±49.4^{b}	PO>ED, DD	NS	P=0.00
18:3n-3	52.3±5.0^{ab}	60.8±4.5^{a}	60.1±2.0^{a}	46.9±4.1^{ab}	52.9±2.8^{ab}	54.7±1.5^{ab}	41.4±4.5^{b}	ED, DD>PO	P=0.04	NS
18:4n-3	48.4±9.2^{b}	50.0±6.2^{b}	44.9±5.2^{b}	77.9±17.1^{ab}	75.2±9.6^{ab}	97.0±13.0^{ab}	129.01±17.7^{a}	PO>ED	P=0.02	NS
20:1n-9	63.0±5.3^{cd}	46.6±5.8^{d}	42.5±0.9^{d}	99.1±5.9^{ab}	60.0±5.6^{cd}	85.1±7.5^{bc}	125.0±7.0^{a}	PO>ED, DD	P=0.00	NS
20:2n-9	61.0±8.5^{b}	31.0±4.2^{b}	42.6±4.7^{b}	344.5±27.5^{a}	40.2±6.0^{b}	223.1±38.1^{a}	254.1±51.7^{a}	PO>DD>ED	NS	P=0.00
20:2n-6	74.9±8.6^{bc}	25.6±4.1^{d}	42.7±5.8^{cd}	93.5±3.9^{b}	67.8±8.3^{bc}	152.3±8.4^{a}	150.5±11.4^{a}	PO>DD>ED	P=0.00	P=0.00
20:3n-6	51.7±13.5^{bc}	22.3±2.3^{c}	30.4±1.1^{c}	173.0±17.0^{a}	45.2±1.9^{c}	116.0±7.4^{abc}	169.3±60.4^{ab}	PO>ED, DD	NS	NS
20:4n-6	42.9±9.1^{bc}	32.8±1.9^{c}	66.0±1.8^{ab}	48.8±7.5^{bc}	47.4±0.6^{bc}	83.7±8.3^{a}	70.4±4.9^{ab}	DD>PO>ED	P=0.00	NS
20:3n-3	36.1±7.9^{c}	17.1±1.6^{c}	17.3±0.1^{c}	71.5±8.0^{b}	20.9±1.9^{c}	68.4±1.6^{b}	164.9±9.2^{a}	PO>DD>ED	P=0.00	P=0.00
20:4n-3	62.0±11.4^{cd}	29.5±1.3^{d}	33.4±1.9^{d}	113.4±17.6^{ab}	35.3±2.4^{d}	80.5±2.4^{bc}	155.9±5.4^{a}	PO>DD>ED	P=0.00	NS
20:5n-3	41.0±11.1	45.5±6.3	44.5±5.9	42.9±11.9	37.6±3.8	47.0±7.6	59.2±13.3	NS	NS	NS
22:1n-9	69.4±5.8^{ab}	62.3±10.8^{b}	46.0±3.8^{b}	76.4±4.2^{ab}	64.5±9.1^{b}	74.8±2.8^{ab}	103.4±9.7^{a}	PO>ED, DD	P=0.00	NS
22:4n-6	30.6±12.9^{bc}	8.0±1.5^{c}	6.1±0.4^{c}	41.7±1.7^{bc}	26.9±3.5^{c}	63.9±5.0^{b}	115.5±13.2^{a}	PO>ED, DD	P=0.00	P=0.00
22:5n-6	26.4±8.1^{bc}	20.0±0.7^{c}	77.3±3.5^{a}	51.7±6.7^{ab}	53.0±1.7^{a}	71.4±7.1^{a}	58.5±5.2^{a}	DD>PO>ED	P=0.00	P=0.00
22:5n-3	46.2±6.3^{b}	61.0±5.3^{b}	32.4±3.7^{b}	75.6±16.6^{ab}	88.8±6.2^{ab}	76.2±7.8^{ab}	135.3±19.5^{a}	PO>DD	P=0.00	NS
22:6n-3	45.4±2.5^{b}	73.0±3.5^{ab}	88.6±6.6^{ab}	74.4±8.8^{ab}	57.9±2.6^{ab}	68.2±5.6^{ab}	101.1±20.0^{a}	NS	NS	NS
n-6 LC-PUFA[1]	43.2±10.0^{bc}	21.0±2.0^{c}	53.0±1.5^{bc}	72.9±6.3^{ab}	49.3±2.3^{bc}	76.6±7.6^{ab}	93.4±12.3^{a}	PO>DD>ED	P=0.00	NS
n-3 LC-PUFA[2]	54.3±15.5	61.3±3.7	73.3±5.7	74.6±15.1	53.1±2.5	77.1±11.4	78.2±28.0	NS	NS	NS

Values (mean ± SE, n=3) with different superscript letters in the same row or separated by > are significantly different (P < 0.05).

4.4.3.4 FILLET FATTY ACID COMPOSITION

Fatty acid composition of fish fillet was similar to whole-fish fatty acid composition, although the levels of some FA, such as n-3 LC-PUFA, were higher in fish fillet, particularly in fish fed FO, ED, and PO diets (Table 4.8). As in fish whole-body, most fatty acids including 14:0, 15:0, 17:0,18:0, 16:1n-7, 18:1n-9, 18:1n-7, 18:3n-3, EPA, 22:1n-9, 22:5n-6, MUFA, n-6, n-9, n-3, n-6/n-3, or EPA/DHA, reflected their respective dietary levels (Table 4.8), whereas 16:0 and ARA levels were not affected by the diet. The highest EPA content was present in fish fed FO diet, followed by those fed ED ($P<0.05$; Table 4.8), while fish fed PO diets showed the lowest content in n-3 LC-PUFA ($P<0.05$; Table 4.8). Reduction of dietary FM decreased 20:1n-9, 20:1n-7, 22:1n-11, 20:3n-6, 16:4n-3, 20:4n-3 and increased 18:2n-6. However, 18:3n-6 was also increased in fillets by the reduction in dietary FM, as well as in fish fed PO diets, in contrast with the reduction of 18:3n-6 in the diet and in concordance with the 18:2n-6 increase. Interestingly, fillet ARA contents were constant, regardless the dietary levels, and, according to the two-way ANOVA, tended to be higher in fish fed DD. Fish fed DD also showed the highest content in n-6 LC-PUFA, particularly n-6 DPA ($P<0.05$; Table 4.8). Besides, fish fed DD, together with those fed ED, also showed the highest contents in DHA ($P<0.05$; Table 4.8).

The PCA analysis showed that PC1 explained 76.5% of the variability in fillet fatty acid composition, whereas PC2 explained only 13.6% (Figure 4.3a). In comparison to whole-body, muscle fatty acid profiles of fish fed PO and FO were more distinctly differentiated by their contents in OA (18:1n-9), LA (18:2n-6) and, particularly, DHA (Figure 4.3a). In contrast, the muscle profiles of fish fed ED and DD were very similar in their contents of these fatty acids but differed more in their EPA and n-6 DPA contents in muscle than in whole-body. Similarly, OA and LA clearly separated fish fed PO, DHA was the strongest influencer differentiating fish fed ED or DD, while EPA was important in distinguishing fish fed FO diet (Figure 4.3b). As for whole-body, PERMANOVA and two-way ANOVA showed that the lipid source significantly affected fillet FA composition ($P<0.05$), but FM content of the diets had no significant effect on general fillet FA composition of fish and no interaction was found between the two variables.

Table 4.8. Fillet fatty acid composition (% total identified FA) of gilthead sea bream fingerlings fed the experimental diets for 74 days

	Diets									
		15% FM			7.5% FM			Two-way ANOVA		
Fatty acid	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
14:0	1.39±0.15^{a}	0.72±0.20^{b}	0.93±0.09^{ab}	0.78±0.02^{b}	0.56±0.08^{b}	1.06±0.13^{ab}	0.80±0.02^{b}	NS	NS	NS
14:1n-7	0.03±0.00	0.04±0.00	0.04±0.01	0.04±0.00	0.03±0.00	0.04±0.01	0.05±0.00	NS	NS	NS
14:1n-5	0.04±0.00^{a}	0.03±0.01^{ab}	0.02±0.00^{ab}	0.02±0.00^{ab}	0.01±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{b}	NS	NS	NS
15:0	0.15±0.01^{a}	0.11±0.01^{b}	0.10±0.01^{b}	0.09±0.00^{b}	0.10±0.01^{b}	0.12±0.01^{ab}	0.09±0.00^{b}	NS	NS	NS
15:1n-5	0.01±0.00	0.02±0.02	0.02±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.00	NS	NS	NS
16:0	10.87±0.28	10.00±0.62	10.47±0.43	10.29±0.24	9.84±0.43	10.85±0.68	10.38±0.06	NS	NS	NS
16:1n-7	2.81±0.16^{a}	1.89±0.33^{b}	2.09±0.12^{ab}	2.37±0.04^{ab}	1.82±0.11^{b}	2.16±0.22^{ab}	2.46±0.08^{ab}	PO>ED	NS	NS
16:1n-5	0.06±0.00^{a}	0.04±0.02^{ab}	0.03±0.00^{ab}	0.03±0.00^{ab}	0.03±0.01^{b}	0.03±0.00^{ab}	0.04±0.00^{ab}	NS	NS	NS
16:2n-4	0.21±0.02^{a}	0.06±0.01^{b}	0.05±0.00^{b}	0.06±0.01^{b}	0.04±0.00^{b}	0.04±0.00^{b}	0.05±0.00^{b}	NS	NS	NS
17:0	0.24±0.02^{a}	0.06±0.01^{b}	0.06±0.00^{b}	0.07±0.00^{b}	0.05±0.00^{b}	0.05±0.00^{b}	0.07±0.00^{b}	NS	NS	NS
16:3n-4	0.16±0.00^{a}	0.11±0.01^{b}	0.10±0.00^{b}	0.13±0.01^{ab}	0.11±0.01^{b}	0.11±0.01^{b}	0.12±0.00^{b}	NS	NS	NS
16:3n-3	0.04±0.01	0.04±0.02	0.02±0.00	0.03±0.01	0.02±0.01	0.02±0.00	0.02±0.00	NS	NS	NS
16:3n-1	0.06±0.00	0.07±0.01	0.06±0.00	0.10±0.03	0.08±0.01	0.04±0.01	0.10±0.03	NS	NS	NS
16:4n-3	0.24±0.01^{a}	0.08±0.01^{bc}	0.06±0.00^{b}	0.10±0.01^{b}	0.08±0.01^{bc}	0.06±0.00^{c}	0.06±0.00^{c}	NS	NS	NS
18:0	3.55±0.15^{a}	3.38±0.03^{ab}	3.30±0.03^{ab}	3.61±0.10^{a}	3.29±0.03^{ab}	3.10±0.07^{b}	3.67±0.08^{a}	PO>ED, DD	NS	NS
18:1n-9	34.35±0.16^{b}	34.54±1.21^{b}	34.06±0.81^{b}	39.16±0.43^{a}	35.27±0.47^{b}	34.53±1.25^{b}	39.18±0.39^{a}	PO>ED, DD	NS	NS
18:1n-7	2.82±0.00^{a}	2.25±0.06^{b}	2.10±0.05^{bc}	2.57±0.03^{a}	2.22±0.04^{b}	1.90±0.12^{c}	2.58±0.01^{a}	PO>ED> DD	NS	NS
18:1n-5	0.08±0.00^{a}	0.08±0.01^{ab}	0.07±0.00^{ab}	0.07±0.00^{abc}	0.06±0.00^{bc}	0.05±0.00^{c}	0.07±0.01^{abc}	NS	P=0.00	NS
18:2n-9	0.56±0.05^{b}	0.55±0.05^{b}	0.54±0.03^{b}	0.98±0.05^{a}	0.57±0.03^{b}	0.67±0.04^{b}	1.12±0.12^{a}	PO>ED, DD	NS	NS
18:2n-6	15.55±0.16^{d}	17.04±0.68^{cd}	16.45±0.40^{cd}	21.20±0.07^{a}	19.13±0.21^{b}	17.73±0.56^{bc}	21.51±0.20^{a}	PO>ED, DD	P=0.00	NS
18:2n-4	0.14±0.00^{a}	0.05±0.02^{b}	0.03±0.00^{b}	0.05±0.00^{b}	0.04±0.00^{b}	0.02±0.00^{b}	0.03±0.00^{b}	NS	NS	NS
18:3n-6	0.65±0.04^{b}	0.66±0.05^{b}	0.65±0.04^{b}	1.31±0.06^{a}	0.79±0.04^{b}	0.87±0.02^{b}	1.49±0.13^{a}	PO>ED, DD	P=0.00	NS
18:3n-4	0.14±0.01^{a}	0.07±0.01^{b}	0.06±0.00^{b}	0.07±0.01^{b}	0.06±0.00^{b}	0.05±0.00^{b}	0.07±0.00^{b}	NS	NS	NS
18:3n-3	4.05±0.05^{a}	3.86±0.10^{a}	3.21±0.07^{b}	4.15±0.06^{a}	4.04±0.08^{a}	3.28±0.12^{b}	4.08±0.08^{a}	PO, ED>DD	NS	NS
18:3n-1	0.01±0.00^{a}	0.00±0.00^{c}	0.00±0.00^{c}	0.01±0.00^{ab}	0.00±0.00^{c}	0.00±0.00^{c}	0.01±0.00^{bc}	NS	NS	NS
18:4n-3	0.82±0.03^{a}	0.44±0.02^{bc}	0.40±0.02^{c}	0.53±0.03^{b}	0.38±0.01^{c}	0.40±0.01^{c}	0.52±0.02^{b}	PO>ED, DD	NS	NS
18:4n-1	0.10±0.00^{a}	0.05±0.02^{b}	0.03±0.00^{b}	0.03±0.01^{b}	0.03±0.01^{b}	0.02±0.00^{b}	0.02±0.00^{b}	NS	NS	NS
20:0	0.37±0.00	0.38±0.04	0.29±0.00	0.34±0.03	0.38±0.03	0.28±0.02	0.30±0.01	NS	NS	NS
20:1n-9	0.28±0.01^{a}	0.27±0.03^{ab}	0.24±0.00^{ab}	0.26±0.02^{ab}	0.17±0.01^{c}	0.15±0.01^{c}	0.20±0.02^{bc}	NS	P=0.00	NS
20:1n-7	1.81±0.02^{a}	1.70±0.16^{ab}	1.48±0.03^{abc}	1.44±0.05^{bc}	1.32±0.06^{cd}	1.04±0.02^{d}	1.28±0.08^{cd}	ED> DD	P=0.00	NS
20:1n-5	0.14±0.01^{a}	0.09±0.01^{b}	0.07±0.01^{b}	0.08±0.01^{b}	0.07±0.01^{b}	0.05±0.00^{b}	0.08±0.01^{b}	NS	NS	NS
20:2n-9	0.29±0.03^{a}	0.30±0.04^{a}	0.26±0.04^{a}	0.27±0.03^{a}	0.19±0.01^{ab}	0.13±0.02^{b}	0.21±0.01^{ab}	NS	P=0.00	NS

Table 4.8. Fillet fatty acid composition (% total identified FA) of gilthead sea bream fingerlings fed the experimental diets for 74 days (continued)

	Diets									
		15% FM			7.5% FM			Two-way ANOVA		
Fatty acid	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
20:2n-6	0.30±0.01^{bc}	0.32±0.02^{ab}	0.30±0.02^{bc}	0.37±0.00^{a}	0.30±0.01^{bc}	0.24±0.01^{c}	0.35±0.01^{ab}	PO>ED, DD	P=0.02	NS
20:3n-6	0.22±0.01^{ab}	0.26±0.05^{ab}	0.28±0.01^{ab}	0.31±0.01^{a}	0.19±0.02^{b}	0.21±0.01^{ab}	0.25±0.02^{ab}	NS	P=0.01	NS
20:4n-6	0.46±0.01	0.44±0.05	0.52±0.01	0.40±0.03	0.48±0.02	0.52±0.05	0.41±0.03	PO>DD	NS	NS
20:3n-3	0.13±0.00^{ab}	0.14±0.01^{ab}	0.11±0.00^{ab}	0.16±0.02^{a}	0.13±0.01^{ab}	0.09±0.01^{b}	0.11±0.01^{ab}	NS	NS	NS
20:4n-3	0.48±0.01^{a}	0.32±0.04^{bc}	0.34±0.00^{b}	0.23±0.02^{cd}	0.26±0.01^{abc}	0.28±0.00^{abc}	0.19±0.01^{d}	ED, DD>PO	P=0.01	NS
20:5n-3	4.68±0.16^{a}	2.97±0.30^{b}	1.39±0.10^{c}	1.39±0.08^{c}	2.80±0.04^{b}	1.09±0.06^{c}	1.34±0.11^{c}	ED>DD, PO	NS	NS
22:1n-11	1.47±0.05^{ab}	1.49±0.32^{a}	1.28±0.01^{abc}	0.97±0.04^{abc}	0.81±0.08^{bc}	0.65±0.06^{c}	0.77±0.11^{c}	NS	P=0.00	NS
22:1n-9	0.53±0.02	0.60±0.15	0.45±0.01	0.48±0.04	0.47±0.08	0.32±0.05	0.41±0.03	NS	NS	NS
22:4n-6	0.12±0.01	0.19±0.08	0.13±0.00	0.11±0.00	0.13±0.01	0.12±0.03	0.11±0.00	NS	NS	NS
22:5n-6	0.21±0.02^{b}	0.48±0.11^{b}	4.03±0.25^{a}	0.30±0.06^{b}	0.53±0.04^{b}	4.14±0.63^{a}	0.38±0.04^{b}	DD>ED, PO	NS	NS
22:5n-3	1.73±0.05^{a}	1.26±0.23^{b}	0.62±0.05^{cd}	0.52±0.02^{d}	1.06±0.05^{bc}	0.48±0.05^{d}	0.49±0.05^{d}	ED>DD, PO	NS	NS
22:6n-3	7.56±0.45^{ab}	12.45±2.41^{a}	13.21±1.36^{a}	4.42±0.33^{b}	12.00±0.71^{a}	12.92±1.93^{a}	4.51±0.44^{b}	ED, DD>PO	NS	NS
Σ SFA	16.59±0.42	14.68±1.78	15.17±0.55	15.19±0.36	14.23±0.52	15.47±0.77	15.31±0.07	NS	NS	NS
Σ MUFA	44.44±0.21^{ab}	43.04±0.87^{b}	41.95±1.01^{b}	47.52±0.52^{a}	42.31±0.60^{b}	40.96±1.32^{b}	47.13±0.23^{a}	PO>ED, DD	NS	NS
Σ n-9	36.01±0.09^{b}	36.25±1.02^{b}	35.56±0.78^{b}	41.14±0.54^{a}	36.68±0.47^{b}	35.80±1.20^{b}	41.12±0.46^{a}	PO>ED, DD	NS	NS
Σ n-6	17.52±0.13^{d}	19.40±0.40^{c}	22.36±0.19^{b}	24.00±0.14^{a}	21.55±0.12^{b}	23.84±0.25^{a}	24.49±0.24^{a}	PO>DD> ED	P=0.00	P=0.02
Σ n-6 LC-PUFA	1.31±0.04^{b}	1.70±0.31^{b}	5.26±0.25^{a}	1.50±0.09^{b}	1.63±0.10^{b}	5.23±0.72^{a}	1.49±0.07^{b}	ED, PO>DD	NS	NS
Σ n-3	19.74±0.61^{a}	21.56±2.93^{a}	19.37±1.42^{a}	11.54±0.34^{bc}	20.77±0.76^{a}	18.63±1.90^{ab}	11.32±0.53^{c}	ED, DD>PO	NS	NS
Σ n-3 LC-PUFA	14.58±0.66^{a}	17.14±2.99^{a}	15.68±1.47^{a}	6.72±0.41^{b}	16.25±0.80^{a}	14.87±2.04^{a}	6.64±0.61^{b}	ED, DD>PO	NS	NS
EPA+DHA	12.24±0.62^{ab}	15.42±2.71^{a}	14.60±1.42^{a}	5.81±0.40^{b}	14.80±0.75^{a}	14.01±1.97^{a}	5.85±0.54^{b}	ED, DD>PO	NS	NS
EPA/ARA	10.08±0.06^{a}	6.74±0.17^{b}	2.66±0.11^{ef}	3.46±0.19^{d}	5.82±0.19^{c}	2.14±0.14^{f}	3.28±0.02^{de}	PO>ED>DD	P=0.00	NS
DHA/EPA	1.62±0.02^{a}	5.81±0.02^{cd}	9.50±0.01^{e}	3.12±0.01^{b}	4.29±0.01^{d}	11.85±0.01^{e}	3.37±0.01^{bc}	PO>ED>DD	NS	NS
n-6/n-3	0.89±0.03^{b}	0.93±0.13^{b}	1.17±0.1^{b}	2.08±0.05^{a}	1.04±0.04^{b}	1.30±0.12^{b}	2.18±0.12^{a}	PO>ED>DD	NS	NS
16:0/16:1	0.10±0.03	0.10±0.00	0.09±0.00	0.09±0.00	0.09±0.00	0.09±0.00	0.09±0.00	NS	NS	NS
18:0/18:1	3.80±0.19^{b}	5.17±0.02^{a}	4.94±0.14^{a}	4.28±0.03^{b}	5.37±0.12^{a}	5.00±0.19^{a}	4.17±0.11^{b}	ED, DD>PO	NS	NS

Values (mean ± SE, n=3) with different superscript letters in the same row or separated by > are significantly different (P < 0.05).

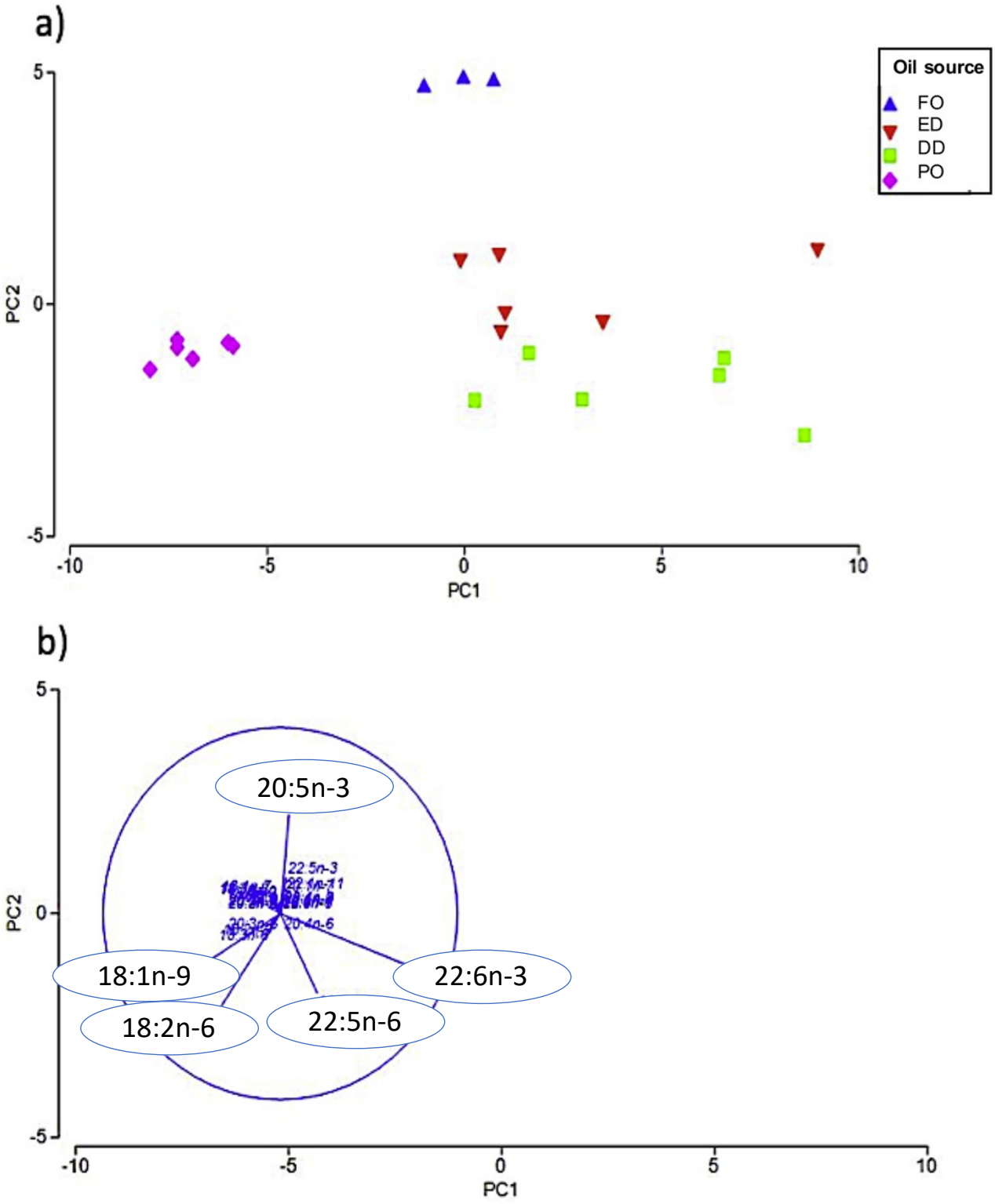

Figure 4.3. Principal components analysis of gilthead sea bream fillet fatty acid composition: a) Plot of the first two principal components (PC1 and PC2) of gilthead sea bream fillet fatty acid profiles; (b) Variables used to form the PCA, in which the circle represents the correlation circle; correlations to PC1 or PC2 are stronger as closer are the arrows to the circle.

4.4.3.5 FILLET LIPID QUALITY INDICES

Hypocholesterolemic (h), hypercholesterolemic (H) FA, and the ratio H/h were similar among fillets of fish fed the different experimental diets (Table 4.9). However, although not statistically different, a tendency to lower AI in fish fed ED than in those fed the FO diet could be observed (Table 4.9). Furthermore, sea bream fed PO diets showed a higher TI than those fed FO, ED, or DD diets, which was confirmed by two-way ANOVA ($P<0.05$; Table 4.9). Additionally, peroxidation index (PI) of fish fed ED and DD diets did not differ from those fed FO but were higher than those fed PO diets ($P<0.05$; Table 4.9).

Table 4.9. Fillet lipid quality indices of gilthead sea bream fingerlings fed the experimental diets for 74 days

Diets										
		15% FM			7.5% FM			Two-way ANOVA		
Index	FO	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
H	12.26±0.40	10.72±1.81	11.40±0.52	11.07±0.25	10.40±0.51	11.91±0.80	11.18±0.07	NS	NS	NS
h	86.96±0.41	88.69±1.76	88.13±0.53	88.42±0.27	89.05±0.49	87.63±1.38	88.36±0.12	NS	NS	NS
h/H	7.11±0.26	8.96±2.01	7.77±0.41	8.00±0.21	8.60±0.45	7.43±0.57	7.90±0.06	NS	NS	NS
AI	0.20±0.02	0.15±0.03	0.17±0.01	0.17±0.00	0.14±0.01	0.18±0.02	0.16±0.02	NS	NS	NS
TI	0.14±0.01	0.13±0.02	0.14±0.01	0.16±0.00	0.12±0.01	0.14±0.01	0.16±0.00	PO>ED, DD	NS	NS
PI	138.12±4.87^{ab}	163.90±23.37^{a}	175.55±12.60^{a}	91.00±3.15^{b}	160.00±6.31^{a}	172.47±19.22^{a}	91.55±3.98^{b}	ED, DD>PO	NS	NS

Values (mean ± SE, n=3) with different superscript letters in the same row or separated by > are significantly different (P < 0.05).

4.5 DISCUSSION

The aquaculture sector is very aware of the economic and environmental bottlenecks of the high use of FM and fish oil in fish feeds. The present study showed that it is possible to completely replace fish oil by algal oils containing mainly DHA or both EPA and DHA, in combination with poultry oil and rapeseed oil in gilthead sea bream fingerlings diets containing 15% FM, without affecting survival, growth, feed utilization and dietary protein efficiency. Our results agree with similar studies in Atlantic salmon (*Salmo salar*) showing good growth when fish oil was completely replaced with algal oil containing both EPA and DHA (Santigosa, 2019) or algae biomass containing mainly DHA (Peterson et al., 2019). The good growth of sea bream fed 15% FM and both algal products suggested that their n-3 LC-PUFA contents (2.8 and 2.4 n-3 LC-PUFA % DW in ED and DD diets, respectively) were sufficient to cover the requirements in juveniles of this species, estimated to be 1.3-1.6 % DW in the diet (Izquierdo, 2005; Houston et al., 2017). However, despite the DHA levels in both diets were higher than the optimum levels (0.6-0.7% DW in diet) determined for juvenile gilthead sea bream, the EPA content in the 15% FM DD diet (0.3 % DW in diet) was lower than the optimum estimated for this fatty acid (0.6-0.9% DW in diet) (Izquierdo, 2015; Houston et al., 2017). This was reflected in a 59% reduction in EPA contents in the whole body of fish fed 15% FM DD diet in comparison to the control fish. EPA play specific and important roles in fish (Izquierdo and Koven, 2011) and in gilthead sea bream it is an important source of eicosanoids such as PGE3, the major prostaglandin produced in plasma of juveniles from this species (Ganga et al., 2005). Therefore, the low dietary EPA values could be responsible for the lower LER and slightly higher FCR in fish fed 15% FM DD in comparison to the control or 15% FM ED. Nevertheless, fish fed 15% FM DD showed a similar utilization of dietary DHA as those fed the 15% FM ED diet, denoted by the identical relative retention values, in agreement with the good growth performance. Interestingly, whole body fatty acid profiles of fish fed DD showed increased 20:3n-3, 20:2n-9, 20:2n-6 or 20:3n-6, which are products from elongation of very long-chain fatty acid 5 protein (Elovl5), a very responsive protein to low dietary levels of EPA and DHA in this species (Houston et al., 2017). Besides, 15% FM DD diet was higher in n-6 DPA than the ED and control diet, and this fact was reflected in the high relative retention of n-6 DPA in the whole body and muscle. Remarkably, there was also a higher relative retention of ARA in fish fed the 15% FM DD diet than in those fed 15% FM ED or control diet, regardless of the dietary ARA levels. These results suggest a potential retro-conversion of 22:5n-6 to 20:4n-6, as also observed in gilthead sea bream larvae fed *Schizochytrium sp.* as a source of n-3 LC-PUFA (Ganuza et al., 2008).

Regarding fillet quality, the high n-3 LC-PUFA contents, particularly DHA, in ED or DD diets, were reflected in fish muscle, thus maintaining the high nutritional value of sea bream

fillet for human consumption. Thus, fish oil replacement by these algal ingredients allowed to maintain very good values for all the indices of lipid quality for human nutrition, particularly TI, that was significantly better than in fish fed PO diets. These results agree with the high DHA deposition in fillet of Atlantic salmon fed the same microalgae oil containing both EPA and DHA (Santigosa, 2019), as well as with the decrease of muscle AI and TI in rainbow trout fed increasing inclusion levels of other microalgae species (*Spirulina platensis,* Teimouri et al., 2016). Several lipid quality indices are common indicators of the potential value of fish nutritional composition for the coronary health of the consumer (Ulbricht and Southgate, 1991). On one hand, the atherogenic index reflects the risk of lipid deposition in blood vessels by considering the ratio between proatherogenic fatty acids, such as SFA and antiatherogenic fatty acids, such as PUFA. On the other hand, the thrombogenic index reflects the probability of forming clots in blood vessels, favoured by the prothrombogenic SFA and prevented by the antithrombogenic MUFA or PUFA. Albeit sea bream in the present trial did not reach commercial size, these indices were calculated in fish fillets to have an estimation of the potential impact of the dietary lipid blends tested, on the fillet quality for the consumer. Thus, the higher TI found in sea bream fed PO diets, suggests a higher MUFA and lower PUFA deposition, and, therefore, a potential increased risk of lipid deposition in blood vessels, decreasing health benefits for the consumer when poultry oil alone replaces fish oil (Turchini et al., 2003; Campos et al., 2019). As it could be expected, values for peroxidation index, indicator of fillet shelf life, were higher in fish fed FO or microalgae oils than in those fed PO, indicating a higher risk of lipid oxidation. These values reflect the high dietary and muscle contents in PUFA, known to be more sensible to oxidation than MUFA (Hulbert et al., 2007).

Furthermore, the present study showed the feasibility of combining these highly valuable sources of n-3 LC-PUFA with more economic lipid ingredients, such as poultry and rapeseed oil, which could constitute a good energy source improving the cost effectiveness of the diet. However, complete replacement of dietary fish oil by poultry oil without supplementation with n-3 LC-PUFA sources led to growth suppression and decreased survival. These results were consistent with the slightly lower growth performance of European sea bass (*Dicentrarchus labrax*) fed a blend of mammal and poultry fat (Monteiro et al., 2018) compared to those fed fish oil, or fed 100% poultry oil compared to those fed 50%fish oil/50%poultry oil (Campos et al., 2019), as well as in yellowtail kingfish (*Seriola lalandi)* when dietary fish oil was replaced by poultry oil at 22ºC (Bowyer et al., 2012). The more marked differences in growth performance in sea bream fed PO diets in the present study compared to previous studies may be related to differences in fish species, fish size or dietary formulations. Moreover, FA composition of fish fed PO diets was markedly distinguished in the PCA by their highest content in OA and LA and their lowest in EPA and DHA in comparison to fish fed the other

diets, in line with results in other fish species where poultry oil replaced fish oil in the diets (Turchini et al., 2009; Campos et al., 2018). Clearly, PO diets contents in n-3 LC-PUFA (0.5-0.7 % DW), DHA (0.3-0.4 % DW) and EPA (0.1-0.2% DW) were not sufficient to fulfill the essential fatty acid requirements of gilthead sea bream juveniles and altered the n-6/n-3 balance, in agreement with previous studies (Izquierdo, 2005). Indeed, survival was positively correlated with the dietary n-3 LC-PUFA and negatively correlated with dietary n-6/n-3 ratio. Increased mortality is often a symptom of EFA-deficiency or an imbalanced dietary fatty acid composition (Bell et al., 1986; Montero et al., 1998; 2003; 2008; 2010). Increase in the dietary n-6/n-3 ratio in gilthead sea bream reduces body lipid content and lipid deposition efficiency (Bandarra et al., 2011), in agreement with the reduced LER values in sea bream fed the PO diets in the present study. Since both n-3 LC-PUFA and n-6 LC-PUFA are precursors of eicosanoids and compete as substrates for the cyclooxygenases and lipoxygenases enzymes, these results may suggest the potential adverse effect of feeding PO diets on fish health due to unbalanced or insufficient dietary n-3 and n-6 LC-PUFA (Samuelsson, 1983). Thus, the poor performance of gilthead sea bream fed PO diets seemed to be related to the low EFA content, to the alteration of n-6/n-3 fatty acids or, more probably, to the influence of more than one of these factors, as shown in other fish species (Takeuchi et al., 1990; Ibeas et al., 1994, 1996; Berge et al., 2009).

The reduced LC-PUFA contents in the PO diets, together with the increase in their precursor n-9, n-6, or n-3 C18 fatty acids led to a high (>100%) relative retentions of fatty acid products from Δ6 desaturase and elongases, including 18:3n-6, 20:2n-9, 20:4n-3 or 20:3n-3 and 20:3n-6, respectively. These results denote the activation of long-chain PUFA synthesis in response to the low n-3 and n-6 LC-PUFA dietary content, in agreement with previous *in vivo* studies in this (Mourente and Tocher, 1993; Izquierdo et al., 2008) and other fish marine species, such as flounder (Izquierdo et al., 1992; Lee et al., 2003; Kim and Lee, 2004), turbot (Owen et al., 1975) or meagre (Carvalho et al., 2018). Retention was also high for DHA and ARA in PO fed-fish compared to those fed the FO diet denoting the selective retention of these important EFA when the dietary levels are below the requirement (Izquierdo et al., 2005), which may be associated to a decreased β-oxidation of DHA, over other FA (Frøyland et al., 1997).

Regardless of the dietary lipid source or n-3 LC-PUFA levels, reduction of FM from 15% to 7.5% markedly reduced dietary nutrients utilization and, consequently, fish growth performance. These results may suggest that the combined replacement of fish oil and FM may also affect growth beyond a palatability issue, previously also associated with a decrease in hepatic insulin growth factor I (IGF-I) expression (Benedito-Palos et al., 2007; Dias et al., 2009) or the effect of the protein quality on lipid metabolism (Dias et al., 2005; 2009). In

agreement, in the present study there was a significant interaction between the dietary lipid source and FM content on sea bream FA relative retention efficiencies. However, further studies are being conducted to understand the mechanisms behind this interaction.

4.6 CONCLUSIONS

In conclusion, the results of the present study showed that a blend of poultry oil and algae oils containing either DHA or both EPA and DHA, are effective in the total replacement of fish oil in practical (15% FM) diets for gilthead sea bream. These blends supported good growth while ensuring high nutritional quality of the fish fillet for the consumer. Both dietary algal lipid sources were effective in increasing fillet DHA content. On the contrary, PO was not able to completely replace fish oil and negatively affected fish performance, in relation to an insufficient dietary n-3 LC-PUFA content.

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CHAPTER 5. OILS COMBINATION WITH MICROALGAL PRODUCTS AS A STRATEGY FOR INCREASING THE N-3 LONG-CHAIN POLYUNSATURATED FATTY ACIDS CONTENT IN FISH OIL-FREE DIETS FOR MEAGRE (*ARGYROSOMUS REGIUS*)

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Keywords: essential fatty acids, fast-growing species, fish oil replacement, novel omega-3 long-chain polyunsaturated fatty acids sources

5.1 ABSTRACT

Diversification of both species and ingredients is essential for the further development of aquaculture production. The present study aimed to corroborate the potential of a blend of microalgae oils and poultry oil as total replacers of fish oil in aquafeeds, a fast-growing species for aquaculture diversification, with a high n-3 LC-PUFA requirement. For that purpose, meagre juveniles (2.7g initial body weight) were fed one of the four experimental diets for 30 days. A control diet (FO diet) was based on 5% fish oil and 7% of rapeseed oil, whereas in the other three experimental diets, fish oil was totally replaced by poultry oil alone (PO diet) or by blending poultry oil and one of two algal oils (ED and DD diets). Growth, tissue proximate and fatty acid composition, and the expression of genes related with fatty acid biosynthesis, antioxidant defence system and immune response were determined. Diets with blends of poultry and microalgae oils supported good growth and feed utilization. Besides, these diets maintained high DHA tissues contents and good fillet lipid quality indices, similar to those from fish fed the control diet containing fish oil. Furthermore, meagre fed ED diet showed the highest expression of *gpx* and *elovl5* in agreement with the highest polyunsaturated fatty acids contents and the highest elongation rates, respectively. A slightly higher hepatic expression of heat shock proteins was related with increasing dietary ARA levels. In contrast, PO was not able to support fish growth performance and flesh contents of n-3 fatty acids, and lead to increased thrombogenic index. Additionally, PO up-regulated hepatic *fads2* expression and down-regulated *gpx* and *hsp*. These results demonstrated the good potential of a blend of microalgae oils and poultry oil to completely replace fish oil in diets for meagre, a fast-growing species with a high n-3 LC-PUFA requirement.

5.2 INTRODUCTION

The limited availability and increased prices of fish meals (FM) and oils (FO), traditional feedstuffs for farmed fish, have promoted the search for sustainable alternative ingredients to reduce the inclusion of FM and FO in aquafeeds (Colombo and Turchini, 2021). Most of the research conducted during the last decades has focused on alternative terrestrial plant or animal ingredients, due to their competitive price and availability, which are nowadays frequently included in fish diets. However, these oils lack n-3 long-chain polyunsaturated fatty acids (LC-PUFA), including docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3), which have important biological functions in fish (Izquierdo and Koven, 2011). Furthermore, n-3 LC-PUFA are essential (EFA) for marine fish species, which have a limited capacity to biosynthesize them through endogenous pathways, and, thus, need to be supplied through the diet (Tocher, 2015). Therefore, whereas the replacement of FM has been successfully achieved (Hodar et al., 2020), the total replacement of FO in modern fish diets, with low contents of FM, faces more challenges. Indeed, high dietary inclusions of plant or animal oils in marine fish diets can reduce fish performance or negatively affect fish health, when EFA requirements of the target species are not met by other sources (Montero and Izquierdo, 2010). In addition, feeding low n-3 LC-PUFA diets also decrease the content of these FA in fish fillets (Nasopoulou and Zabetakis, 2012). Thus, the nutritional value of the final product for the consumer, as well as the beneficial effects associated to fish consumption for human nutrition could be reduced since n-3 LC-PUFA are known to be important in the prevention of human metabolic, cardiovascular, and coronary disorders (Connor, 2000; Simopoulos, 2012).

Last year's studies have focus on the search for novel lipid sources containing n-3 LC-PUFA, that would not only meet fish requirements and maximize productive parameters, but also guarantee aquaculture sustainability and produce fish with a high nutritional quality for human consumption. Among the several novel sources identified, including oils from microorganisms, krill or genetically modified plants (Naylor et al., 2009; Adarme-Vega et al., 2014; Betancor et al., 2016a,b; Tocher et al., 2019), microalgae highlight for their potential. Microalgae are advantageous compared with other conventional or novel ingredients for their simple nutritional composition, higher content in EPA and/or DHA, and lower footprint (Tibbetts, 2018; Shah et al., 2018). For instance, microalgae from the genus *Crypthecodinium*, *Thraustochytrium* or *Schizochytrium* are very rich in DHA (Qiu et al., 2001; Ganuza and Izquierdo, 2007), whereas those from *Phaeodactylum* or *Nannochloropsis* are rich in EPA (Atalah et al., 2007; Eryalçin et al., 2015), and effective EFA sources in the replacement of FO in weaning diets for gilthead sea bream (*Sparus aurata*) (Eryalçin et al., 2015). However,

despite their high nutritional potential, the use of microalgae as lipid sources in fish feeds has been constricted by their high production costs and their still limited availability (Tibbetts, 2018; Sarker et al., 2016). For this reason, their inclusion in fish diets has been mostly limited to highly valuable fish species or during early developmental stages or specific phases of the grow-out cycle, when DHA and EPA requirements are higher. Furthermore, lately, the use of extracted oils rather than algal biomass presents advantages such as their high digestibility and easier combination with lower-cost vegetable or animal oils. Moreover, the combine use of microalgae oils would allow the optimization of the feed's fatty acid profiles, providing higher flexibility in the formulation. Recently, combinations of microalgae oils with poultry and vegetable oils effectively replaced FO without adversely affecting growth, utilization of dietary fatty acids, or the nutritional quality of fish fillet for the consumer (Carvalho et al., 2020). Thus, blends of these oils could be also interesting in farmed species with different nutritional requirements and nutrient utilization capacity. This is particularly important in emerging species that allow the diversification of the European farmed fish, for instance the fast-growing meagre (*Argyrosomus regius*). Recently, the n-3 LC-PUFA requirements of juveniles from this species have been found to reach 2% DM, with an EPA / DHA proportion of 0.9 (Carvalho et al., 2018), a requirement considerable higher than those of other marine species with lower growth rates.

Therefore, the general objective of this study was to determine if the blend of microalgae oils with lower-cost lipid sources (poultry and vegetable oils) constitutes an effective lipid source for meagre juveniles with potential to totally replace FO in modern aquaculture feeds. The effects of practical combinations with the tested lipid sources were assessed on fish growth, nutrient utilization, tissue fatty acid deposition, nutritional quality of flesh and the expression of health-related genes.

5.3 MATERIAL AND METHODS

5.3.1 EXPERIMENTAL DIETS

Four experimental diets were formulated with 15% of fish meal to contain similar amounts of protein, lipid, and energy. A control diet (FO diet) was based on 5% fish oil and 7% of rapeseed oil. In the other experimental diets, fish oil was totally replaced by blending poultry oil and one of two algal oils extracted from the microalga *Shyzochrytium sp*. (ED and DD diets) or poultry oil alone (PO diet) (Table 5.1). One algal oil (Veramaris algal oil, Veramaris, The Netherlands) contained both EPA and DHA (ED diet), whereas the other algal oil (DHA Natur Oil, Archer Daniels Midland, USA) contained mainly DHA and n-6 DPA (DD diet). Both diets with microalgae approximately reached the n-3 LC-PUFA level of the control FO diet (in % of dry weight) but differed in their specific EPA and DHA contents as well as in their EPA/ARA and EPA/DHA ratios (Table 5.2). Feeds were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) at a pellet size of 1 mm and shipped to the ECOAQUA Institute facilities (Canary Islands, Spain), where they were analyzed for proximate (Table 5.1) and fatty acid composition (Table 5.2) following the methods described in Section 2.3. All diets were kept at 10 °C before and during the feeding trial.

Table 5.1 Formulation and proximate composition of the experimental diets

	Diets			
Ingredients (%)	FO	ED	DD	PO
Fish meal[a]	15.00	15.00	15.00	15.00
Wheat [a]	12.30	12.43	12.13	11.43
Corn gluten [a]	6.58	6.12	6.20	10.00
Hi-pro soya [a]	5.00	5.00	5.00	5.00
Wheat gluten [a]	17.71	18.02	17.92	15.38
Soya protein concentrate [a]	25.00	25.00	25.00	25.00
Faba beans [a]	5.00	5.00	5.00	5.00
Fish oil [a]	5.28	0.00	0.00	0.00
Rapseed oil [a]	7.92	7.60	5.69	7.29
Veramaris algal oil[b]	0.00	2.46	0.00	0.00
DHA Natur oil [c]	0.00	0.00	3.67	0.00
Poultry oil [f]	0.00	3.16	3.92	5.70
Vitamin premix[d]	0.10	0.10	0.10	0.10
Mineral premix[e]	0.10	0.10	0.10	0.10
Microalgae:PO	-	1:1.3	1:1.1	-
Proximate analysis (%)				
Crude protein	49.31	48.35	48.06	50.18
Crude lipids	18.19	17.53	18.23	17.50
Neutral lipids	89.32	89.91	90.15	91.96
Polar lipids	10.68	10.09	9.85	8.04
Moisture	6.60	8.23	7.85	8.28
Ash	4.66	4.51	4.70	4.64
Energy (MJ kg^{-1})	22.52	22.06	22.23	22.14
Protein / Energy (g MJ^{-1})	21.9	21.92	21.62	22.66
Starch (theorical value)	10.03	10.04	10.18	9.87
Fiber (theorical value)	2.53	2.53	2.53	2.54

[a] Skretting AS (Norway)

[b] Veramaris algal oil (Veramaris, The Netherlands)

[c] DHA Natur oil (ADM Animal Nutrition, USA)

[d,e] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC,

[f] Poultry oil: Sonac. B.V. The Netherlands

Table 5.2. Fatty acid composition of the experimental diets (% total identified FA)

	Diets			
Fatty acid	FO	ED	DD	PO
14:0	2.05	0.73	1.33	0.72
14:1n-7	0.40	0.59	0.34	0.08
14:1n-5	0.37	0.52	0.35	0.03
15:0	0.45	0.41	0.35	0.10
15:1n-5	0.29	0.42	0.36	0.03
16:0ISO	0.37	0.49	0.40	0.03
16:0	9.72	8.80	10.51	11.59
16:1n-7	2.51	1.24	1.42	1.90
16:1n-5	0.31	0.64	0.38	0.06
16:2n-4	0.44	0.59	0.52	0.07
17:0	0.49	0.00	0.41	0.06
16:3n-4	0.28	0.68	0.35	0.11
16:3n-3	0.31	0.65	0.49	0.04
16:3n-1	0.28	0.42	0.46	0.05
16:4n-3	0.54	0.55	0.44	0.13
18:0	2.72	2.48	2.70	3.40
18:1n-9	30.01	28.16	29.26	39.78
18:1n-7	2.68	1.94	1.73	2.38
18:1n-5	0.37	0.47	0.47	0.07
18:2n-9	0.41	0.49	0.39	0.00
18:2n-6	15.74	15.52	16.36	23.16
18:2n-4	0.32	0.62	0.41	0.03
18:3n-6	0.00	0.94	0.52	0.08
18:3n-4	0.34	0.53	0.49	0.10
18:3n-3	5.02	4.30	3.61	5.29
18:3n-1	0.29	0.47	0.46	0.00
18:4n-3	0.94	0.57	0.53	0.39
18:4n-1	0.27	0.42	0.47	0.03
20:0	0.68	0.73	0.50	0.52
20:1n-9	0.37	0.41	0.43	0.18
20:1n-7	2.33	2.20	1.91	1.96
20:1n-5	0.47	0.47	0.41	0.17
20:2n-9	0.30	0.56	0.35	0.05
20:2n-6	0.32	1.10	0.56	0.27
20:3n-6	0.32	0.66	0.58	0.11
20:4n-6	0.59	0.80	0.48	0.32
20:3n-3	0.29	0.60	0.46	0.10
20:4n-3	0.44	0.72	0.64	0.11
20:5n-3	5.30	3.33	1.54	1.24
22:1n-11	2.37	2.26	1.56	1.94
22:1n-9	0.56	0.56	0.58	0.39
22:4n-6	0.36	1.08	1.32	0.15
22:5n-6	0.47	1.11	3.05	0.13
22:5n-3	1.31	1.04	1.09	0.31
22:6n-3	5.36	8.72	9.04	2.11
∑ SFA	16.11	13.15	15.80	16.39
∑ MUFA	43.02	39.89	39.20	48.96
∑ n-6	17.79	21.21	22.88	24.29
∑ n-6 LC-PUFA	2.06	4.75	5.99	0.98
∑ n-3	19.51	20.49	17.84	9.72
∑ n-3 LC-PUFA	12.70	14.41	12.77	3.87
EPA+DHA	10.66	12.05	10.58	3.35
EPA/ARA	8.91	4.18	3.22	3.88
DHA/EPA	1.0	2.6	5.9	1.7
n-6/n-3	0.91	1.04	1.28	2.50

5.3.2 EXPERIMENTAL FISH AND FEEDING TRIAL CONDITIONS

Meagre juveniles (*Argyrosomus regius*), with an initial weight of 2.74 ± 0.01 g (mean ± SE) were distributed in 12 tanks of 500 L, at a density of 0.6 kg m^{-3} (55 fish per tank). The experimental tanks were filled with seawater (37 mg L^{-1}) at an open flow of 500 L h^{-1}, maintaining the dissolved oxygen over 8 mg L^{-1} and with natural temperature (23.6 ± 0.3 ºC) and photoperiod (12:12 h light/dark). Fish were fed until apparent satiety 3 times a day from Monday to Saturday for 30 days. Each experimental diet was randomly assigned to an experimental tank and tested in triplicate (n=3). Feed delivery was measured daily, and uneaten pellets were collected in a net by opening the water outlet 30 min after each meal, dried in an oven for 24 h and weighed to estimate feed intake (FI, feed $fish^{-1}$ day^{-1}).

5.3.3 SAMPLING PROCEDURES

Individual weight and length were assessed at the starting point and after the feeding period. Prior to sampling, fish were fasted for 24 h. Productive parameters were calculated following standard equations:

Specific growth rate (SGR, % day^{-1}) = 100* (ln BW_1−ln BW_0) / nº days of the trial, where BW_0 and BW_1 corresponded to fish body weight (g) at the beginning and at the end of the trial;

Allometric coefficient corresponded to the slope (*b*) of the potential regression between the wet weight (W) and the total length (TL) of all fish in each tank ($W = a*TL^b$) at the end of the experiment.

Thermal growth coefficient (TGC) = ($BW_1^{1/3} - BW_0^{1/3}$) / (Temperature * days);

Biological feed conversion ratio (FCR_b) = Feed delivered ($t_1 - t_0$)/ (Biomass t_1 – Biomass t_0 + $Biomass_{harvested}$ + $Biomass_{lost}$), where t_0 and t_1 corresponded at the beginning and end of the trial, respectively.

Efficiency of protein retention (% of protein intake) =100* (BW_1 (g) * protein in whole-$body_1$ (%))– initial weight (g) * protein in whole-$body_0$ (%) / FI (g) * dietary protein (%)

Efficiency of lipid retention (% of lipid intake) =Efficiency of protein retention (% of lipid intake) =100* (BW_1 (g) * lipid in whole-$body_1$ (%))– initial weight (g) * lipid in whole-$body_0$ (%) / FI (g) * dietary lipid (%)

Efficiency of energy retention (% of energy intake) =Efficiency of energy retention (% of energy intake) =100* (BW_1 (g) * energy in whole-$body_1$ (%))– initial weight (g) * energy in whole-$body_0$ (%) / FI (g) * dietary energy (%)

Furthermore, flesh lipid quality indices for human nutrition (Chen and Liu, 2020) were estimated as follow:

Hypercholesterolemic fatty acids (H) = 12:0 + 14:0 + 16:0

Hypocholesterolemic fatty acids (h) = 18:0 + Σ MUFA + Σ PUFA

Atherogenic index (AI) = (12:0 + 4 * 14:0 + 16:0) / (Σ MUFA + Σ n-3 PUFA + Σ n-6 PUFA)

Thrombogenic index (TI) = (14:0 + 16:0 + 18:0) / (0.5* Σ 18:1) + (0.5* Σ MUFA) + (0.5* Σ n-6 PUFA) + (3* Σ n-3 PUFA) + (Σ n-3 PUFA / Σ n-6 PUFA)

Peroxidation index (PI) = 0.025 × (Σ monoenoic fatty acids) + 1 × (Σ dienoic fatty acids) + 2 × (Σ trienoic fatty acids) + 4 × (Σ tetraenoic fatty acids) + 6 × (Σ pentaenoic fatty acids) + 8 × (Σ hexaenoic fatty acids).

Therefore, at the end of the experiment, fish were euthanized with an excess of clove oil and samples of whole-body, liver and muscle from 6 fish per tank were collected and pooled (per tissue and per tank) for proximal and fatty acids compositions, which were frozen at −80 °C until analysis. Additionally, livers from 5 fish per tank were also collected and conserved in RNA later (Sigma-Aldrich, Madrid, Spain) at -80 ºC until gene expression analysis.

5.3.4 BIOCHEMICAL ANALYSIS

All samples were homogenized prior to analysis. Moisture, ash, and protein contents were determined according to Association of Official Analytical Chemists (AOAC), 2000. Lipids were extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1989). FAMES were then separated by gas liquid chromatography (Izquierdo et al., 1990), quantified by a flame ionization detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified comparing with previously characterized standards.

5.3.5 GENE EXPRESSION

Total RNA was extracted from livers using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, samples were homogenized with the TissueLyzer-II (Qiagen) with TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA) and centrifuged with chloroform at 12000 g for 15 min, at 4 °C. The RNA phase was mixed with 75% ethanol and transferred into a RNeasy spin column, using RW1 and RPE buffers (Qiagen) to purify RNA bonded to a membrane obtaining purified RNA which was then eluted with 25 µL of RNase-free water. The NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Gel Red™ staining (Biotium Inc., Hayward, CA) on 1.4% agarose electrophoresis gel were used to determine the quantity and integrity of RNA, respectively. Complementary DNA (cDNA) was synthetized using iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions using an iCycler thermal cycler (Bio-Rad).

Liver *mRNA* levels of *fatty acyl desaturase 2* (*fads2)* (Monroig et al., 2013), *fatty acyl elongase 5* (*elovl5*) (Monroig et al., 2013), *heat shock proteins 70* and *90* (*hsp70* and *hsp90)* (Ruiz et al., 2019), *glutathione peroxidase* (*gpx*) (Ruiz et al., 2019) and *superoxide dismutase* (*sod*) (Ruiz et al., 2019) were determined by RT-qPCR in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad). *Elongation factor 1 alpha (ef1a)* (Ruiz et al., 2019) was used as a housekeeping gene. Primer sequences are detailed in Table 5.3. RT-PCR conditions used were the following: a first step of 3 min 30 s at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 60.5 °C, 30 s at 72 °C, 1 min at 95 °C, and a final 81 cycles of 10s from 55 °C to 95 °C. Reactions were carried out in in a final volume of 15 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA at 1:10 dilution and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. Each run ended with an analysis of the melting curve leading to a melting peak specific for the amplified target DNA.

Table 5.3. Sequences of primers used for running RT-PCR analysis gene expression in meagre livers

Genes	Forward	Reverse	Temperature
ef1a	GGTGCTGGACAAACTGAAGG	GAACTCACCAACACCAGCAG	59ºC
fads2	TGACTGGGTGACAATGCAG	TGGTGCTAACTTTGTGCCCT	60.5ºC
elovl5	CATCACACAGTTACAGCTGGT	GAATTGTGTGCACGGTTTCT	60.5ºC
hsp70	AACGTTCAGGACTTGCTGCT	CCCTTCGTAGACCTGGATGA	56.9ºC
hsp90	AAAAGGCCGAGAAGGAAGAG	GGCTTGGTCTTGTTCAGCTC	61ºC
gpx	AAGCAGTTTGCCGAGTCCTA	GCTGGTCTTTCAGCCACTTC	57ºC
sod	GGCCCTCACTTCAATCCCTA	TCCTTTTCCCAGATCGTCGG	59ºC

5.3.6 STATISTICAL ANALYSIS

For all statistical analysis carried out, each tank was considered as a replicate (n=3 for each treatment). All data were tested for normal distribution and homogeneity of variances using Shapiro–Wilk and Levene's tests, respectively. A one-way analysis of variance (ANOVA) was applied to all data and means were compared with Tukey's multiple range test (Tukey, 1949). When necessary, transformations to normalize data were applied (Sokal and Rohlf, 1981). If normalization was not possible, the data were analyzed using the non-parametric Kruskall-Wallis test. Principal component analysis (PCA) was performed for the overall performance and physiology parameters to obtain a more integrated interpretation of the main effects produced in the fish by the different diets. The first two components that explained more than 50% of total variance among the different diets were selected and graphed. Confidence levels were established at 95% ($P<0.05$). When appropriate, data were also subjected to the best-fit regressions (linear, exponential, or logarithmic) or correlation matrixes (Pearson's coefficients), which were checked for significance. Statistical analyses were done using the SPSS 21.0 or Prism9 software packages.

5.3.7 ETHICAL STATEMENT

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (reference OEBA_ULPGC_23/2019).

5.4 RESULTS

5.4.1 GROWTH PERFORMANCE AND FEED UTILIZATION

After 30 days of feeding, meagre juveniles fed the diets FO, ED and DD showed similar growth performance (total length, body weight, WG, SGR, TGC), that was significantly ($P<0.05$) or tendentially ($P<0.09$) higher than meagre fed PO (Table 5.4). Indeed, according to the PCA analysis, SGR was one of the variables that most explained the variability between the treatments (Figure 5.2a), separating particularly fish fed ED for its highest SGR from those fed PO (Figure 5.2b). Furthermore, fish fed ED diets presented a tendency to show the highest allometric exponent, particularly when compared with those fed PO ($P<0.09$). In the same way, fish fed FO, ED and DD diets showed lower ($P<0.05$) FCR than those fed PO, despite the similar feed intake among all (Table 5.4). This was also reflected in similar ways in efficiency of protein and energy retention ($P<0.05$; Table 5.4). Interestingly, meagre fed DD diets present lower efficiency of lipid retention, showing intermediate values between fish fed FO and ED diets and those fed PO (Table 5.4).

Table 5.4. Growth performance of meagre juveniles fed the experimental diets for 30 days

	Diets			
	FO	ED	DD	PO
Performance parameters				
Total length (mm)	8.77 ± 0.12	8.59 ± 0.09	8.76 ± 0.03	7.82 ± 0.07*
Body weight (g)	9.28 ± 0.29^{a}	9.18 ± 0.16^{a}	9.12 ± 0.32^{a}	6.73 ± 0.10^{b}
Weight gain (g)	6.55 ± 0.28	6.43 ± 0.17	6.38 ± 0.33	4.00 ± 0.10
SGR (% day^{-1})	3.90 ± 0.05	3.94 ± 0.07	3.96 ± 0.13	2.95 ± 0.04*
TGC	1.00 ± 0.04	0.97 ± 0.02	0.98 ± 0.04	0.71 ± 0.01*
Allometric exponent	2.85 ± 0.12	3.09 ± 0.05*	2.88 ± 0.08	2.74 ± 0.04
FCR	0.67 ± 0.05^{b}	0.71 ± 0.02^{b}	0.75 ± 0.04^{b}	1.03 ± 0.07^{a}
Feed intake (% BW day^{-1})	2.44 ± 0.16	2.53 ± 0.09	2.69 ± 0.11	2.89 ± 0.17
Nutrient retention efficiencies				
Efficiency of protein retention (% of protein intake)	45.05 ± 3.52^{a}	43.91 ± 2.67^{a}	42.99 ± 2.52^{a}	29.39 ± 2.61^{b}
Efficiency of lipid retention (% of lipid intake)	38.17 ± 4.46^{a}	31.82 ± 4.20^{a}	24.93 ± 7.24^{ab}	15.82 ± 1.90^{b}
Efficiency of energy retention (% of energy intake)	37.23 ± 3.42^{a}	34.59 ± 3.11^{a}	32.06 ± 0.86^{a}	21.66 ± 1.78^{b}

Superscripts with lowercase letters indicate significant differences with $P<0.05$. *Denotes a tendency ($P<0.09$).

5.4.2 TISSUE BIOCHEMICAL AND FATTY ACID COMPOSITION

Dietary oils combinations unaffected the proximate composition of meagre whole-body and muscle. However, fish fed PO presented slightly lower protein and lipid in whole-body when compared to those fed FO, ED and/or DD diets (Table 5.5). In contrast, meagre fed FO, ED or DD diets presented a trend to lower liver lipids and higher water content ($P<0.09$) than those fed PO diet (Table 5.5).

Regarding fish tissue fatty acid composition, 93% of them consisted of the SFA 16:0 and 18:0, the MUFA 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-7 and 22:1n-11, and the PUFA 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3 and 22:6n-3 in all tissues (Tables 5.6-5.8). In general, tissue FA composition reflected that of the diets. For instance, meagre fed DD diet presenting the highest content in n-6 DPA in all tissues, and the lowest EPA/DHA ratio ($P<0.05$; Tables 5.6-5.). In contrast, fish fed PO diet presented the highest 18:2n-6 and n-6/n-3 ratio in muscle and liver ($P<0.05$), as well as the same tendency for whole-body (Tables 5.6-5.8). Indeed, muscle n-6/n-3, along with other FA, including the n-6 PUFA in muscle, and the liver contents in 16:2n-4, 16:1n-5, 20:0, 20:1n-5 were the FA that most drove variability among fish fed the different diets according to the PCA analysis (Figure 5.2a). EPA was the highest in livers and fillets from fish fed FO diet, followed by those fed ED diets, and the lowest in fish fed DD or PO diets ($P<0.05$; Tables 5.7 and 5.8), whereas in whole-body the same tendency was observed (Table 5.6). In contrast, DHA was the highest in livers meagre fed ED or DD diets, particularly compared with those fed PO ($P<0.05$; Table 5.8), and in whole-body and muscle, a similar tendency was observed (Tables 5.6 and 5.7).

For some FA, a contrary trend to the dietary pattern was noticed. For instance, the contents of 18:2n-9, 20:2n-9, 18:3n-6, 20:3n-6, all products from FA desaturation, were the highest ($P<0.05$) in in all tissues of meagre fed PO, contrary to the lowest dietary contents of these FA (Tables 5.6-5.8). The similar trend was also observed for some elongation products, 20:1n-5, 20:1n-9, 22:1n-9, 20:2n-6 and 22:4n-6, being higher ($P<0.05$) in fish fed PO than in fish fed FO, ED or DD diets, despite the lowest concentrations of these FA in PO diet (Tables 5.6-5.8). Besides, hepatic ARA was also the highest in livers of meagre fed DD diets, despite the second lowest dietary level of this FA. Additionally, the dietary contents of 16:3n-3 were the highest in ED and DD diets whereas that of 18:3n-3 were the lowest (Table 5.2). However, meagre tissues, particularly in whole-body ($P<0.05$) fed these diets contrasted with the dietary composition, and 16:3n-3 showed the lowest concentration whereas 18:3n-3 presented similar contents compared to FO and PO (Table 5.6). The PCA results showed a good separation of treatment groups from some specific fatty acids of liver, fatty acid ratios of muscle and liver and TI, together with growth and some antioxidant enzymes as a function of diets, explaining 84.21% of the variance among the different treatments (Figure 5.2 a,b).

Regarding flesh lipid quality indices, AI or PI indices were unaffected by the diet but meagre fed PO presented a significantly higher TI than those fed FO, ED or DD diets ($P<0.05$; Table 5.7). Interestingly, TI was significantly positively correlated with 18:0, 18:2n-6, whereas negatively correlated with 20:5n-3 and 22:6n-3 in muscle ($P<0.05$; Pearson's coefficient).

Table 5.5. Whole-body, muscle and liver biochemical composition (% ww) of meagre juveniles fed the experimental diets for 30 days

	Diets			
	FO	ED	DD	PO
Whole-body				
Protein	14.09 ± 0.36	14.10 ± 0.31	14.46 ± 0.03	13.84 ± 0.22
Lipid	3.94 ± 0.12	3.49 ± 0.31	3.22 ± 0.84	2.64 ± 0.14
Ash	1.22 ± 0.03	1.26 ± 0.02	1.35 ± 0.10	1.32 ± 0.01
Water	79.78 ± 0.41	79.98 ± 0.87	79.71 ± 0.69	81.44 ± 0.14
Muscle				
Protein	18.76 ± 0.06	18.56 ± 0.11	18.62 ± 0.12	18.66 ± 0.03
Lipid	1.69 ± 0.09	1.64 ± 0.13	1.67 ± 0.09	1.52 ± 0.10
Ash	1.18 ± 0.15	1.24 ± 0.17	1.20 ± 0.17	1.06 ± 0.18
Water	78.38 ± 0.10	78.72 ± 0.36	78.51 ± 0.09	78.72 ± 0.23
Liver				
Lipid	9.20 ± 1.46	9.37 ± 1.12	8.52 ± 0.87	17.75 ± 3.03*
Water	73.12 ± 1.65	73.12 ±0.68	73.53 ± 0.23	67.71 ± 2.08*

*Denotes a tendency (P<0.09)

Table 5.6. Whole-body fatty acid composition (% total identified fatty acids) of meagre juveniles fed the experimental diets for 30 days

	Diets			
Fatty acid	FO	ED	DD	PO
14:0	1.22±0.41	0.20±0.05	0.58±0.17	0.27±0.13
14:1n-7	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.00
14:1n-5	0.05±0.01	0.01±0.00	0.02±0.00	0.01±0.00
15:0	0.22±0.05	0.09±0.01	0.12±0.02	0.08±0.03
15:1n-5	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00
16:0ISO	0.04±0.01	0.01±0.00	0.01±0.01	0.02±0.00
16:0	13.13±1.80	10.45±0.94	11.92±1.33	9.62±1.73
16:1n-7	3.11 ± 0.36^{a}	1.50 ± 0.08^{b}	1.85 ± 0.14^{ab}	1.88 ± 0.47^{ab}
16:1n-5	0.09±0.01	0.03±0.00	0.04±0.00	0.05±0.01
16:2n-6	0.01±0.00	0.00±0.00	0.00±0.00	0.01±0.00
16:2n-4	0.23±0.01	0.06±0.00	0.07±0.00	0.07±0.02
17:0	0.16±0.00	0.05±0.00	0.06±0.00	0.06±0.01
16:3n-4	0.18 ± 0.01^{a}	0.12 ± 0.01^{b}	0.12 ± 0.01^{b}	0.15 ± 0.01^{ab}
16:3n-3	0.10 ± 0.01^{a}	0.04 ± 0.00^{b}	0.05 ± 0.00^{b}	0.07 ± 0.01^{ab}
16:3n-1	0.32±0.02	0.31±0.04	0.31±0.03	0.57±0.06
16:4n-3	0.14 ± 0.02^{b}	0.16 ± 0.01^{b}	0.17 ± 0.01^{b}	0.27 ± 0.02^{a}
16:4n-1	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
18:0	4.92±0.32	4.91±0.46	4.38±0.50	7.45±1.07
18:1n-9	35.48±1.16	38.33±1.09	34.17±0.84	37.50±2.18
18:1n-7	2.69±0.19	2.71±0.13	2.37±0.17	3.14±0.21
18:1n-5	0.10 ± 0.00^{ab}	0.08 ± 0.01^{b}	0.09 ± 0.00^{b}	0.11 ± 0.01^{a}
18:2n-9	0.05 ± 0.00^{a}	0.04 ± 0.00^{ab}	0.03 ± 0.01^{b}	0.05 ± 0.01^{a}
18:2n-6	16.77 ± 0.62^{b}	19.94 ± 0.60^{a}	18.73 ± 0.75^{ab}	19.52 ± 0.67^{a}
18:2n-4	0.14±0.01	0.06±0.00	0.05±0.01	0.08±0.01
18:3n-6	0.11±0.01	0.07±0.01	0.13±0.03	0.08±0.02
18:3n-4	0.15±0.04	0.07±0.01	0.07±0.01	0.09±0.01
18:3n-3	3.32±0.36	3.56±0.38	2.97±0.34	2.39±0.26
18:3n-1	0.05±0.03	0.02±0.01	0.03±0.01	0.02±0.01
18:4n-3	0.47±0.10	0.23±0.04	0.32±0.05	0.24±0.05
18:4n-1	0.06±0.01	0.03±0.00	0.03±0.00	0.04±0.01
20:0	0.42±0.02	0.45±0.03	0.36±0.04	0.45±0.09
20:1n-9	0.20±0.01	0.20±0.01	0.23±0.02	0.30±0.05
20:1n-7	2.19±0.07	2.20±0.08	2.15±0.14	2.70±0.39
20:1n-5	0.15 ± 0.00^{a}	0.09 ± 0.00^{bc}	0.08 ± 0.00^{c}	0.13 ± 0.02^{ab}

Table 5.6. Whole-body fatty acid composition (% total identified fatty acids) of meagre juveniles fed the experimental diets for 30 days (continued)

	Diets			
Fatty acid	FO	ED	DD	PO
20:2n-9	0.05±0.00	0.04±0.00	0.04±0.01	0.07±0.01
20:2n-6	0.30±0.01b	0.34±0.01^{b}	0.32±0.02^{b}	0.51±0.05^{a}
20:3n-9	0.04±0.01	0.01±0.00	0.01±0.01	0.02±0.00
20:3n-6	0.08±0.02^{ab}	0.08±0.00^{b}	0.14±0.00^{a}	0.11±0.01^{ab}
20:4n-6	0.78±0.19	0.66±0.05	0.80±0.05	0.75±0.07
20:3n-3	0.08±0.01	0.09±0.01	0.09±0.00	0.09±0.00
20:4n-3	0.26±0.05	0.17±0.02	0.23±0.03	0.16±0.01
20:5n-3	3.35±0.93	2.33±0.49	1.68±0.27	1.96±0.33
22:1n-11	1.46±0.07	1.44±0.04	1.73±0.15	1.79±0.26
22:1n-9	0.54±0.03	0.53±0.01	0.51±0.05	0.78±0.10
22:4n-6	0.26±0.15	0.18±0.05	0.17±0.03	0.18±0.04
22:5n-6	0.32±0.07^{b}	0.34±0.03^{b}	2.97±0.36^{a}	0.26±0.03^{b}
22:5n-3	1.26±0.35	0.76±0.07	0.57±0.05	0.64±0.12
22:6n-3	4.94±1.65	7.01±1.36	9.18±1.66	5.25±0.92
EPA+DHA	8.28±2.59	9.34±1.85	10.86±1.93	7.21±1.24
EPA/ARA	4.62±1.24	3.62±0.86	2.08±0.23	2.58±0.29
EPA/DHA	0.71±0.05^{a}	0.33±0.01^{b}	0.18±0.01^{c}	0.38±0.02^{b}
SFA	20.10±2.57	16.16±1.43	17.44±2.03	17.95±0.98
MUFA	46.07±1.45	47.13±1.40	43.25±1.49	48.41±2.67
n-9	36.33±1.15	39.13±1.11	34.99±0.91	38.69±2.30
n-6	18.64±0.62^{b}	21.61±0.55^{ab}	23.26±1.16^{a}	21.42±0.61^{ab}
n-6 LC-PUFA	1.75±0.36	1.60±0.09	4.39±0.40	1.81±0.12
n-3	13.91±3.26	14.35±2.32	15.27±2.36	11.08±1.63
n-3 LC-PUFA	9.88±2.80	10.35±1.92	11.75±1.97	8.11±1.34
n-6/n-3	1.46±0.27	1.57±0.21	1.59±0.20	2.02±0.30*
H	14.35±2.20	10.65±0.97	12.50±1.49	9.89±1.84
H	84.82±2.26	88.74±1.00	86.93±1.55	89.50±1.81
h/H	6.32±1.32	8.51±0.95	7.18±0.94	9.90±2.30
AI	0.23±0.05	0.14±0.01	0.18±0.03	0.13±0.03
TI	0.21±0.04	0.16±0.02	0.18±0.03	0.19±0.01
PI	104.29±22.26	112.69±15.38	139.80±18.95	94.38±11.16

Superscripts with lowercase letters indicate significant differences with P <0.05. *Denotes a tendency (P<0.09)

Table 5.7. Fillet fatty acid composition (% total identified fatty acids) of meagre juveniles fed the experimental diets for 30 days

	Diets			
Fatty acid	FO	ED	DD	PO
14:0	0.94±0.18^{a}	0.46±0.04^{ab}	0.75±0.09^{ab}	0.42±0.05^{b}
14:1n-7	0.01±0.00^{b}	0.01±0.00^{ab}	0.01±0.00^{a}	0.01±0.00^{ab}
14:1n-5	0.03±0.00	0.02±0.00	0.03±0.01	0.04±0.01
15:0	0.19±0.02^{a}	0.15±0.01^{ab}	0.15±0.01^{ab}	0.12±0.01^{b}
15:1n-5	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
16:0ISO	0.02±0.00	0.01±0.00	0.01±0.00	0.02±0.00
16:0	12.61±0.52	13.41±0.47	13.61±0.18	12.90±0.31
16:1n-7	1.99±0.31	1.24±0.12	1.44±0.13	1.41±0.10
16:1n-5	0.08±0.01^{a}	0.03±0.00^{ab}	0.04±0.00^{b}	0.05±0.00^{b}
16:2n-6	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00
16:2n-4	0.14±0.03	0.04±0.01	0.04±0.01	0.03±0.01
17:0	0.12±0.02	0.05±0.00	0.05±0.00	0.05±0.00
16:3n-4	0.13±0.01^{a}	0.11±0.00^{b}	0.11±0.00^{ab}	0.12±0.00^{ab}
16:3n-3	0.09±0.01	0.07±0.01	0.07±0.01	0.07±0.00
16:3n-1	0.42±0.05^{b}	0.45±0.01^{b}	0.40±0.05^{b}	0.70±0.07^{a}
16:4n-3	0.45±0.07	0.52±0.07	0.48±0.06	0.53±0.04
16:4n-1	0.07±0.01	0.07±0.01	0.06±0.01	0.06±0.03
18:0	5.34±0.22^{b}	5.71±0.32^{b}	5.28±0.43^{b}	7.37±0.36^{a}
18:1n-9	28.12±1.96	29.67±0.81	27.98±1.34	29.50±0.78
18:1n-7	2.99±0.05^{a}	2.73±0.05^{b}	2.46±0.05^{c}	2.80±0.06^{ab}
18:1n-5	0.08±0.00^{ab}	0.07±0.00^{c}	0.08±0.00^{bc}	0.09±0.00^{a}
18:2n-9	0.05±0.00^{a}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±0.00^{b}
18:2n-6	16.56±0.76^{c}	18.90±0.25^{b}	17.88±0.51^{bc}	21.37±0.32^{a}
18:2n-4	0.13±0.01	0.05±0.00	0.06±0.02	0.05±0.00
18:3n-6	0.11±0.01	0.08±0.00	0.12±0.01	0.10±0.01
18:3n-4	0.11±0.00	0.06±0.00	0.06±0.00	0.07±0.00
18:3n-3	2.92±0.32	2.70±0.26	2.45±0.16	2.05±0.15
18:3n-1	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00
18:4n-3	0.34±0.05^{a}	0.15±0.02^{ab}	0.20±0.03^{ab}	0.12±0.01^{b}
18:4n-1	0.06±0.00	0.03±0.00	0.03±0.00	0.03±0.00
20:0	0.41±0.03^{ab}	0.40±0.01^{a}	0.34±0.01^{b}	0.34±0.01^{b}
20:1n-9	0.16±0.01	0.14±0.01	0.15±0.01	0.16±0.01
20:1n-7	2.05±0.08	1.85±0.02	1.85±0.02	1.80±0.06
20:1n-5	0.13±0.01	0.07±0.01	0.06±0.01	0.08±0.00
20:2n-9	0.04±0.01^{a}	0.03±0.00^{ab}	0.02±0.00^{b}	0.03±0.00^{ab}
20:2n-6	0.37±0.04	0.38±0.02	0.36±0.02	0.47±0.02
20:3n-9	0.02±0.00^{a}	0.01±0.00^{b}	0.00±0.00^{b}	0.01±0.00^{b}
20:3n-6	0.10±0.01^{ab}	0.09±0.01^{b}	0.14±0.02^{a}	0.12±0.00^{ab}
20:4n-6	1.04±0.16	0.97±0.08	1.05±0.09	1.07±0.07
20:3n-3	0.13±0.01	0.13±0.01	0.12±0.02	0.14±0.00
20:4n-3	0.27±0.00^{a}	0.15±0.01^{c}	0.19±0.01^{b}	0.12±0.00^{c}
20:5n-3	4.98±0.40^{a}	3.08±0.11^{ab}	2.16±0.09^{b}	2.40±0.10^{b}
22:1n-11	1.05±0.04	0.86±0.05	1.02±0.07	1.02±0.07
22:1n-9	0.56±0.05	0.51±0.03	0.54±0.04	0.63±0.03
22:4n-6	0.13±0.02^{ab}	0.11±0.01^{ab}	0.11±0.00^{b}	0.14±0.00^{a}
22:5n-6	0.68±0.15^{b}	0.63±0.06^{b}	3.55±0.50^{a}	0.46±0.04^{b}
22:5n-3	1.56±0.27^{a}	0.88±0.03^{ab}	0.60±0.03^{b}	0.75±0.02^{b}
22:6n-3	12.20±2.53	12.89±0.74	13.83±1.20	10.13±0.48
EPA+DHA	17.18±2.89	15.97±0.78	16.00±1.29	12.53±0.58
EPA/ARA	4.87±0.35^{a}	3.24±0.34^{ab}	2.08±0.08^{bc}	2.25±0.05^{c}
EPA/DHA	0.43±0.05^{a}	0.24±0.02^{ab}	0.16±0.01^{b}	0.24±0.00^{b}
SFA	19.64±0.60	20.19±0.62	20.19±0.17	21.22±0.47
MUFA	37.26±2.11	37.21±0.93	35.68±1.55	37.59±1.00
n-9	28.93±1.91	30.37±0.80	28.73±1.36	30.35±0.82
n-6	18.99±0.42^{c}	21.16±0.10^{b}	23.21±0.18^{a}	23.74±0.30^{a}
n-6 LC-PUFA	2.31±0.37	2.17±0.17	5.21±0.63	2.26±0.12

Table 5.7. Fillet fatty acid composition (% total identified fatty acids) of meagre juveniles fed the experimental diets for 30 days (continued)

	Diets			
Fatty acid	FO	ED	DD	PO
n-3	22.93±2.87^{ab}	20.56±0.63^{a}	20.10±1.23^{ab}	16.32±0.47^{b}
n-3 LC-PUFA	19.13±3.18	17.12±0.78	16.91±1.33	13.54±0.59
n-6/n-3	0.86±0.11^{b}	1.03±0.03^{b}	1.16±0.06^{ab}	1.46±0.05^{a}
H	13.55±0.69	13.87±0.45	14.36±0.26	13.32±0.33
H	85.71±0.70	85.52±0.45	85.09±0.27	86.15±0.33
h/H	6.36±0.39	6.18±0.22	5.93±0.13	6.48±0.19
AI	0.21±0.02	0.19±0.01	0.21±0.01	0.19±0.01
TI	0.17±0.01^{b}	0.18±0.01^{b}	0.19±0.00^{b}	0.21±0.00^{a}
PI	176.50±24.47	166.30±6.44	183.17±12.95	140.68±4.90

Superscripts with lowercase letters indicate significant differences with P <0.05. *Denotes a tendency (P <0.09)

Table 5.8. Liver fatty acid composition (% total identified fatty acids) of meagre juveniles fed the experimental diets for 30 days

	Diets			
Fatty acid	FO	ED	DD	PO
14:0	0.88±0.09^{a}	0.49±0.05^{b}	0.76±0.02^{a}	0.36±0.03^{b}
14:1n-7	0.01±0.00	0.01±0.00	0.01±0.00	0.00±0.00
14:1n-5	0.04±0.01^{a}	0.01±0.00^{ab}	0.02±0.00^{b}	0.02±0.00^{b}
15:0	0.18±0.00^{a}	0.15±0.02^{ab}	0.15±0.00^{b}	0.10±0.01^{b}
15:1n-5	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00
16:0ISO	0.04±0.01	0.02±0.00	0.02±0.00	0.02±0.00
16:0	13.49±0.22^{a}	13.78±0.98^{a}	13.67±0.23^{a}	10.05±0.54b
16:1n-7	3.35±0.25^{a}	2.39±0.09^{ab}	2.60±0.14^{b}	2.75±0.07^{b}
16:1n-5	0.10±0.01	0.04±0.00	0.05±0.00	0.05±0.01
16:2n-6	0.01±0.00	0.00±0.00	0.01±0.00	0.00±0.00
16:2n-4	0.13±0.03^{a}	0.03±0.00^{ab}	0.04±0.00^{ab}	0.02±0.00^{b}
17:0	0.11±0.01^{a}	0.07±0.00^{b}	0.07±0.00^{b}	0.05±0.00^{b}
16:3n-4	0.22±0.01^{a}	0.17±0.00^{b}	0.16±0.01^{b}	0.20±0.01^{a}
16:3n-3	0.06±0.01	0.03±0.00	0.03±0.00	0.04±0.00
16:3n-1	0.06±0.03	0.16±0.12	0.05±0.01	0.03±0.00
16:4n-3	0.14±0.06	0.18±0.07	0.18±0.03	0.05±0.01
16:4n-1	0.02±0.01	0.02±0.01	0.02±0.00	0.01±0.00
18:0	7.36±0.81	6.29±0.40	5.47±0.26	7.61±0.35
18:1n-9	37.54±0.78^{bc}	39.05±0.94^{ab}	34.23±0.88^{c}	42.06±0.28^{a}
18:1n-7	3.05±0.08^{ab}	2.62±0.03^{b}	2.31±0.01^{c}	2.86±0.03^{a}
18:1n-5	0.11±0.01^{ab}	0.08±0.00^{b}	0.09±0.01^{ab}	0.12±0.00^{a}
18:2n-9	0.13±0.02	0.09±0.03	0.08±0.02	0.08±0.01
18:2n-6	16.43±0.81^{c}	19.19±0.36^{ab}	18.28±0.47^{bc}	21.55±0.45^{a}
18:2n-4	0.13±0.02	0.04±0.00	0.04±0.00	0.04±0.00
18:3n-6	0.23±0.02	0.18±0.04	0.22±0.02	0.18±0.02
18:3n-4	0.12±0.02^{a}	0.05±0.01^{b}	0.07±0.00^{ab}	0.07±0.01^{b}
18:3n-3	2.68±0.07	2.63±0.22	2.36±0.05	2.35±0.14
18:3n-1	0.01±0.00	0.01±0.00	0.01±0.01	0.00±0.00
18:4n-3	0.25±0.04	0.11±0.01	0.15±0.02	0.08±0.01
18:4n-1	0.04±0.01	0.01±0.00	0.03±0.02	0.01±0.00
20:0	0.27±0.02^{ab}	0.24±0.00^{a}	0.20±0.01^{b}	0.21±0.00b
20:1n-9	0.19±0.02^{ab}	0.16±0.01^{b}	0.17±0.01^{ab}	0.23±0.01^{a}
20:1n-7	2.53±0.10	2.36±0.04	2.35±0.10	2.60±0.15
20:1n-5	0.14±0.01	0.09±0.00	0.08±0.00	0.10±0.01
20:2n-9	0.06±0.01	0.04±0.01	0.04±0.01	0.04±0.00
20:2n-6	0.49±0.02^{c}	0.54±0.01^{bc}	0.60±0.02^{b}	0.69±0.02^{a}
20:3n-9	0.01±0.00	0.00±0.00	0.00±0.00	0.01±0.00

Table 5.8. Liver fatty acid composition (% total identified fatty acids) of meagre juveniles fed the experimental diets for 30 days (continued)

	Diets			
Fatty acid	FO	ED	DD	PO
20:3n-6	0.10±0.00^{b}	0.09±0.01^{b}	0.16±0.01^{a}	0.12±0.00^{b}
20:4n-6	0.66±0.17^{ab}	0.65±0.07^{ab}	1.02±0.14^{a}	0.42±0.02^{b}
20:3n-3	0.11±0.00	0.11±0.01	0.14±0.01	0.12±0.01
20:4n-3	0.18±0.03	0.10±0.02	0.17±0.02	0.10±0.02
20:5n-3	1.72±0.03^{a}	1.08±0.13^{b}	0.80±0.08^{bc}	0.57±0.08^{c}
22:1n-11	0.77±0.06^{ab}	0.65±0.04^{b}	0.69±0.01^{b}	0.91±0.04^{a}
22:1n-9	0.86±0.07	0.77±0.08	0.74±0.03	0.85±0.05
22:4n-6	0.10±0.01	0.09±0.01	0.11±0.00	0.10±0.01
22:5n-6	0.24±0.06^{b}	0.24±0.03^{b}	2.66±0.17^{a}	0.07±0.01^{c}
22:5n-3	0.63±0.04^{a}	0.43±0.03^{b}	0.35±0.02^{b}	0.27±0.05^{b}
22:6n-3	4.01±1.31^{ab}	4.43±0.21^{a}	8.55±0.77^{a}	1.79±0.28^{b}
EPA+DHA	5.73±1.31^{ab}	5.51±0.32^{ab}	9.36±0.82^{a}	2.36±0.36^{b}
EPA/ARA	2.93±0.61	1.73±0.32	0.81±0.11	1.36±0.19
EPA/DHA	0.52±0.13^{a}	0.24±0.02^{ab}	0.09±0.01^{b}	0.32±0.02^{ab}
SFA	22.34±0.93^{a}	21.04±1.10^{ab}	20.33±0.10^{a}	18.41±0.55^{b}
MUFA	48.70±1.32^{a}	48.24±0.92^{a}	43.33±1.13^{b}	52.58±0.45^{a}
n-9	38.78±0.83^{bc}	40.11±0.91^{ab}	35.27±0.93^{c}	43.27±0.30^{a}
n-6	18.24±1.02^{b}	20.98±0.32^{ab}	23.05±0.55^{a}	23.13±0.46^{a}
n-6 LC-PUFA	1.58±0.24	1.61±0.11	4.54±0.29	1.40±0.05
n-3	9.78±1.23^{a}	9.10±0.49^{ab}	12.74±0.86^{a}	5.37±0.57^{b}
n-3 LC-PUFA	6.66±1.29^{ab}	6.16±0.36^{ab}	10.02±0.84^{a}	2.86±0.43^{b}
n-6/n-3	1.90±0.13^{b}	2.32±0.15^{b}	1.83±0.13^{b}	4.39±0.41^{a}

Superscripts with lowercase letters indicate significant differences with P <0.05. *Denotes a tendency (P <0.09)

5.4.3 GENE EXPRESSION

Meagre fed ED diet presented higher (P<0.05) hepatic *gpx* mRNA levels than those fed PO (Figure 5.1). In contrast, PO diet induced a hepatic upregulation of *fads2* compared to FO or DD (P<0.1; Figure 5.1). Indeed, *gpx* and *fads2* expressions were two of the most explaining variables in the PCA analysis, contributing for separating fish fed particularly these two treatments from each other (Figure 5.2). Furthermore, *gpx* was positively correlated with hepatic EPA contents, whereas *fads2* expression was negatively correlated with dietary n-3 LC-PUFA and positively correlated with diet 18:1n-9, diet and hepatic 18:2n-6 contents and diet n-6/n-3 ratio (P<0.05; Pearson's coefficient). Expression of *sod* and *elovl5* were not affected by the different treatments, (Figure 5.1). Similarly, no significant differences were detected in the expression of *hsp90* and *hsp70* among fish fed the different diets, but fish fed PO showed slightly lower values of expression of both genes compared to those fed the other diets (Figure 5.1). Furthermore, *hsp90* was positively correlated with dietary EPA/ARA contents (P<0.05; Pearson's coefficient), whereas *hsp70* was positively correlated with hepatic ARA (P<0.05; Pearson's coefficient).

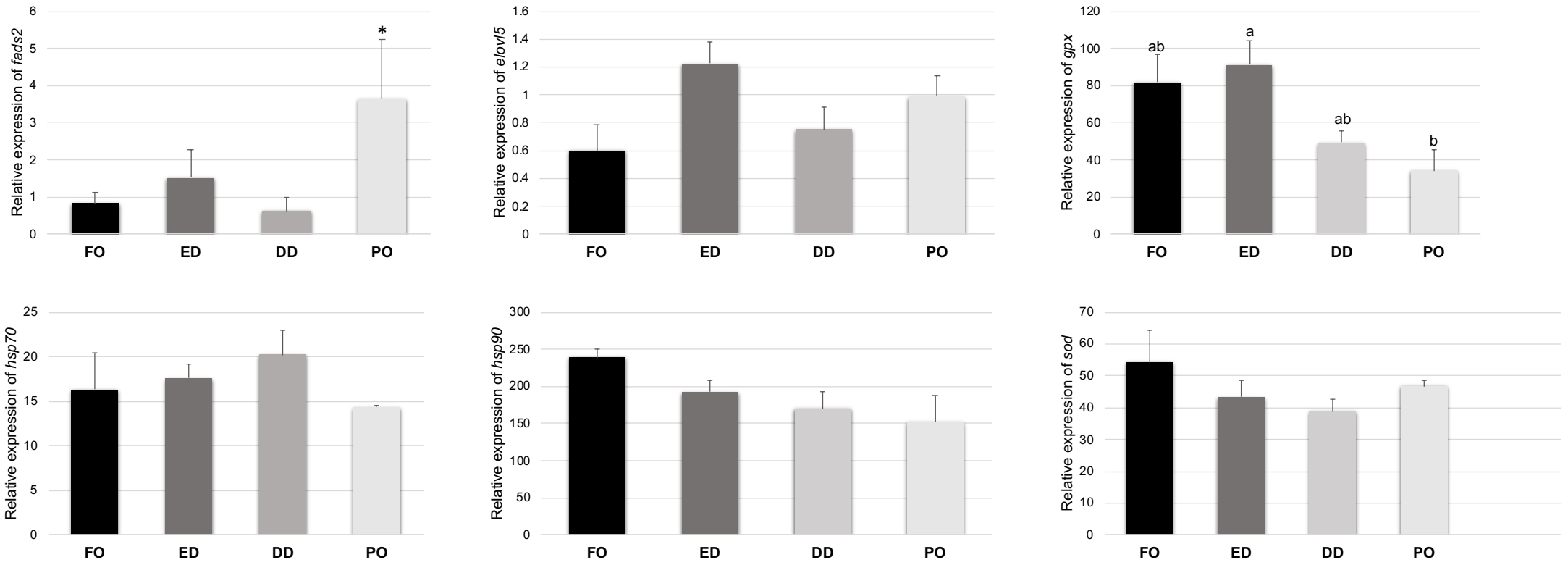

Figure 5.1. Relative-expression ($2^{-\Delta\Delta ct}$) of health-related genes in livers of meagre juveniles fed the experimental diets for 30 days; * indicates a difference with 0.05 < P < 0.10. Lower case subindices indicate P<0.05; (*fads2, fatty acyl desaturase 2; elovl5 fatty acyl elongase 5; hsp70/hsp90, heat shock proteins 70 and 90; gpx, glutathione peroxidase; sod, superoxide dismutase).*

(a)

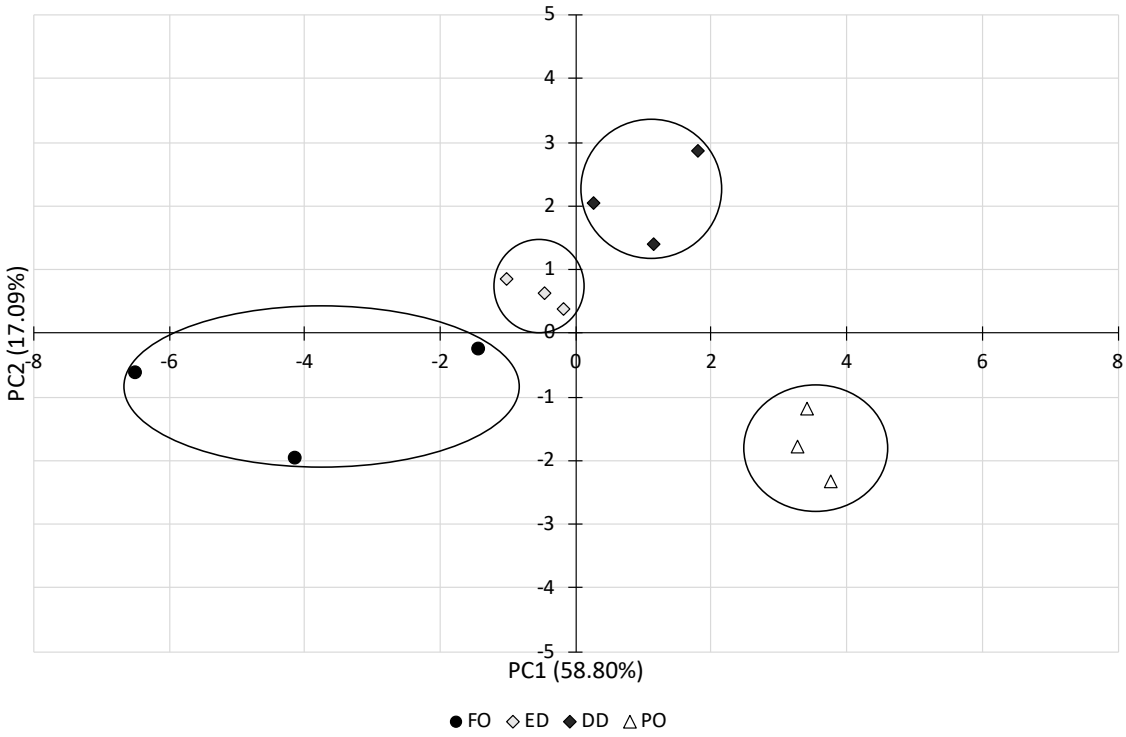

Figure 5.2a. Principal component analysis (PCA) of overall performance, gene expression and tissue composition of meagre juveniles fed the experimental diets for 30 days: plot of the scores of the cases (three replicates of the four treatments: FO, DD, ED and PO).

(b)

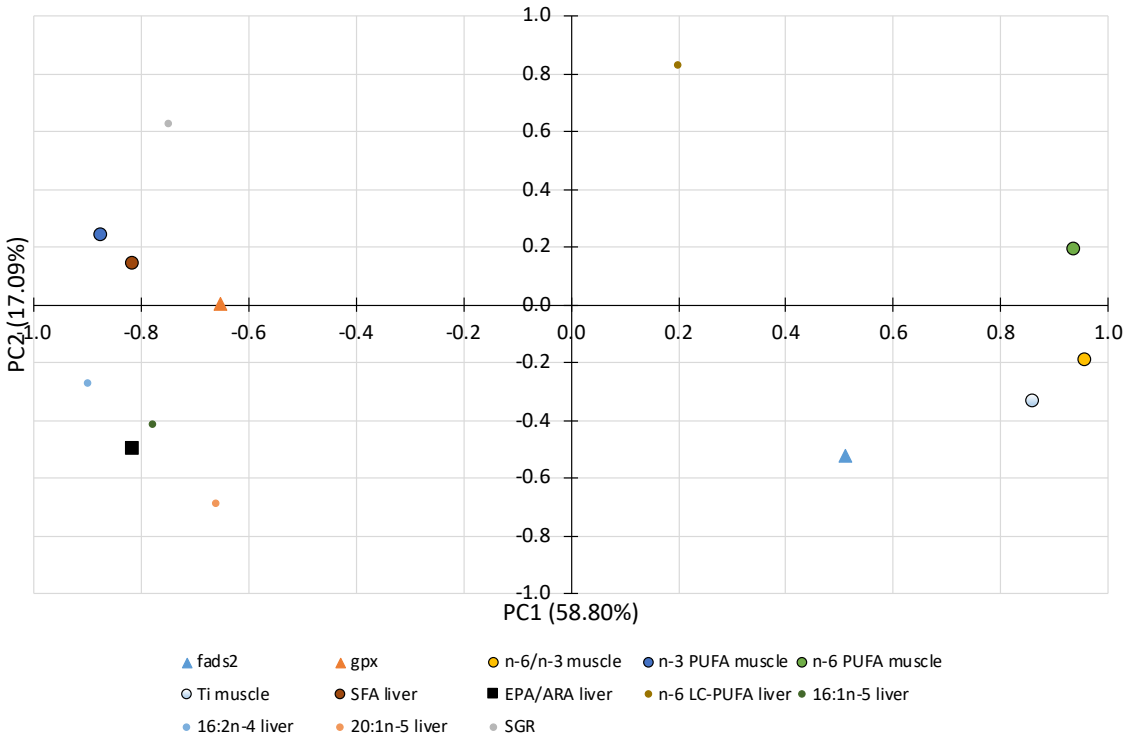

Figure 5.2b. Principal component analysis (PCA) of overall performance, gene expression and tissue composition of meagre juveniles fed the experimental diets for 30 days: projection of the variables in the principal components 1 and 2 (factor loadings).

5.5 Discussion

Aquaculture diversification is an effective strategy to meet industry and market demands by both increasing the number of farmed species and the ingredients used in aquafeeds. In this context, on one hand, meagre is one of the most promising species to diversify aquaculture products (Soares et al., 2018). On the other hand, microalgae oil is one of the top potential ingredients to totally replace dietary FO and achieve aquaculture sustainability, maximize fish production, and guarantee a safe and good nutritional quality product for the consumer (Pratiwy and Pratiwy, 2020; Colombo and Turchini, 2021). In the present study, the combinations of two commercial microalgal oils (Veramaris algal oil and DHA Natur oil) with other conventional lipid sources used in modern fish diets (rapeseed oil and poultry oil) were effective in supporting the growth of meagre juveniles, feed utilization and nutrient retention, as much as meagre fed a traditional FO diet. These results indicate that the microalgae oils supplemented are capable of meeting meagre relatively high n-3 LC-PUFA requirements (Carvalho et al., 2018), as recently demonstrated for gilthead sea bream (*Sparus aurata*) (Carvalho et al., 2020). Being a new species for aquaculture, meagre n-3 LC-PUFA requirement for maximum growth performance was recently estimated to be around 2.1% in dry weight of the diet (Carvalho et al., 2018), a value that was indeed covered by diets containing FO, Veramaris algal oil and DHA Natur oil as lipid sources. However, despite both microalgal oils had a high DHA content, the largest difference between them was their EPA content, which was more than double in the diet containing Veramaris algal oil than in that with DHA Natur oil. The dietary EPA could, at least, partially explain the best lipid retention efficiency in fish fed the first diet compared with those fed the latter, similar to what was suggested in sea bream fed different microalgal diets (Carvalho et al., 2020). In contrast, when FO was totally replaced by PO as the single oil in diet and without further n-3 LC-PUFA supplementation, the growth, feed utilization and nutrient retention efficiency (lipid, energy, and protein) of fish were negatively affected. These results denote that the residual amount of n-3 LC-PUFA coming from the 15% FM in the diets, was insufficient to cover meagre EFA requirements and support a balanced performance. Although the present study did not include an economic analysis of the different dietary formulations used here, it is expected that the blend of these microalgal oils with lower-cost sources as poultry and rapeseed oils could be a reasonable cost-effective tool for total FO replacement in meagre diets. Most importantly, those replacement mixes are a good strategy for increasing the n-3 LC-PUFA contents in aquaculture products under an "ocean-friendly" approach (low FM and FO-free diets).

Indeed, dietary fatty acid composition of aquaculture feeds is usually reflected in fish cells and body tissues. In the present study, liver fatty acid profile revealed more significant

differences among fish fed the different treatments, whereas in muscle or whole-body, these differences were smaller although followed the same tendency. This confirms also in meagre that liver is a highly sensitive tissue to differences in dietary composition and highlights the high activity metabolism of fatty acids in liver (Bradbury, 2006). Furthermore, fillet n-3 LC-PUFA deposition is of pivotal importance for meeting consumers needs and expectations. In the present study, meagre fed the microalgae diets showed the highest DHA content in body tissues, including muscle, in line with the dietary composition of the diets. However, microalgal oils were not able to deposit EPA contents as FO due to the lower dietary EPA contents. Indeed, the replacement of FO by these microalgae oils reduced by a 38 and 57% EPA in muscle in ED and DD diets, respectively, compared to meagre fed FO diet. However, muscle from meagre fed the former diet presented higher EPA than those from fish fed the latter, even considering that Veramaris algal oil was included at lower percentage in the diet than DHA Natur oil. Increasing the inclusion of Veramaris algal oil in the diet would probably significantly rise the EPA levels in fish body since this oil has approximately 21% of EPA of the total FA content, compared to 16% of FO. Additionally, the similar lipid quality indices, including AI and TI, found among fish fed the control FO diet and those fed the microalgae diets, denote the high nutritional value for the consumer of fillets from meagre fed these oil combinations.

In contrast, the PO diet increased TI of meagre fillets, which is in line with the higher 18:0 and a slightly higher SFA and MUFA contents of fish fillets fed this diet, which are prothrombogenic fatty acids. In contrast, it also agrees with the reduced contents in those fillets of EPA and DHA, known to have antithrombogenic properties (Ulbricht and Southgate, 1991). These results, along with the highest n-6/n-3 found in these fillets, denote that complete replacement of FO by PO negatively affect the nutritional quality of fish products to the consumer, related with a higher risk of lipid deposition on blood vessels (thrombogenicity), in agreement with other studies in fish fed this animal fat (Turchini et al., 2003; Campos et al., 2019; Carvalho et al., 2020). Indeed, livers of fish fed PO showed significant higher lipid content than those fed the other experimental diets, suggesting a deficient lipid mobilization from hepatocytes. This condition is often a symptom of n-3 LC-PUFA deficiency (Verreth et al., 1994), since these FA are known to strongly participate in the formation of lipoproteins and, therefore, in the exportation of lipids from outside the liver (Caballero et al., 2004; Kjaer et al., 2008). In agreement, in a previous study with meagre, levels of n-3 LC-PUFA below the requirement (2.0%) were associated to an excessive hepatic lipid accumulation, jeopardizing liver normal function, and increasing hepatic inflammation (Carvalho et al., 2018). Further morphological analysis are being conducted to evaluate the health condition of the hepatic tissue in meagre fed this diet. Indeed, PO and other low n-3 LC-PUFA sources, for instance

vegetable oils, caused a severe hepatic steatosis by increasing lipogenic activity in livers of sea bream juveniles (Houston et al., 2017; Carvalho et al., 2021).

Additionally, meagre fed DD diet also presented the highest n-6 DPA in body tissues, including muscle, due to the highest dietary content of this FA in *Schizochytrium sp*., which is found at 20% of the level of DHA (Sprague et al., 2017). This agrees with other studies in fish that used this microalgal species (Kousoulaki et al., 2020; Carvalho et al., 2020). N-6 DPA competes with DHA for the incorporation in brain phospholipids membranes (García-Calatayud et al., 2005). Therefore, this FA has been associated with neural and behaviour alterations in mammals, particularly at early stages of life (García-Calatayud et al., 2005). However, studies conclude that a higher intake though fish consumption with higher contents of this FA, for instance, does not impact DHA accumulation in adult human brain and, thus, does not constitute a problem for human health (Nauroth et al. 2010; Sprague at al., 2017).

Although the tissue contents of most of FA reflected the diet, some desaturases products through 18:1n-9 or 18:2n-6 were increased in fish fed PO, contrary to their low levels in the diet. These results, together with the up-regulation of *fads2* expression in livers of these fish, suggest an activation of the desaturation processes in a response to a deficient n-3 LC-PUFA diet. This agrees well with the high availability of n-9 (18:1n-9) and n-6 (18:2n-6) substrates. This metabolic compensatory response to the decrease in n-3 LC-PUFA was reported previously in meagre (Carvalho et al., 2018) and several other marine species for who's these FA are essential (Kim and Lee, 2004; Houston et al., 2017; Carvalho et al., 2020). Indeed, FADS2 from meagre was characterized by having a typical Δ6 and Δ8 functional activity (Monroig et al., 2013). While Δ6 pathway typically involves the initial desaturation of 18:3n-3 or 18:2n-6 to 18:4n−3 and 18:3n−6, respectively, the Δ8 pathway comprises first the elongation of 18:3n-3 or 18:2n-6 to 20:3n-3 or 20:2n-6, respectively, which are further desaturated to 20:4n-3 and 20:3n-6. Furthermore, whereas an apparent desaturation towards 18:1n-9 and 18:2n-6 was observed, the same tendency was not observed through n-3 substrate (18:3n-3), which was presented at much lower dietary quantity than 18:1n-9 or 18:2n-6. These results suggest that, despite the higher affinity of Δ6 desaturase for n-3 FA, the availability of the substrate seem to have a larger influence on the desaturation products produced (Sissener et al., 2017). Indeed, in the present study, *fads2* expression was positively correlated with 18:1n-9 and 18:2n-6. In addition, different studies reported that, under n-3 LC-PUFA deficiency, *elovl5* can be either (Carvalho et al., 2018) or not (Houston et al., 2017; Carvalho et al., 2020) up-regulated. In fact, despite the lack of statistical differences, meagre fed PO showed higher absolute values of expression of *elovl5* if we compared to those fed FO, which is also in concordance with the apparent increase of the elongation products in

body tissue of these fish. Indeed, meagre ELOVL5 has a functional activity towards C18 and C20 FA while a lower activity towards C22 FA, similar to other fish species, but with the particularity of the action towards C16 as well (Monroig et al., 2013). Therefore, meagre was the first marine fish species found able to convert 16:3n-3 to 18:3n-3 at a higher rate than other marine species (Monroig et al., 2013). This is in line with the highest *elovl5* expression found in meagre fed the microalgae diets, with the highest dietary 16:3n-3. Consequently, meagre fed these microalgae diets, characterized by the lowest 18:3n-3 content, showed similar values of this FA in fish body, probably due to the elongation of 16:3n-3. This highlights the added value of microalgal products rich in 16:3n-3 as alternative sources for aquafeeds to increase also 18:3n-3 contents in fish tissues.

In addition, the highest dietary PUFA and, consequently, the increase in the risk of peroxidation and oxidative stress could be the reason behind the higher basal expression of *gpx* in meagre fed ED diet compared with those fed PO. This was indeed correlated with the hepatic EPA contents of fish. This enzyme is responsible for protecting biosystems from oxidative damage due to reactive oxygen species (Kutluyer et al., 2017). Similarly, although no significant differences were detected among fish fed the different treatments in mRNA levels of SOD, another antioxidant enzyme, *sod* expression was also correlated with dietary highly unsaturated fatty acids, such as the n-6 LC-PUFA 22:5n-6, and the DHA. In agreement, previous studies in fish have also reported a higher basal activity of different antioxidant enzymes in response to high PUFA diets (Mourente et al., 2002; Rueda-Jasso et al., 2004). Furthermore, a previous study with meagre also reported a higher basal *cat* expression, another gene encoding an antioxidant enzyme, which supposed a more controlled stress response to handling (Carvalho et al., 2019). Therefore, this higher basal expression of *gpx* in meagre fed ED diets might constitute a compensatory condition for the higher pro-oxidant environment. Furthermore, despite the lack of significant effects of the dietary treatment in the expression of HSP in the present study, the expressions of *hsp90* and *hsp70* were positively correlated with dietary EPA and EPA/ARA and hepatic ARA, respectively. These results suggest a potential effect of EPA and ARA on HSP metabolism, as described in mammals and fish (Jurivich et al., 1994; Montero et al., 2015). HSP's are conserved proteins expressed in all living organisms under normal conditions, and function as chaperons for maintaining homeostasis and are involved in the response to several stressors to avoid and repair cellular damage (Iwama et al., 1998). In agreement with the present results, an increasing tendency was observed also between *hsp70* expression and dietary ARA in European sea bass (*Dicentrarchus labrax*) larvae, with the highest basal expression found in fish fed the highest level (1.2% dry weight), suggesting a better preparation of the tissues for dealing with stressful situations (Montero et al., 2015). Furthermore, Senegalese sole (*Solea senegalensis*) fed

vegetable oils low in ARA, presented a reduced expression of *hsp90* and *hsp70* (Benitez-Dorta et al., 2013), like meagre fed PO of the present study. Therefore, the slightly higher basal expression *of hsp* in meagre fed with higher dietary ARA and/or EPA diets might be translated into an optimized and adapted health condition of fish compared to lower dietary ARA and EPA levels (as for instance in PO diet), improving fish welfare. These basal higher expressions of antioxidant defence system and heat shock protein in fish fed higher LC-PUFA diets might be particularly important when dealing with stressful events and can favoured the animal by avoiding great alterations in tissue biochemistry and, consequently, cell damage. Therefore, studying the response of these genes under stress in fish fed microalgal diets would be of interest in the future.

5.5 CONCLUSIONS

The combination of two commercial microalgae oils (Veramaris algal oil and DHA Natur oil diets) with other conventional lipid sources used in modern fish diets (rapeseed oil and poultry oil) effectively supported growth, feed utilization, DHA tissue levels and high fillet lipid quality indices of meagre juveniles, in a similar way to fish fed a FO diet. Furthermore, meagre juveniles fed the ED diet were more able to convert 16:3n-3 to 18:3n-3 and showed the highest expression of *elovl5*. In addition, the ED diet led to an increased *gpx* expression, which might constitute a compensatory condition for a higher pro-oxidant environment and could protect the fish under stressful conditions to avoid oxidative damage. A slightly higher hepatic expression of *hsp's* was related with increasing dietary ARA levels. In contrast, PO was not able to support fish growth performance and n-3 LC-PUFA flesh contents, and increased thrombogenic index in fillet. Additionally, PO up-regulated hepatic *fads2* expression in response to n-3 LC-PUFA deficiency and downregulated *gpx* and *hsp*, which could suggest a less optimized health condition.

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CHAPTER 6. HEPATIC BIOCHEMICAL, MORPHOLOGICAL AND MOLECULAR EFFECTS OF FEEDING MICROALGAE AND POULTRY OILS TO GILTHEAD SEA BREAM (*SPARUS AURATA*)

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Keywords: Fatty acids; Lipid metabolism; Novel lipid sources

6.1 ABSTRACT

The present work investigated how the combination of poultry oil with microalgae oils, rich in eicosapentaenoic acid and docosahexaenoic acid (ED diets) or n-6 docosapentaenoic acid and n-3 docosahexaenoic acid (DD diets) modulates hepatic lipid metabolism in gilthead sea bream juveniles. Diets were tested using two different fish meal contents (15% and 7.5%) and compared against a fish oil-based diet (CTRL) and two negative control diets based on poultry oil as lipid source (PO diets). After 74 days of feeding, sea bream fed 15% FM ED or DD diets showed similar daily growth index to those fed CTRL, while those fed PO diets caused reduced growth. Fish livers reflected the highest contents in n-3 long-chain polyunsaturated fatty acids when fed CTRL, ED, or DD diets, which down-regulated *fas*, *scd-1a*, *fads2*, *lpl* and *cpt1*, reducing hepatic lipid accumulation and hepatocytes size. In contrast, fish fed PO diets showed the lowest deposition of n-3 long-chain polyunsaturated fatty acids and the highest oleic acid in liver, leading with the highest hepatosomatic index due to increased liver lipids. Therefore, these fish revealed a severe hepatic steatosis associated with an increased expression of lipogenesis-related genes, particularly *fas*, *lpl* and *sbrep1.* Furthermore, PO diets seemed to activate desaturation pathways in fish livers, reflected by the highest accumulation of fatty acids that are products from desaturases and the highest *fads2* and *scd-1a* expressions. The reduction of the dietary fish meal content to 7.5% lowered fish growth, although hepatic lipid metabolism seemed to be more affected by FO replacement than FM replacement. Combining microalgae with poultry oil could be an alternative lipid and essential fatty acid source to fish oil in marine fish diets.

6.2 INTRODUCTION

It is recognized worldwide that the replacement of fish-derived meals (FM) and oils (FO) in aquaculture feeds is mandatory for aquaculture sustainability, both from an economical and an environmental point of view. Therefore, in the past decades, finding alternative ingredients has justified extensive research, in which plant raw materials have been in the forefront and have been reviewed elsewhere (Turchini et al., 2009; Montero and Izquierdo, 2010; Nasopoulpou and Zabetakis, 2012). However, in relation to FO, its replacement by vegetable oils (VO) markedly reduces the dietary n-3 and n-6 long-chain polyunsaturated fatty acids (LC-PUFA), particularly docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6). Especially in marine fish, such as gilthead sea bream (*Sparus aurata*), these FA are considered essential (EFA) and play important structural and functional roles in fish metabolism. This essentiality results from the inefficient biosynthetic activities of desaturases and elongases in saltwater species (Izquierdo and Koven, 2011). Indeed, DHA, EPA and ARA are of pivotal physiological importance in vertebrates and their deficiency is associated with different health problems, from retarded development and growth to changes in lipid metabolism that cause nutritional diseases (Simopoulos, 2012; Spisni et al., 1998). For these reasons, the high inclusion of lipid sources devoid of n-3 LC-PUFA in fish diets often reduces growth, increases lipid accumulation, particularly in liver, and, in severe cases, may lead to fish death (Spisni et al., 1998; Caballero et al., 2004). Furthermore, liver plays a central role in physiology, regulating lipid synthesis and catabolism (reviewed in Bradbury, 2006). Therefore, lipid homeostasis requires maintaining the balance between lipogenesis and lipolysis, that is achieved by a range of transcription factors and genes, which in turn, can be regulated by dietary fatty acids (Morais et al., 2011; Houston et al., 2017).

To reduce the dependence on FM and FO and counterbalance the negative effects of some VO in farmed fish, novel cost-effective lipid sources are being obtained from microorganisms, genetically modified plants, or animal fats (Naylor et al., 2009; Adarme-Vega et al., 2014; Betancor et al., 2016a,b; Tocher et al., 2019; Campos et al., 2019). Microalgae are among the most promising lipid sources, mainly due to its high EPA and/or DHA content (Shah et al., 2018). However, their high production costs constrain their use in fish diets to very early life stages, when EFA requirements are higher (Eryalcyn et al., 2015). Successful replacement of FO was achieved with microalgae in salmonids at later stages of development (Santigosa, 2019; Kousoulaki et al., 2020). Other studies have successfully replaced FO, completely or partially by microalgae in different fish species, such as European sea bass (*Dicentrarchus labrax*) (Tibaldi et al., 2015; Haas et al., 2016), olive flounder (*Paralichthys olivaceus*) (Qiao et al., 2014) or Nile tilapia (*Oreochromis niloticus*) (Sarker et al., 2016).

However, in gilthead sea bream juveniles or older stages, the studies have focused more on the use of microalgae as additives or as FM replacers, than as FO substitutes (Cerezuela et al., 2012; Vizcaíno et al., 2014).

The consumer demand for n-3 LC-PUFA has resulted in an increased need of fish products. The growing quest to maintain the economic sustainability of aquaculture likely forces the use of microalgae products combined with other cheaper and more available sources such as poultry oil (PO). PO shows a competitive price and availability and has a high potential as energy source given its high saturated (SFA) and monounsaturated fatty acids (MUFA) contents but lacks n-3 LC-PUFA (Rosenlund et al. 2001; Higgs et al. 2006; Hatlen et al., 2015). It has been incorporated as supplemental lipid source or partial replacer of FO in diets for some aquaculture species (Higgs et al., 2006; Hatlen et al., 2015; Turchini et al., 2013; Liland et al., 2015; Salini et al., 2015; Campos et al., 2019). A recent study by our research group found similar growth performance and enhanced fillet DHA deposition in gilthead sea bream fed a blend of microalgae oils and PO when compared to those fed FO (Carvalho et al., 2020). However, how these two lipid sources or their combination modulate hepatic lipid metabolism pathways in fish has not yet been investigated. Therefore, the aim of the present study was to investigate the nutritional regulation effects of totally replacing FO by a blend of microalgae oils, either DHA and EPA or DPA-rich, with PO on hepatic tissue of gilthead sea bream (*Sparus aurata*) juveniles. For that, biochemical, histological, and molecular responses were assessed.

The few studies conducted on the concomitant replacement of FM and FO reported an interaction between dietary protein and lipid sources on fish metabolism (Dias et al., 2005; 2009; Benedito-Palos et al., 2007; Torrecillas et al., 2017). Thus, we further investigated the possible role of the simultaneous replace of FM and FO on fish hepatic lipid metabolism, the former by increasing the dietary content of vegetable meals and the latter using the same microalgae and PO combinations as replacers of FO.

6.3 MATERIALS AND METHODS

6.3.1 DIETS

A total of 7 experimental diets were formulated with similar lipid and protein contents. A control diet (CTRL) was formulated to mimic practical diets used in gilthead sea bream culture, containing 15% of FM and 71.5% of a blend of vegetable meals, as well as 5% of FO and 7.92% of rapeseed oil. Three other experimental diets were based on CTRL diet but totally replaced FO by a combination of PO and one of two microalgae oils, either rich in EPA and DHA (15% FM ED) or DHA and n-6 docosapentaenoic acid (DPA) (15% FM DD) (Gren Oil, Veramaris, Netherlands and DHA Natur Oil, Archer Daniels Midland, USA, respectively). Three other experimental diets were formulated with the same lipid sources, in parallel with the replacement of 50% FM (7.5% FM ED, 7.5% FM DD or 7.5% FM PO), by increasing the vegetable meals contents. For each level of FM, a diet replacing FO by only PO was formulated to evaluate the effects of the PO as sole replacer of FO (15% FM PO and 7.5% FM PO). Feeds were manufactured by Skretting ARC Feed Technology Plant (Stavanger, Norway) at a pellet size of 1 mm and shipped to the ECOAQUA Institute laboratories (Canary Islands, Spain). Diets compositions are detailed in Table 6.1 and fatty acid profiles are presented in Table 6.2.

Table 6.1. Formulation and proximate composition of the experimental diets

	Diets						
		15% FM			7.5% FM		
Ingredients (%)	CTRL	ED	DD	PO	ED	DD	PO
Fish meal[a]	15.00	15.00	15.00	15.00	7.50	7.50	7.50
Wheat [a]	12.30	12.43	12.13	11.43	9.90	9.52	10.88
Corn gluten [a]	6.58	6.12	6.20	10.00	10.00	10.00	10.00
Hi-pro soya [a]	5.00	5.00	5.00	5.00	8.00	8.00	5.00
Wheat gluten [a]	17.71	18.02	17.92	15.38	18.25	17.00	17.72
Soya protein concentrate [a]	25.00	25.00	25.00	25.00	27.50	29.00	30.00
Faba beans [a]	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil [a]	5.28	0.00	0.00	0.00	0.00	0.00	0.00
Rapseed oil [a]	7.92	7.60	5.69	7.29	7.52	5.58	7.57
Veramaris algal oil[b]	0.00	2.46	0.00	0.00	2.76	0.00	0.00
DHA Natur oil [c]	0.00	0.00	3.67	0.00	0.00	4.11	0.00
Poultry oil [f]	0.00	3.16	3.92	5.70	3.38	4.09	6.12
Vitamin premix[d]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral premix[e]	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Microalgae:PO	-	1:1.3	1:1.1	-	1:1.2	1:1	-
Proximate analysis (%)							
Crude protein	49.31	48.35	48.06	50.18	49.31	49.14	50.38
Crude lipids	18.19	17.53	18.23	17.50	17.92	17.32	17.58
Neutral lipids	89.32	89.91	90.15	91.96	90.37	90.00	94.17
Polar lipids	10.68	10.09	9.85	8.04	9.63	10.00	5.83
Moisture	6.60	8.23	7.85	8.28	8.05	7.09	7.67
Ash	4.66	4.51	4.70	4.64	4.60	4.19	3.94
Starch (theorical value)	10.03	10.04	10.18	9.87	8.64	8.52	9.05
Fiber (theorical value)	2.53	2.53	2.53	2.54	2.78	2.85	2.81

[a] Skretting AS (Norway)

[b] Veramaris algal oil (Veramaris, The Netherlands)

[c] DHA Natur oil (ADM Animal Nutrition, USA)

[d, e] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC,

[f] Poultry oil: Sonac. B.V. The Netherlands

Table 6.2. Fatty acid composition of the experimental diets (% total identified FA)

	Diets						
		15% FM			7.5% FM		
Fatty acid	FO	ED	DD	PO	ED	DD	PO
14:0	2.05	0.73	1.33	0.72	0.39	1.30	0.82
14:1n-7	0.40	0.59	0.34	0.08	0.16	0.05	0.08
14:1n-5	0.37	0.52	0.35	0.03	0.19	0.06	0.03
15:0	0.45	0.41	0.35	0.10	0.16	0.15	0.08
15:1n-5	0.29	0.42	0.36	0.03	0.16	0.02	0.02
16:0ISO	0.37	0.49	0.40	0.03	0.19	0.07	0.03
16:0	9.72	8.80	10.51	11.59	9.41	12.09	13.08
16:1n-7	2.51	1.24	1.42	1.90	0.87	1.43	2.13
16:1n-5	0.31	0.64	0.38	0.06	0.15	0.02	0.03
16:2n-4	0.44	0.59	0.52	0.07	0.17	0.10	0.11
17:0	0.49	0.00	0.41	0.06	0.17	0.05	0.05
16:3n-4	0.28	0.68	0.35	0.11	0.24	0.09	0.10
16:3n-3	0.31	0.65	0.49	0.04	0.26	0.07	0.02
16:3n-1	0.28	0.42	0.46	0.05	0.32	0.05	0.01
16:4n-3	0.54	0.55	0.44	0.13	0.22	0.06	0.07
18:0	2.72	2.48	2.70	3.40	2.92	2.95	3.50
18:1n-9	30.01	28.16	29.26	39.78	33.58	34.87	40.78
18:1n-7	2.68	1.94	1.73	2.38	2.09	1.88	2.38
18:1n-5	0.37	0.47	0.47	0.07	0.15	0.08	0.05
18:2n-9	0.41	0.49	0.39	0.00	0.18	0.02	0.02
18:2n-6	15.74	15.52	16.36	23.16	19.44	19.69	24.58
18:2n-4	0.32	0.62	0.41	0.03	0.20	0.03	0.00
18:3n-6	0.00	0.94	0.52	0.08	0.19	0.15	0.11
18:3n-4	0.34	0.53	0.49	0.10	0.23	0.06	0.06
18:3n-3	5.02	4.30	3.61	5.29	4.63	4.04	5.27
18:3n-1	0.29	0.47	0.46	0.00	0.26	0.03	0.03
18:4n-3	0.94	0.57	0.53	0.39	0.29	0.23	0.20
18:4n-1	0.27	0.42	0.47	0.03	0.21	0.03	0.03
20:0	0.68	0.73	0.50	0.52	0.85	0.41	0.42
20:1n-9	0.37	0.41	0.43	0.18	0.21	0.14	0.11
20:1n-7	2.33	2.20	1.91	1.96	1.77	1.29	1.36
20:1n-5	0.47	0.47	0.41	0.17	0.22	0.10	0.07
20:2n-9	0.30	0.56	0.35	0.05	0.23	0.04	0.06
20:2n-6	0.32	1.10	0.56	0.27	0.32	0.13	0.16
20:3n-6	0.32	0.66	0.58	0.11	0.24	0.14	0.07
20:4n-6	0.59	0.80	0.48	0.32	0.50	0.40	0.21
20:3n-3	0.29	0.60	0.46	0.10	0.40	0.11	0.04
20:4n-3	0.44	0.72	0.64	0.11	0.38	0.22	0.07
20:5n-3	5.30	3.33	1.54	1.24	3.65	1.01	0.69
22:1n-11	2.37	2.26	1.56	1.94	1.45	1.02	0.93
22:1n-9	0.56	0.56	0.58	0.39	0.37	0.27	0.26
22:4n-6	0.36	1.08	1.32	0.15	0.25	0.11	0.06
22:5n-6	0.47	1.11	3.05	0.13	0.46	3.97	0.22
22:5n-3	1.31	1.04	1.09	0.31	0.68	0.31	0.13
22:6n-3	5.36	8.72	9.04	2.11	10.62	10.67	1.46
∑ SFA	16.11	13.15	15.80	16.39	13.90	16.96	17.95
∑ MUFA	43.02	39.89	39.20	48.96	41.35	41.21	48.23
∑ n-6	17.79	21.21	22.88	24.29	21.40	24.59	25.41
∑ n-6 LC-PUFA	2.06	4.75	5.99	0.98	1.77	4.75	0.72
∑ n-3	19.51	20.49	17.84	9.72	21.12	16.73	7.96
∑ n-3 LC-PUFA	12.70	14.41	12.77	3.87	15.73	12.32	2.39
EPA+DHA	10.66	12.05	10.58	3.35	14.27	11.68	2.15
EPA/ARA	8.91	4.18	3.22	3.88	7.35	2.51	3.28
DHA/EPA	1.0	2.6	5.9	1.7	2.9	10.6	2.1
n-6/n-3	0.91	1.04	1.28	2.50	1.01	1.47	3.19

6.3.2 FEEDING TRIAL AND FISH PERFORMANCE

Gilthead sea bream fingerlings (initial body weight of 2.50 ± 0.01 g, mean ± SE) were allocated, at a stocking density of 0.55 kg m^{-3}, in 21 fiberglass cylindrical tanks with 250 L volume supplied with filtered sea water, continuous air infusion and constant temperature (23.06 ± 0.33 º C) and dissolved oxygen (6.36 ± 0.18 mg L^{-1}). One experimental diet was randomly assigned to each tank. Triplicate tanks were fed each diet (n=3). Fish were fed to visual apparent satiety 3 times a day, 6 days per week, for 74 days. At the end of the experimental trial, all fish were individually weighed to calculate the Daily Growth Index (DGI= (Final body weight$^{1/3}$ – Initial body weight$^{1/3}$) / days * 100). Other details on growth performance, nutrient utilization and fillet fatty acid deposition of sea bream fed these diets containing microalgae and PO were previously described (Carvalho et al., 2020). The present study focused on the effect of these lipid sources on hepatic lipid metabolism, which in turn, could contribute to explain fish growth performance. For that purpose, fish were fasted for 24 h, and then 15 fish per tank were euthanized to collect liver samples for different analysis. Livers were weighed to calculate hepatosomatic index in each fish using the following formula: Hepatosomatic index (HSI, %) = (liver weight / body weight) * 100.

6.3.3 DIETS AND HEPATIC BIOCHEMICAL COMPOSITION

For diet samples, protein, ash, and moisture contents were additionally analysed according A.O.A.C. (2000). Liver samples from 5 animals per tank were collected and pooled. All samples were homogenized, and lipids from diet and liver tissue were extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957). Fatty acid methyl esters (FAMES) were obtained by transmethylation of lipids (Christie, 1989), which were then separated by gas liquid chromatography (Izquierdo et al., 1990), quantified by a flame ionization detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified comparing with previously characterized standards.

6.3.4 GENE EXPRESSION IN LIVER

6.3.4.1 RNA EXTRACTION, QUANTIFICATION AND COMPLEMENTARY DNA SYNTHESIS

A central portion of liver tissue from 5 fish per tank were collected in RNA later (Sigma-Aldrich, Madrid, Spain) and stored at -80º C until analysis. Total RNA was extracted from livers using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, samples were homogenized with the TissueLyzer-II (Qiagen) with TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA) and centrifuged with chloroform at 12000g for 15 min, at 4 °C. The RNA phase was mixed with 75% ethanol and transferred into a RNeasy spin column, using RW1 and RPE buffers (Qiagen) to purify RNA bonded to a membrane obtaining purified RNA which was then eluted with 25 µL of RNase-free water. The NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Gel Red™ staining (Biotium Inc., Hayward, CA) on 1.4% agarose electrophoresis gel were used to determine the quantity and integrity of RNA, respectively.

Complementary DNA (cDNA) was synthetized using iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions using an iCycler thermal cycler (Bio-Rad).

6.3.4.2 RT-QPCR ANALYSIS

Liver *mRNA* levels of lipid metabolism-related genes, including *fatty acyl desaturase 2* (*fads2*), *fatty acyl elongase 5* (*elovl5*), *stearoyl-CoA desaturase-1 a* (*scd-1a*), *lipoprotein lipase* (*lpl*), *fatty acid synthase* (*fas*), *sterol regulatory element-binding protein 1 and 2* (*srebp-1*, *srebp-2*), *carnitine palmitoyl transferase I* (*cpt-1*) and *peroxisome proliferator-activated receptor alpha* (*ppar*-α), were determined by RT-qPCR in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad). *Elongation factor 1 alpha* (*ef1*α) was used as a housekeeping gene. All primers sequences are described in Table 6.3. RT-PCR conditions used were the following: a first step of 3 min 30 s at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 60.0 °C, 30 s at 72 °C, 1 min at 95 °C, and a final denaturing step from 58 °C to 95 °C for 10 s. All PCR reactions were carried out in in a final volume of 15 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA (1:10 dilution) and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. Each run was ended with an analysis of melting curve leading to a melting peak specific for the amplified target DNA.

Table 6.3. Primers sequences used for RT-PCR analysis of lipid metabolism related-gene expression of gilthead sea bream hepatic tissue

Genes	Forward	Reverse	Gene Bank Access nº
*ef1*α	CATGGTTGTGGAGCCCTTCT	TCCTGCACGACCATTCATTTC	AF184170
fads2	GCAGGCGGAGAGCGACGGTCTGTTCC	AGCAGGATGTGACCCAGGTGGAGGCAGAAG	AY055749
elovl5	CCTCCTGGTGCTCTACAAT	GTGAGTGTCCTGGCAGTA	AY660879
scd-1a	GGAGGCGGAGGCGTTGGAGAAGAAG	AGGGAGACGGCGTACAGGGCACCTATATG	JQ277703
lpl	CGTTGCCAAGTTTGTGACCTG	AGGGTGTTCTGGTTGTCTGC	AY495672.2
fas	TGCCATTGCCATAGCACTCA	ACCTTTGCCCTTTGTGTGGA	JQ277708.1
srebp-1	AGGGCTGACCACAACGTCTCCTCTCC	GCTGTACGTGGGATGTGATGGTTTGGG	JQ277709
srebp-2	GCTCACAAGCAAAATGGCCT	CAAAACTGCTCCCTTCCCCA	AM970922.1
cpt-1	GTGCCTTCGTTCGTTCCATGATC	TGATGCTTATCTGCTGCCTGTTTG	JQ308822
ppar α	TCTCTTCAGCCCACCATCCC	ATCCCAGCGTGTCGTCTCC	AY590299

Ef1 α, *elongation factor 1 alpha; fads2, fatty acyl desaturase 2; elovl5, fatty acyl elongase; scd-1a, stearoyl-CoA desaturase-1 a; lpl, lipoprotein lipase; fas, fatty acid synthase; srebp1, 2, sterol regulatory element-binding protein 1 and 2; cpt-1, carnitine palmitoyl transferase I; ppar-α, peroxisome proliferator-activated receptor alpha*

6.3.5 Hepatic tissue histomorphology

For histological examination of hepatic tissue morphology, the livers of 5 fish per tank were collected, dehydrated in a graded ethanol series, and embedded in paraffin wax. Paraffin blocks were made and cut with a Leica microtome (Mod. Jung Autocut 2055; Leica, Nussloch, Germany) in 4 µm sections, which were placed in slides and stained with haematoxylin and eosin (H&E) (Martoja and Martoja-Pierson, 1970) to visualization under a light microscope (BX51TF, Olympus, Tokyo, Japan).

6.3.5.1 Semi-quantitative histological examination

All slides were firstly blinded evaluated by three different researchers for describing general histomorphology of the hepatic tissue. A semi-quantitative score was used to evaluate hepatocyte lipid vacuolization: score 0–1 was considered as normal morphology of liver tissue, with none or very small lipid vacuoles within the hepatocytes; score 1-2 was considered as moderate hepatic steatotic alterations associated to a moderate lipid infiltration; and score 2–3 as severe steatotic alterations referring to a high lipid infiltration and large vacuoles within the hepatocytes.

6.3.5.2 Histometric analysis

Micrographs from each slide were taken using a Nikon Microphot-FXA microscope (Nikon Instruments Inc., Melville, NY, USA) incorporated with an Olympus DP50 camera

(Olympus Optical Co. LTD, Shinjuku-ku, Tokyo, Japan), The total area of 25 hepatocytes per specimen (375 hepatocytes per experimental diet) were measured, as well as the maximum and minimum length of these hepatocytes considering the hepatocyte nucleus as reference point and using arbitrary units. All the measurements were done with Image Pro-Plus software for Windows.

6.3.6 STATISTICAL ANALYSIS

All data are presented as mean ± SE, and each tank was treated as a replicate (n=3 per treatment). Shapiro–Wilk and Levene's tests were used to test data normality and homogeneity of variances, respectively. A one-way ANOVA was applied to compare the experimental diets with the control diet. Additionally, a two-way ANOVA was used, excluding the control diet, to evaluate the effect of the oil source and fish meal independently in the novel diets and their potential interaction. Significant differences were detected when P-value was below 0.05 ($P<0.05$) and if applicable, means were compared with Tukey's as post-hoc test (Tukey, 1949), except for steatosis degree scores in which a Mann-Whitney U test was applied. Data were also subjected to the best fit regressions (linear, exponential, or logarithmic), which were also checked for significance at $P<0.05$. Statistical treatment of the data was carried out using SPSS 21.0 software for macOS 10.15.

6.3.7 ETHICAL APPROVAL

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). All procedures were approved by the Canary Island Council of Agriculture, Fishing and Water and the Bioethical Committee of the University of Las Palmas de Gran Canaria (reference OEBA-ULPGC-21/ 2018).

6.4 RESULTS

6.4.1 FEEDING TRIAL AND FISH PERFORMANCE

After 74 days of feeding the experimental diets, sea bream fed both 15% FM ED and DD diets showed a similar growth than those fed CTRL diet, as indicated by the comparable daily growth indexes (DGI) among them (Figure 6.1). However, fish fed 15 % FM PO diet showed lower DGI than those fed CTRL, ED, or DD diets ($P<0.05$). Similar tendency was observed when comparing diets with 7.5% FM among them ($P<0.05$; Table 6.4). However, a lowering effect in DGI occurred when FM was reduced to 7.5% FM ($P<0.05$; Table 6.4). Fish fed the PO diets showed the highest ($P<0.05$) HSI, whereas those fed ED or DD diets, at either 15 or 7.5% FM, presented similar HSI than those fed CTRL diet (Table 6.4). Similarly, the two-way ANOVA showed a significant effect of the lipid source on HSI ($P<0.05$; Table 6.4). However, neither the dietary FM content nor the interaction between both dietary variables had a significant effect on HSI (Table 6.4). In addition, HSI linearly decreased with the increase of dietary n-3 LC-PUFA in % DW ($r^2=0.98$ and $P=0.00$).

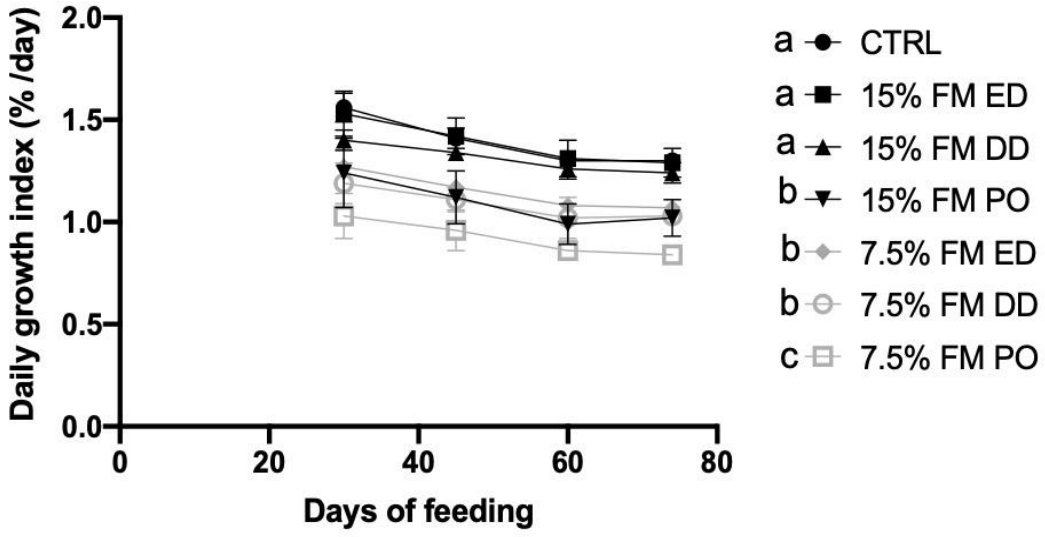

Figure 6.1. Daily growth indices of gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%), during the whole feeding period.

6.4.2 LIVER BIOCHEMICAL COMPOSITION

Lipid contents in liver were significantly higher in fish fed the PO diets than in those fed any of the other diets ($P<0.05$; Table 6.4). Thus, liver lipid contents linearly decreased with the increase of dietary n-3 LC-PUFA in % DW ($r^2=0.89$ $P=0.00$). Besides, HSI linearly increased with liver lipids ($r^2=0.88$ $P=0.01$).

Liver fatty acid composition showed a tendency to mirror the dietary profiles for most fatty acids, including oleic acid (OA, 18:1n-9), EPA, n-6, and n-3 DPA, DHA or the sum of major groups of FA, such as SFA, MUFA, n-3 LC-PUFA, n-9, n-6, or n-3 FA (Table 6.4). Briefly, sea bream fed PO diets showed the highest hepatic OA and MUFA contents ($P<0.05$), while sea bream fed CTRL, ED or DD diets showed the highest DHA and n-3 LC-PUFA contents ($P<0.05$). Fish fed CTRL or ED diets showed higher hepatic EPA and n-3 DPA contents than those fed DD or PO diets ($P<0.05$), while those fed DD diets showed the highest n-6 DPA ($P<0.05$). In contrast, some fatty acids in fish liver did not mirror the dietary pattern as expected (Table 6.4). This was the case for some products of different desaturases, for instance the higher values of 16:1n-5 and 18:1n-5, products of SCD-1a found in fish fed PO than in those fed ED or DD diets, when compared with the lower values in the respective diets ($P<0.05$). Accordingly, PO fish showed a lower 16:0/16:1 ratio than those fed DD or ED diets ($P<0.05$). The same tendency was noted on 18:2n-9, 20:2n-9, 18:3n-6, 20:3n-6, and, to a slighter extent on 18:4n-3, all potential products of FADS2. Besides, despite dietary linoleic acid (LA, 18:2n-6) levels were higher in PO diets, fish fed these diets did not show significant differences in LA in comparison to those fed ED or DD diets. The same was true for linolenic acid (LNA, 18:3n-3), which was the highest in 7.5% FM PO diet, but was the second lowest in livers of fish fed this diet. Furthermore, a lower 18:1n-9/18:2n-9 ratio was found in fish fed PO diets, mostly in those fed 7.5% FM PO ($P<0.05$), and a tendency to present lower 18:3n-3/18:4n-3 ratio in these fish as well. However, these tendencies were not so marked for elongation products, such as 20:2n-6 or 20:3n-3, where contents in fish liver reflect the dietary patterns (Table 6.4). Interestingly, sea bream fed DD diets presented the highest hepatic 20:4n-6 contents despite the low dietary levels ($P<0.05$; Table 6.4).

The dietary FM content significantly influenced some liver FA, increasing 18:3n-6, 20:4n-6 and 22:5n-6 in fish fed 7.5% FM diets, which resulted in an increasing of total n-6 and n-6 LC-PUFA in these fish ($P<0.05$; Table 6.4). On the contrary, it decreased the contents of 20:3n-6, 20:5n-3, 22:1n-11 and 22:5n-3 in sea bream fed 7.5% FM diets when compared to those fed 15% FM ($P<0.05$; Table 6.4). However, there was not a significant combined effect of the dietary FM content and the lipid source in liver fatty acid composition of fish (Table 6.4).

Table 6.4. Hepatosomatic index (%), hepatic lipid content (% ww) and liver fatty acid composition (% total fatty acids) of gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%)

Diets										
		15% FM			7.5% FM			Two-way ANOVA		
	CTRL	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
HSI (%)	1.47±0.09 ^{ab}	1.44±0.09^{ab}	1.48±0.08^{ab}	1.70±0.16^{a}	1.32±0.06^{b}	1.42±0.07^{ab}	1.77±0.08^{a}	ED^{b} DD^{b} PO^{a}	NS	NS
Liver lipids (% ww)	20.44±1.08 ^{b}	20.24±0.88^{b}	20.01±1.53^{b}	29.16±1.16^{a}	21.05±0.65^{b}	19.38±2.38^{b}	26.97±2.19^{a}	ED^{b} DD^{b} PO^{a}	NS	NS
Fatty acids (% total FA)										
14:0	0.91±0.16^{ab}	0.67±0.01^{a}	0.64±0.02^{a}	0.65±0.01^{a}	0.61±0.01^{ab}	0.63±0.03^{ab}	0.50±0.02^{b}	NS	P=0.04	NS
14:1n-7	0.01±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00	NS	NS	NS
14:1n-5	0.03±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	NS	NS	NS
15:0	0.11±0.01^{a}	0.09±0.00^{abc}	0.08±0.00^{bcd}	0.07±0.00^{cd}	0.10±0.00^{ab}	0.08±0.00^{bcd}	0.06±0.00^{d}	ED^{a} DD^{b} PO^{c}	NS	NS
15:1n-5	0.01±0.00	0.02±0.01	0.01±0.01	0.02±0.00	0.01±0.00	0.01±0.00	0.01±0.00	NS	NS	NS
16:0ISO	0.03±0.00^{a}	0.01±0.00^{ab}	0.01±0.01^{ab}	0.01±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{b}	0.01±0.00^{b}	NS	NS	NS
16:0	10.52±0.73	10.740.22±	9.95±0.24	9.58±0.13	10.60±	9.42±0.21	9.13±0.09	NS	NS	NS
16:1n-7	2.35±0.14^{a}	1.77±0.00^{ab}	1.71±0.04^{ab}	1.96±0.01^{b}	1.73±^{ab}	1.64±0.03^{b}	1.72±0.04^{ab}	ED^{ab} DD^{b} PO^{a}	P=0.00	P=0.03
16:1n-5	0.10±0.01^{a}	0.04±0.00^{b}	0.05±0.00^{b}	0.07±0.01^{b}	0.05±^{b}	0.05±0.01^{b}	0.07±0.00^{b}	NS	NS	NS
16:2n-4	0.13±0.00^{a}	0.04±0.00^{b}	0.03±0.00^{b}	0.03±0.00^{b}	0.03±^{b}	0.03±0.00^{b}	0.03±0.00^{b}	NS	NS	NS
17:0	0.16±0.02^{a}	0.06±0.00^{b}	0.05±0.00^{b}	0.07±0.01^{b}	0.05±^{b}	0.05±0.00^{b}	0.06±0.01^{b}	NS	NS	NS
16:3n-4	0.18±0.01^{a}	0.13±0.00^{bcd}	0.12±0.00^{cd}	0.14±0.00^{b}	0.13±^{bc}	0.11±0.00^{d}	0.12±0.00^{bcd}	ED^{a} DD^{b} PO^{a}	P=0.02	P=0.03
16:3n-3	0.05±0.01^{a}	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.01^{b}	0.02±^{b}	0.02±0.91^{b}	0.02±0.00^{b}	NS	NS	NS
16:4n-3	0.07±0.01	0.03±0.00	0.02±0.00	0.03±0.01	0.21±	0.02±0.00	0.02±0.00	NS	NS	NS
18:0	6.16±0.40	5.47±0.09	5.70±0.00	5.52±0.10	5.38±	5.33±0.22	5.99±0.28	NS	NS	NS
18:1n-9	39.38±1.47^{bc}	39.39±0.34^{bc}	38.62±0.00^{c}	43.30±0.31^{ab}	39.93±^{abc}	38.56±1.18^{c}	43.77±0.59^{a}	ED^{b} DD^{b} PO^{a}	NS	NS
18:1n-7	3.41±0.18	2.58±0.03	2.56±0.00	2.97±0.03	2.25±	2.38±0.12	1.98±0.99	NS	NS	NS
18:1n-5	0.11±0.01^{a}	0.08±0.00^{bc}	0.07±0.08^{bc}	0.09±0.00^{ab}	0.08±^{bc}	0.06±0.00^{c}	0.08±0.00^{bc}	ED^{b} DD^{b} PO^{a}	NS	NS
18:2n-9	1.80±0.28^{b}	1.58±0.11^{b}	1.96±0.67^{b}	3.28±0.41^{a}	1.87±^{b}	1.84±0.08^{b}	4.10±0.44^{a}	ED^{b} DD^{b} PO^{a}	NS	NS
18:2n-6	14.00±0.37^{c}	16.70±0.33^{ab}	15.36±0.07^{bc}	17.50±0.39^{ab}	18.24±^{a}	16.86±0.46^{ab}	17.12±0.74^{ab}	NS	NS	NS
18:2n-4	0.17±0.01^{a}	0.04±0.00^{b}	0.03±0.00^{b}	0.04±0.00^{b}	0.03±^{b}	0.03±0.00^{b}	0.03±0.00^{b}	NS	P=0.00	NS
18:3n-6	2.19±0.37^{b}	2.19±0.13^{b}	2.53±0.17^{b}	4.24±0.45^{a}	2.89±^{b}	2.70±0.02^{b}	4.86±0.12^{a}	ED^{b} DD^{b} PO^{a}	P=0.03	NS
18:3n-4	0.13±0.00^{a}	0.06±0.00^{b}	0.05±0.66^{b}	0.07±0.00^{b}	0.05±^{b}	0.05±0.00^{b}	0.06±0.00^{b}	NS	NS	NS
18:3n-3	2.83±0.16^{abc}	3.11±0.06^{a}	2.37±0.00^{c}	2.61±0.05^{abc}	2.93±^{ab}	2.47±0.06^{bc}	2.40±0.19^{bc}	ED^{a} DD^{b} PO^{b}	NS	NS
18:4n-3	0.80±0.19	0.63±0.06	0.59±0.10	0.84±0.12	0.62±	0.60±0.00	0.79±0.01	ED^{ab} DD^{b} PO^{a}	NS	NS
18:4n-1	0.08±0.01	0.03±0.01	0.02±0.01	0.02±0.00	0.22±	0.01±0.00	0.02±0.04	NS	NS	NS
20:0	0.25±0.04	0.23±0.03	0.19±0.09	0.19±0.01	0.18±	0.19±0.03	0.19±0.01	NS	NS	NS
20:1n-9	0.35±0.04^{a}	0.26±0.01^{bc}	0.27±0.01^{ab}	0.29±0.02^{ab}	0.16±^{d}	0.17±0.01^{cd}	0.21±0.01^{bcd}	ED^{b} DD^{ab} PO^{a}	P=0.00	NS

Table 6.4. Hepatosomatic index (%), hepatic lipid content (% ww) and liver fatty acid composition (% total fatty acids) of gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%) (continued)

	Diets									
		15% FM			7.5% FM			Two-way ANOVA		
	CTRL	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
20:1n-7	1.41±0.20^{a}	1.21±0.05^{ab}	1.09±0.02^{abc}	0.94±0.05^{bc}	0.83±0.07^{bc}	0.80±0.07^{bc}	0.77±0.03^{c}	ED^{a} DD^{ab} PO^{b}	P=0.00	NS
20:1n-5	0.16±0.03^{a}	0.08±0.00^{b}	0.08±0.00^{b}	0.09±0.00^{b}	0.07±0.00^{b}	0.08±0.00^{b}	0.09±0.00^{b}	ED^{b} DD^{b} PO^{a}	NS	NS
20:2n-9	0.59±0.04^{ab}	0.63±0.08^{a}	0.57±0.01^{ab}	0.54±0.02^{ab}	0.27±0.03^{b}	0.26±0.07^{b}	0.48±0.07^{ab}	NS	P=0.00	NS
20:2n-6	0.42±0.07	0.42±0.04	0.39±0.01	0.36±0.03	0.77±0.42	0.32±0.03	0.34±0.03	NS	NS	NS
20:3n-9	0.03±0.01^{a}	0.01±0.00^{ab}	0.00±0.02^{b}	0.01±0.01^{ab}	0.00±0.00^{b}	0.00±0.00^{b}	0.01±0.00^{ab}	NS	NS	NS
20:3n-6	0.37±0.01^{ab}	0.41±0.05^{ab}	0.46±0.01^{a}	0.33±0.02^{ab}	0.24±0.02^{b}	0.32±0.05^{ab}	0.30±0.05^{ab}	NS	P=0.01	NS
20:4n-6	0.49±0.04^{b}	0.47±0.01^{b}	0.71±0.14^{a}	0.28±0.01^{c}	0.53±0.01^{b}	0.78±0.01^{a}	0.29±0.03^{c}	ED^{b} DD^{a} PO^{c}	P=0.02	NS
20:3n-3	0.16±0.02^{a}	0.16±0.01^{a}	0.12±0.02^{ab}	0.10±0.01^{b}	0.14±0.01^{ab}	0.10±0.01^{b}	0.10±0.01^{b}	ED^{a} DD^{b} PO^{b}	NS	NS
20:4n-3	0.39±0.03	0.31±0.02	0.31±0.00	0.15±0.01	0.52±0.34	0.24±0.02	0.11±0.02	NS	NS	NS
20:5n-3	2.44±0.58^{a}	1.78±0.08^{ab}	0.82±0.06^{bc}	0.51±0.02^{c}	1.45±0.15^{abc}	0.72±0.09^{bc}	0.38±0.07^{c}	ED^{a} DD^{b} PO^{c}	P=0.02	NS
22:1n-11	0.96±0.17^{a}	0.72±0.07^{ab}	0.71±0.03^{ab}	0.52±0.02^{bc}	0.35±0.05^{bc}	0.40±0.07^{bc}	0.32±0.01^{c}	ED^{ab} DD^{a} PO^{b}	P=0.00	NS
22:1n-9	0.63±0.13^{a}	0.48±0.02^{ab}	0.46±0.01^{ab}	0.47±0.01	0.33±0.05^{b}	0.37±0.04^{ab}	0.40±0.01^{ab}	NS	P=0.00	NS
22:4n-6	0.09±0.01	0.09±0.01	0.09±0.04	0.07±0.01	0.08±0.03	0.10±0.01	0.06±0.00	NS	NS	NS
22:5n-6	0.14±0.01^{b}	0.24±0.01^{b}	2.87±0.07^{a}	0.07±0.01^{b}	0.44±0.14^{b}	3.16±0.15^{a}	0.14±0.02^{b}	ED^{b} DD^{a} PO^{b}	P=0.03	NS
22:5n-3	0.99±0.17^{a}	0.74±0.05^{ab}	0.38±0.04^{c}	0.17±0.01^{c}	0.47±0.04^{bc}	0.31±0.04^{c}	0.14±0.03^{c}	ED^{a} DD^{b} PO^{c}	P=0.00	P=0.02
22:6n-3	4.39±0.57^{bc}	6.24±0.31^{ab}	7.86±0.02^{ab}	1.71±0.01^{c}	5.04±0.62^{abc}	8.68±1.79^{a}	1.68±0.16^{c}	ED^{a} DD^{b} PO^{c}	NS	NS
∑ SFA	18.14±0.81^{a}	17.28±0.18^{ab}	16.62±0.01^{ab}	16.09±0.01^{ab}	16.94±0.68^{ab}	15.70±0.36^{b}	15.95±0.37^{ab}	ED^{a} DD^{ab} PO^{c}	NS	NS
∑ MUFA	48.91±2.03^{ab}	46.65±0.41^{ab}	45.66±0.11^{ab}	50.76±0.33^{a}	45.83±0.81^{ab}	44.56±1.47^{b}	49.45±0.58^{ab}	ED^{b} DD^{b} PO^{a}	NS	NS
∑ n-9	42.76±1.38^{b}	42.34±0.37^{b}	41.88±0.05^{b}	47.89±0.24^{a}	42.56±0.63^{b}	41.21±1.25^{b}	48.96±0.97^{a}	ED^{b} DD^{b} PO^{a}	NS	NS
∑ n-6	17.71±0.49^{c}	20.53±0.25^{b}	22.41±0.99^{ab}	22.85±0.18^{ab}	23.20±0,65^{a}	24.24±0.43^{a}	23.10±0.73^{a}	ED^{ab} DD^{a} PO^{b}	P=0.00	NS
∑ n-6 LC-PUFA	1.51±0.06^{b}	1.64±0.08^{b}	4.52±0.20^{a}	1.10±0.03^{b}	2.06±0.53^{b}	4.68±0.10^{a}	1.12±0.11^{b}	ED^{b} DD^{a} PO^{c}	NS	NS
∑ n-3	12.12±1.64^{a}	13.01±0.48^{a}	12.49±1.13^{a}	6.14±0.07^{bc}	11.41±1.02^{ab}	13.15±2-06^{a}	5.64±0.51^{c}	ED^{a} DD^{a} PO^{b}	NS	NS
∑ n-3 LC-PUFA	8.37±1.29^{a}	9.23±0.44^{a}	9.49±1.16^{a}	2.64±0.11^{bc}	7.63±0.86^{ab}	10.04±1.93^{a}	2.41±0.29^{c}	ED^{a} DD^{a} PO^{b}	NS	NS
EPA+DHA	6.83±1.11^{ab}	8.02±0.38^{a}	8.68±1.06^{a}	2.21±0.10^{bc}	6.49±0.77^{abc}	9.39±1.89^{a}	2.06±0.22^{c}	ED^{a} DD^{a} PO^{b}	NS	NS
EPA/ARA	4.86±0.87^{a}	3.79±0.18^{ab}	1.15±0.09^{cd}	1.81±0.06^{cd}	2.75±0.32^{bc}	0.92±0.12^{d}	1.30±0.12^{cd}	ED^{a} DD^{c} PO^{b}	P=0.00	NS
EPA/DHA	0.54±0.09^{a}	0.29±0.01^{b}	0.10±0.00^{c}	0.30±0.02^{b}	0.29±0.01^{b}	0.08±0.01^{c}	0.22±0.02^{bc}	ED^{a} DD^{b} PO^{a}	P=0.02	P=0.04
n-6/n-3	1.51±0.21^{b}	1.58±0.07^{b}	1.83±0.22^{b}	3.72±0.02^{a}	2.08±0.25^{b}	1.93±0.29^{b}	4.15±0.27^{a}	ED^{b} DD^{b} PO^{a}	NS	NS
16:0/16:1	4.30±0.17^{d}	5.93±0.13^{a}	5.66±0.08^{ab}	4.73±0.06^{cd}	5.94±0.15^{a}	5.56±0.12^{ab}	5.12±0.16^{bc}	ED^{a} DD^{b} PO^{c}	NS	NS
18:0/18:1	0.14±0.00	0.13±0.00	0.14±0.00	0.12±0.00	0.13±0.00	0.13±0.00	0.13±0.01	NS	NS	P=0.03
18:1/18:2(n-9)	23.45±5.09^{ab}	25.11±1.61^{a}	20.00±1.74^{abc}	13.58±1.59^{bc}	21.48±1.29^{abc}	21.06±1.18^{abc}	10.89±0.94^{c}	ED^{a} DD^{b} PO^{c}	NS	NS
18:3/18:4(n-3)	3.97±0.93	5.03±0.54	4.03±0.25	3.22±0.43	4.77±0.30	4.25±0.49	3.05±0.18	NS	NS	NS

Different letters denote significant differences among the experimental groups at the end of the trial (P<0.05).

6.4.3 GENE EXPRESSION OF LIPID METABOLISM-RELATED MARKERS

6.4.3.1 LIPOGENIC PATHWAY

Sea bream fed CTRL, ED and DD diets presented similar relative expression of *srebp1* and *fas* (Figure 6.2), whereas fish fed PO diets showed significant higher ($P<0.05$) *fas* expression, particularly those fed 7.5% FM PO diet, and a tendency to higher *srebp1* expression (78% higher than fish fed 7.5% ED for instance) (Figure 6.2). Indeed, *srebp1* and *fas* linearly decreased with the increase of the dietary n-3 LC-PUFA ($r^2=0.75$ $P=0.01$ and $r^2=0.85$ $P=0.00$, respectively) and DHA ($r^2=0.75$ $P=0.01$ and $r^2=0.77$ $P=0.01$, respectively). No significant differences were observed for *srebp2* expression among sea bream fed the different diets (Figure 6.2).

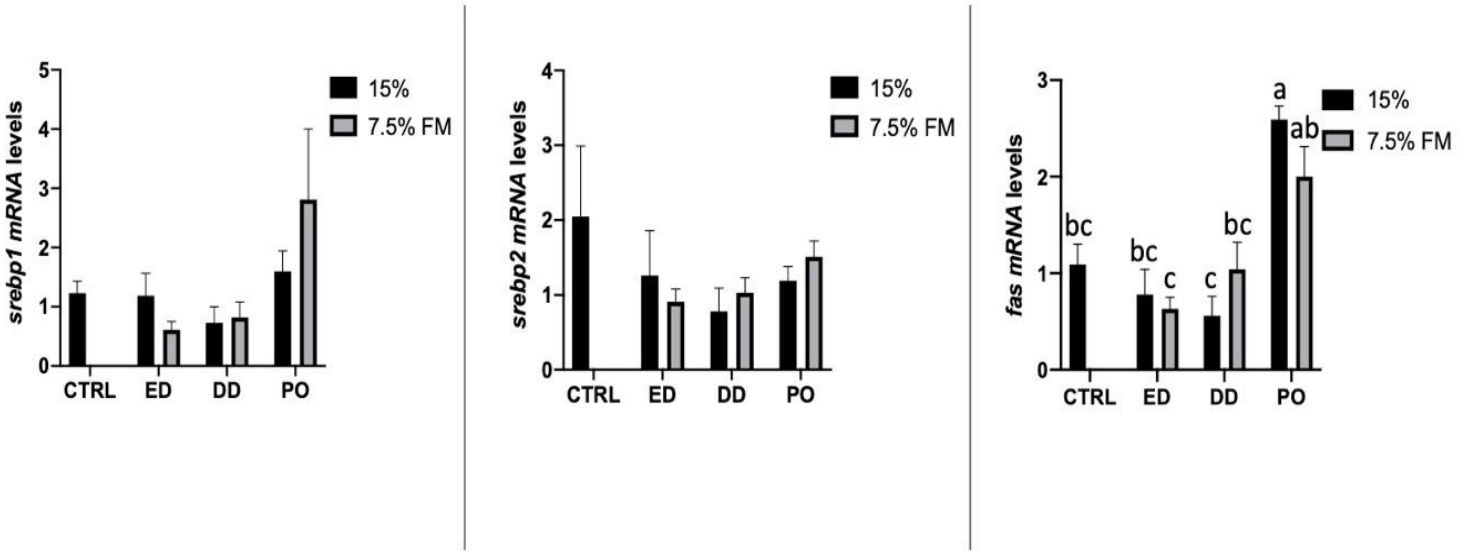

Figure 6.2. Hepatic relative expression of lipogenic pathway related genes in gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%).

6.4.3.2 ELONGATION AND DESATURATION PATHWAYS

Sea bream fed the ED or DD diets showed similar *scd-1a* or *fads2* relative expression compared to those fed the CTRL diet (Figure 6.3). In contrast, in fish fed PO diets, *scd-1a* was significantly up-regulated ($P<0.05$). Although no significant differences were detected, probably due to the large inter-variation in fish gene expression, a tendency to present higher (70-92%) *mRN*A levels of *fads2* was also noted in fish fed PO when compared to those fed CTRL, ED, or DD diets (Figure 6.3). Indeed, both *scd-1a* and *fads2* presented a significant logarithmic correlation with the dietary dry weight of n-3 LC-PUFA ($r^2=0.86$, $P=0.00$ for *scd-1a*; $r^2=0.73$, $P=0.01$ for *fads2).* Additionally, *scd-1a* was particularly highly correlated with the

dietary 16:0 (r^2=0.77 P=0.01) and 18:0 (r^2=0.95 P=0.00), but also logarithmic with the dietary DHA (r^2=0.73 P=0.01), whereas *fads2* was positively correlated with the dietary OA (r^2=0.80 P=0.01), LA (r^2=0.84 P=0.00), ARA (r^2=0.86 P=0.00) and EPA (r^2=0.73 P=0.01). In contrast, the tendency was not so clear for *elovl5* expression (Figure 6.3).

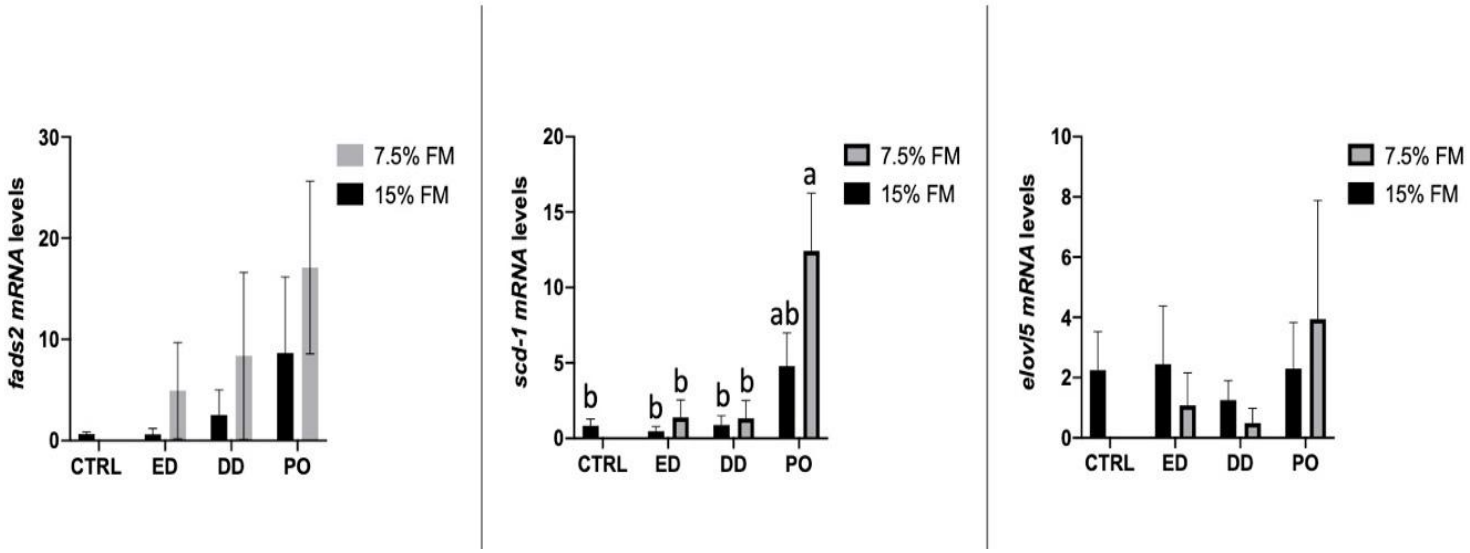

Figure 6.3. Hepatic relative expression of elongation and desaturation pathway related genes in gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%).

6.4.3.3 LIPOLYTIC PATHWAY

Expression of *lpl* was 4-5 times higher in fish fed 7.5% FM PO than in fish fed the CTRL diet, despite the lack of significant differences (P>0.05). (Figure 6.4). Additionally, *lpl* was linearly increased with the dietary OA (r^2=0.89 P=0.01) and LA (r^2=0.84 P=0.00) (r^2=0.86 P=0.00). For *cpt1* expression, similar *mRNA* values were shown among CTRL, ED, and DD diets, but a higher relative expression in fish fed 7.5% FM PO than in those fed the other diets could be noted (P<0.05; Figure 6.4). No significant differences were observed regarding *ppar*α.

For all the different genes studied, the two-way ANOVA showed that the dietary oil source was the major driver in the differences found in gene expression (Table 6.5), whereas the expressions of the studied genes were not affected by the dietary content of FM (Table 6.5). In addition, no interaction effect was observed between these two dietary variables in any of the genes determined (Table 6.5).

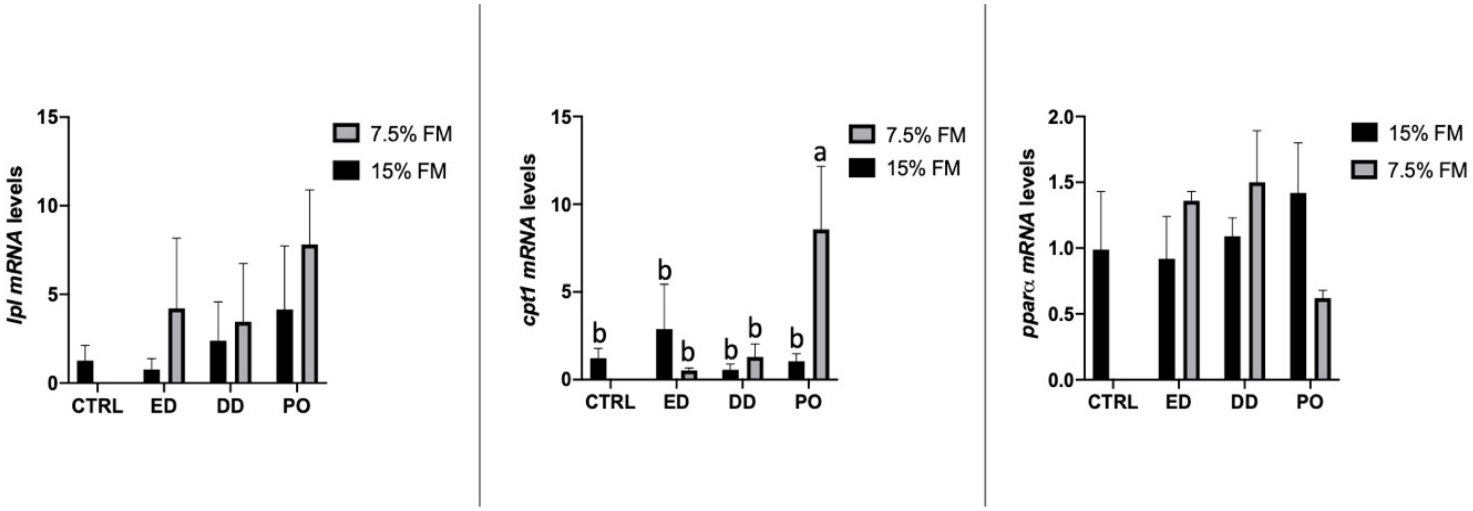

Figure 6.4. Hepatic relative expression of lipolytic pathway related genes in gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%).

Table 6.5. Independent and interacted effects in gene expression of feeding gilthead sea bream with novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%)

	Two-way ANOVA		
	Lipid source	%FM	Lipid source x %FM
Gene expression			
srebp1	NS	NS	NS
srebp2	NS	NS	NS
fas	ED^{b} DD^{b} PO^{a}	NS	NS
scd-1a	ED^{b} DD^{b} PO^{a}	NS	NS
fads2	NS	NS	NS
elovl5	NS	NS	NS
lpl	NS	NS	NS
cpt1	NS	NS	NS
pparα	NS	NS	NS

6.4.4 HEPATIC TISSUE HISTOMORPHOLOGY

Morphologically, sea bream fed ED and DD diets showed lower cytoplasmatic lipid vacuolization that, when present, was in the form of micro-vacuolization, with smaller eosinophilic hepatocytes and prominent basophilic nuclei (Figure 6.5). This hepatocyte vacuolization was tendentially increased (P=0.05) in those fish fed CTRL diet as showed by the slightly higher degree of steatosis and hepatocyte size, but more significantly (P=0.00) in those fed PO diets, which showed a strong macro-vacuolization leading to the highest steatosis level and the largest hepatocytes (Table 6.5; Figure 6.5). Indeed, hepatocyte size increase linearly with the increase of liver lipids (r^2=0.93 P=0.00), as well with the decrease of the dietary n-3 LC-PUFA (r^2=0.88 P=0.00) and the increase of the dietary OA (r^2=0.78 P=0.01). Furthermore, both hepatocyte size and liver lipids were strongly correlated and linearly increased with the expression of *fas* (r^2=0.90 P=0.00 and r^2=0.89 P=0.00, respectively) and logarithmic correlated with the expression of *scd-1a* (r^2=0.86 P=0.01 and r^2=0.74 P=0.00, respectively). Similarly, hepatocyte size also showed a linear increased with the increase in *srebp1* expression (r^2=0.69 P=0.02).

Again, the two-way ANOVA showed only significant differences for lipid source, adding strength to the results already observed for one-way ANOVA, with FM content or an interaction being less influencers of liver morphology (Table 6.6).

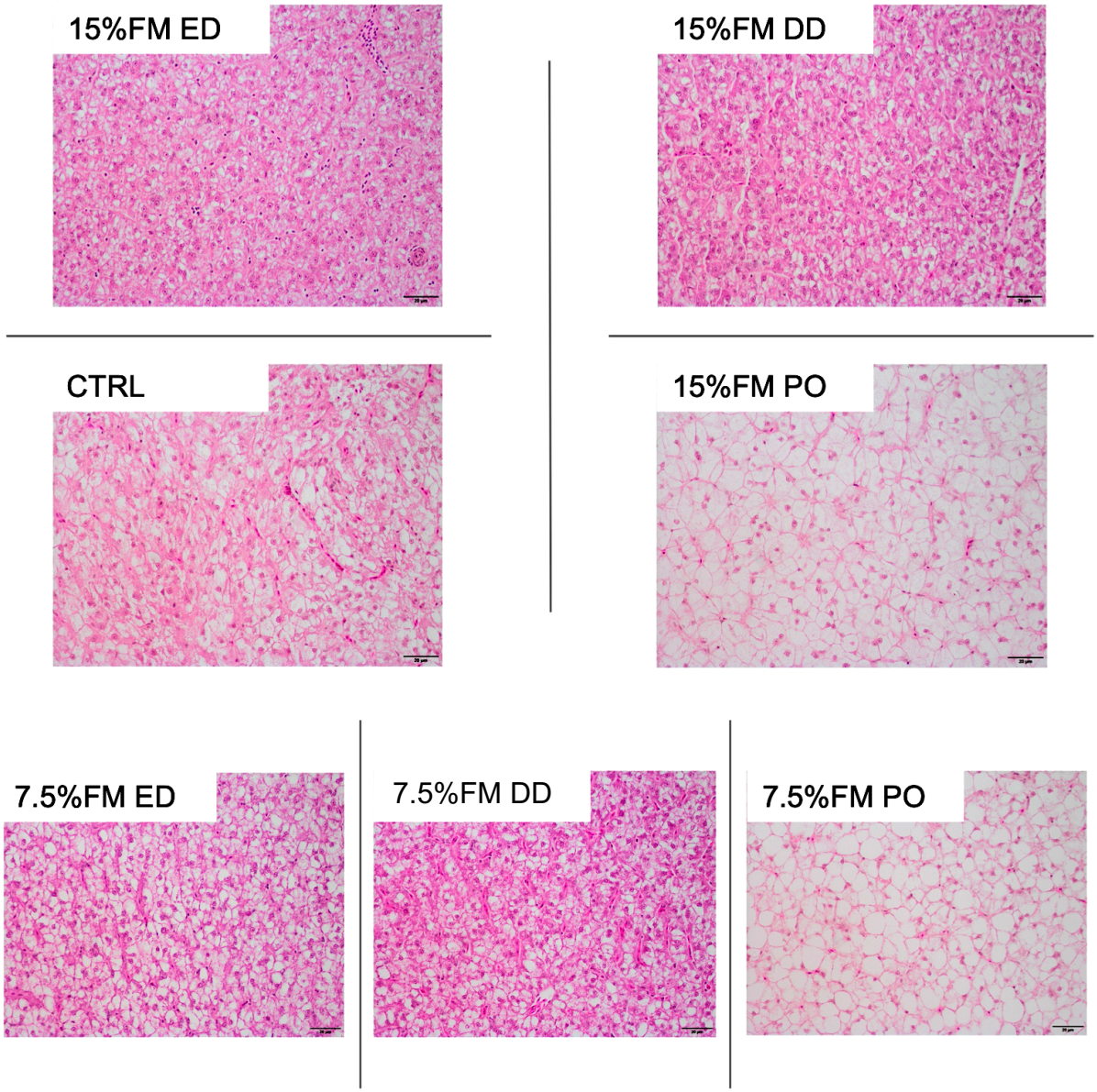

Figure 6.5. Liver histological appearance of gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%) (stained with H&E; scale 20 μm).

Table 6.6. Histological parameters of hepatic tissue of gilthead sea bream fed novel lipid sources (microalgae and poultry oils) as replacers of FO under two dietary contents of fish meal (15 and 7.5%)

Diets										
		15% FM			7.5% FM			Two-way ANOVA		
	CTRL	ED	DD	PO	ED	DD	PO	Lipid source	%FM	Lipid source x %FM
Steatosis degree	2.30±0.13^{b}	2.05±0.14^{b}	2.04±0.14^{b}	2.38±0.14^{b}	2.08±0.17^{b}	2.04±0.17^{b}	2.75±0.10^{a}	-	-	-
Hepatocyte area	45.82±1.98^{bc}	35.67±2.61^{c}	35.63±2.81^{c}	70.97±7.19^{ab}	42.00±2.90^{c}	39.51±0.54^{c}	71.14±10.64^{a}	ED^{b} DD^{b} PO^{a}	NS	NS

Different letters denote significant differences among the experimental groups at the end of the trial (P<0.05).

6.5 Discussion

The decrease in dietary n-3 LC-PUFA in feeds based on low FO and high VO inclusions affects fish metabolism and health, particularly in marine species (reviewed in Montero and Izquierdo, 2010). Therefore, novel n-3 LC-PUFA sources are necessary to promote aquafeeds production and aquaculture sustainability. In fact, EFA-deficiency causes many alterations in metabolism, including different lipid pathways, which may negatively affect fish performance. Optimum dietary n-3 LC-PUFA levels for gilthead sea bream juveniles were previously estimated at 1.3-1.6 % DW (Izquierdo, 2005; Houston et al., 2017). Specifically, optimum dietary levels for this species were estimated to be 0.6-0.7% DW for DHA and 0.6-0.9% DW for EPA (Izquierdo, 2005). Accordingly, in the present study, FO, ED, and DD diets covered the specific requirements for n-3 LC-PUFA and DHA of gilthead sea bream. Even though, microalgae diets (ED and DD) presented EPA contents under the optimum levels estimated for the species, particularly in DD diets. Despite this, similar growth was observed among sea bream fed either 15% FM microalgae diets (ED or DD) and FO diet. These results agree with the effectiveness of DHA as a growth promoter in comparison to EPA (Watanabe et al., 1993; Izquierdo, 1996, 2005). Furthermore, the present results indicated the potential of microalgae oils, rich in DHA, as effective n-3 LC-PUFA sources for sea bream juveniles, as it was recently found for salmonids juveniles (Peterson et al., 2019; Santigosa, 2019; Kousoulaki et al., 2020).

In addition, it is well recognized that fish tissue fatty acid composition usually reflects the dietary composition (Cowey and Sargent, 1972). In the present study, livers from sea bream fed ED or DD diets mirrored the high dietary DHA contents, while those from fish fed FO (CTRL diet) showed the highest content of EPA. Therefore, the highest hepatic DHA contents of sea bream fed microalgae oils in the present study seemed to be the main reason for the smallest alterations in fish hepatic histology. Noticeable, fish fed ED or DD diets showed a very low lipid infiltration, reflected in a microvacuolization of the hepatocytes and the lowest hepatocyte area. This was also consistent with the lowest steatosis degree, liver lipid contents and HSI found in these fish, as reported in other marine species fed diets meeting their EFA requirements (Ibeas et al., 1994; Kjaer et al., 2008; Carvalho et al., 2018). Indeed, n-3 LC-PUFA has been related to a reduced hepatic lipid infiltration by maintaining lipid metabolism homeostasis, since they reduce triacylglycerides synthesis (Berge et al., 1999). Furthermore, n-3 LC-PUFA increase the expression of microsomal transfer protein or apolipoprotein A1 (Kjaer et al., 2008), whereas down-regulate adipophilin expression, which is a molecular marker of intracellular lipid accumulation (Leaver et al., 2008). In agreement, all the genes related with lipogenesis and lipolytic pathways metabolism analyzed in the present study, showed similar relative expression among sea bream fed microalgae oils and FO. This suggest that the total replacement of FO is possible by blending microalgae oils with other

EFA-devoid lipid sources, usually cheaper and more available, such as PO, with no major impact in sea bream hepatic lipid metabolism. Given the still high production costs of microalgae, a detailed economical study of the experimental diets containing both microalgae and poultry oils would be of interest. Furthermore, the use of microalgae oils could even improve liver health status by reducing lipid vacuolization, even when comparing with FO (CTRL diet). Further studies are needed to determine the potential positive long-term effects of microalgae sources. Besides, the highest n-6 DPA contents were found in livers of sea bream fed DD diets, a FA reported in high amounts in *Schizochytrium sp.* (Jiang et al., 2004; Zangh et al., 2017). Interestingly, these fish also presented the highest liver ARA contents, contrary to their lowest dietary levels, suggesting a retro-conversion of the excessive n-6 DPA to ARA, as reported previously in gilthead sea bream larvae fed this microalga (Ganuza and Izquierdo, 2007).

The total replacement of FO by PO alone led to the lowest sea bream growth in the present study, probably associated to an EFA-deficiency. In fact, the replacement by PO was not sufficient to maintain n-3 LC-PUFA optimum levels for this species, being deficient in both DHA and EPA (Izquierdo, 2005). This agrees with the increased HSI and liver lipids in these fish, common indicators of EFA-deficiency in teleost (Verreth et al., 1994). Hepatomegaly associated to an increase in liver lipids can be the result of an excess in dietary lipids, surpassing the liver storage capacity, but also of an inadequate dietary fatty acid composition by altering hepatic lipid metabolism and leading to steatosis (Spisni et al., 1998). This pathologic condition compromises fish metabolic utilization of nutrients and energy and, consequently, fish health and performance (Spisni et al., 1998; Verreth et al., 1994; Montero and Izquierdo, 2010). In line, sea bream of the present study developed a severe steatosis, characterized by a high lipid macrovacuolization and the highest hepatocyte area. Indeed, hepatocyte size, hepatic lipids and HSI were all strongly linearly correlated with the dietary n-3 LC-PUFA. Likewise, livers from fish fed PO diets reflected the lowest dietary n-3 LC-PUFA and the highest hepatic OA, in agreement with previous studies in other fish species fed poultry fats (Turchini et al., 2009; Campos et al., 2019). The accumulation of both OA and LA were shown to be major FA influencers of the appearance of hepatic steatosis in fish (Caballero et al., 2004). Furthermore, due to the high molecular weight of OA, the influx of this FA inside the hepatocyte was sufficient to create a large lipid droplet (Bradury, 2006), which was observed in fish fed PO diets. In addition, OA seems to be slowly metabolized in liver of marine fish, since the excess of this FA is mainly degraded in peroxisomes and marine fish has a lower capacity to replicate this organelle, contributing to the lipid accumulation in the hepatocytes (Caballero et al., 2002).

Lipid metabolism homeostasis is achieved by an equilibrium between anabolic and catabolic pathways. In the present study, the total replacement of FO by PO significant altered lipid metabolism in liver of sea bream, particularly in relation to lipogenic processes. PO induced an up-regulation of hepatic *sbrep1* and *fas*, both genes involved in lipid synthesis. Srebp1 regulates *de novo* synthesis of FA, having a wide range of targets, including *fas*, whose enzymatic product synthetizes SFA. Similarly in previous studies, low n-3 LC-PUFA diets (based on VO) were associated to a higher expression of *srebp1* and/or *fas*, including in gilthead sea bream (Houston et al., 2017), black sea bream (*Spondyliosoma cantharus*) (Jin et al., 2017) or Atlantic Salmon (*Salmo salar*) (Morais et al, 2011). Indeed, the expressions of both genes in the present study were linearly correlated with the dietary n-3 LC-PUFA, particularly with DHA, suggesting that the low dietary contents of these FA are responsible for the up-regulation. The high correlations between the expression of *srebp1* and *fas* and fish hepatocyte size suggest that the increased regulation of these genes in sea bream fed PO might have contributed to the increased lipid deposition in liver resulting in a severe steatosis situation, which is in in agreement with previous studies (Morais et al., 2011; Houston et al., 2017).

Additionally, sea bream fed PO diets showed higher contents of palmitoleoyl-CoA (16:1) and oleyol-CoA (18:1), desaturation products of SCD-1a on palmitoyl-CoA (16:0) and stearoyl-CoA (18:0), respectively. The same was true for 18:2n-9, 20:2n-9, 18:3n-6, 20:3n-6, all potential products of FADS2. These results, added to the decreased desaturation index 18:1n-9/18:2n-9 and the highest *scd-1a* and *fads2* expression in these fish, suggest an increased Δ9 and Δ6 desaturase activity in response to an EFA-deficiency. These results denote a Δ6 activity on n-9 FA in gilthead sea bream, as previously suggested (Houston et al., 2017). An *in vivo* up-regulation *fads2* was commonly observed in other marine species fed low EFA-diets, such as meagre (*Argyrosomus regius*) (Carvalho et al., 2018), turbot (*Scophthalmus maximus*) (Owen et al., 1975) or flounder (Lee et al., 2003; Kim and Lee, 2004). However, synthesis of DHA and EPA also requires Δ5 and, possibly, Δ4 desaturases in the last steps of the desaturation pathway, which are usually poorly activated in marine species (Tocher, 2015). Thus, the negative impact of PO diets on fish growth and liver health status denoted that the production of LC-PUFA was not sufficient to cover gilthead sea bream essential fatty acid requirements, despite the *fads2* up-regulation. Furthermore, the possible higher activity of Fads2 on n-9 and n-6 precursors (OA and LA, respectively) rather than on n-3 precursors (LNA) could be probably associated with a lower availability of the latter substrate than the formers, despite the higher affinity of Δ6 for n-3 fatty acids (Sissener et al., 2017). Interestingly, previous studies also related low dietary n-3 LC-PUFA diets to an up-regulation of *elovl5* in marine fish (Carvalho et al., 2018). However, in gilthead sea beam in the present

study, *elovl5* expression was not significantly up-regulated. These results agree with the minor effect of dietary VO on hepatic expression of *elovl5* in a previous study on gilthead sea bream (Houston et al., 2017).

In addition, lipolytic processes seemed less affected by the substitution of FO by PO. LPL is the enzyme responsible for hydrolysing TAG of lipoproteins making FA available for uptake into organs (Fielding and Frayan, 1998). Previous studies suggested that OA, alone or in combination with LA increased *lpl* expression in liver of red sea bream (*Pragus major*) (Liang et al., 2002), in agreement with the up-regulation tendency of this gene in gilthead sea bream fed 7.5% FM PO of the present study. Indeed, *lpl* expression showed a linear correlation with the dietary OA and LA. Additionally, *cpt1* was up-regulated in these fish. Although several studies suggested a regulatory mechanism of *lpl* and *cpt1* gene expression by PPARs in mammals (Simopoulos, 1996), no significant effects were found in *ppar*α expression in the present study. Both *ppar*α and *cpt1* are two important genes involved in β-oxidation of FA in peroxisomes and mitochondria, respectively, but are regulated by independent pathways (Ayisi et al., 2018). Similarly, in yellow croaker (*Larimichthys crocea*) dietary composition caused no difference in the *ppar*α expression (Dong et al., 2017). However, *ppar*α and/or *cpt1* were down-regulated in gilthead sea bream and Japanese sea bass (*Lateolabrax japonicus*) in response to low dietary n-3 LC-PUFA (high VO diets), while upregulated in rainbow trout (*Oncorhynchus mykiss*) (Houston et al., 2017; Dong et al., 2017). The discrepancy of these results may indicate that FA β-oxidation can be influenced by several other stimuli rather than only dietary fatty acid composition (Houston et al., 2017).

Few studies have been conducted on the concomitant replacement of FM and FO, some reported an interaction between dietary protein and lipid sources on fish metabolism (Dias et al., 2005; 2009; Benedito-Palos et al., 2007; Torrecillas et al., 2017). In agreement, the concomitant replacement of FM and FO in the present study negatively affected growth performance. This was denoted by the lowest weight in fish fed 7.5% FM compared to those fed 15%. However, little effect of FM level was shown in hepatic indices, liver lipid metabolism associated gene expression or histological status. Furthermore, no interactions were found between the dietary content of FM and lipid source, suggesting that both factors affected independently sea bream performance and metabolism, with FO replacement exerting a much stronger effect than FM content.

6.6 CONCLUSIONS

The present study demonstrates that the total replacement of FO by a blend of microalgae oils, rich in n-3 LC-PUFA, and PO, have no major impact on growth and hepatic lipid metabolism of gilthead sea bream juveniles. In turn, PO as sole source of FO replacement negatively affected fish lipid metabolism, up-regulating lipogenic-related genes (*srebp1* and *fas*), as well as *lpl*. Consequently, these fish showed an increased liver lipid deposition that led to a severe hepatic steatosis and reduced growth. The high dietary contents in n-3 LC-PUFA of FO and microalgae oils diets down-regulated genes involved in FA biosynthetic pathway, including *scd-1a* and *fads2.*

Both protein and lipid sources seemed to independently affect metabolism and performance of fish, with FO replacement exerting a much stronger in lipid metabolism than FM replacement.

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CHAPTER 7. DIETARY NOVEL OILS MODULATE NEURAL FUNCTION AND PRESERVE LOCOMOTOR RESPONSE IN GILTHEAD SEA BREAM (*SPARUS AURATA*) JUVENILES BY REGULATING SYNTHESIS AND CONTENTS OF FATTY ACIDS IN BRAIN

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7.1 ABSTRACT

N-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly docosahexaenoic acid (22:6n-3, DHA), are the most important fatty acids with physiological significance in brain function of all vertebrates. Recently, novel lipid sources are available for the industry, including products obtained from microorganisms. Dietary fatty acid profiles may affect tissue composition and cell functioning. To determine the effect of novel lipid sources on behaviour and neural function in gilthead sea bream (*Sparus aurata*), juveniles were fed three isoproteic and isoenergetic diets containing either (FO), a microalgae oil combined with poultry oil (DD) or only poultry oil (PO). Behaviour response, brain fatty acid composition and relative expression of neurogenesis and neural activity-related genes in telencephalon were evaluated during 5 months of feeding with the experimental diets. Brain of sea bream fed diet PO showed the highest DHA content as well as increased desaturation and elongation products, contrary to the dietary pattern. This, together with an increased telencephalic *fads2* expression denoted the effective activation of LC-PUFA synthesis and retention as a compensatory mechanism for a dietary deficiency. Furthermore, *neurod6*, *bdnf* and *nos1* were proportionally upregulated in relation to the neural DHA content. Locomotor performance during cruising and escape responses was not affected by the experimental diets. However, there was a tendency for escape latency to be longest in fish fed PO and DD diets, and a relationship between escape latency and brain n-6 docosapentaenoic acid (DPA) contents was suggested. Indeed, the relation of n-6 DPA with behavioural deficits was shown previously in mammals and might deserve further attention and corroboration in fish as well in the future.

7.2 INTRODUCTION

N-3 long-chain polyunsaturated fatty acids (LC-PUFA) are involved in several metabolic pathways, such as energy production, membrane structure and function, control of lipid homeostasis and production of eicosanoids (Tocher, 2003). Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) have particularly significant biological roles and are essential fatty acids (EFA) for marine species, due to their limited desaturases (FADS) and elongases (ELOVL) activities, necessary for their endogenous synthesis (Izquierdo and Koven, 2011; Tocher, 2015). Despite the well-known positive effects of DHA and EPA on vertebrate growth, reproduction, and disease prevention, these FA are also main components of polar lipids, which play critical roles in the regulation of membrane integrity and function. Moreover, they strongly participate in neural development and performance (Sargent et al., 1995; Izquierdo and Koven, 2011). Indeed, DHA is the most important fatty acid with physiological significance for brain function in vertebrates, including in fish (Bourre, 2004; Marszalek and Lodish, 2005). This fatty acid is especially relevant during very early life stages, accounting for 30–40% of fatty acids contained in neural cell membranes (Lauritzen et al., 2001). Despite being less studied, EPA may also play important roles in neural function, by enhancing brain development in fish (Furuita et al., 1998), whereas in mammals, EPA has been shown to have a neuro-protective role (Joffre et al., 2019). The high demand for n-3 LC-PUFA from the brain in early stages of life is one of the most significant reasons behind the considerable higher DHA and EPA requirements in larvae than in juveniles or sub-adult fish (Izquierdo, 2005). However, despite these important functions in fish neuromodulation, there is still little information regarding the effects of dietary n-3 LC-PUFA on brain function of teleosts. Indeed, composition of neural cells can directly influence brain activity that, in turn, modulates fish behaviour (Benitez-Santana et al., 2012, 2014). Behaviour is a consequence of the connection between the perception of the environment by the animal and its nervous system that translate the sensory perception into a specific reaction (Lund et al., 2014). Therefore, normal fish behaviour is associated with a normal neural function and consequently, closely related with fish welfare and health status. Like in the natural environment, that fish faces several stressors, for instance, predators, in captivity, fish also experience stressful situations, including confinement due to high density, chasing due to fish netting, etc. These types of external stimuli are usually translated into typical behavioural response patterns that need to be monitored for fish welfare. For instance, previous studies with gilthead sea bream larvae (*Sparus aurata*) provided the first evidence regarding the effects of n-3 LC-PUFA on Mauthner neurons and escape behaviour (Benítez-Santana et al., 2012; 2014). DHA-deficient diets were shown to greatly reduce DHA incorporation in larval brain tissue and, consequently, delaying escape response to a sound stimulus (Benítez-

Santana et al., 2012; 2014). Additionally, EFA-deficient diets also have short (28 days) and long-term (112 days) negative consequences in behaviour of pikeperch (*Sander lucioperca*) larvae (Lund et al., 2014). These unsuccessful or altered behavioural responses could be derived from a failure of neuronal activity or a low perception of the stimulus. Escape responses are natural mechanisms for avoiding stressors. They consist of both non-locomotor, for instance escape latency, as well as locomotor variables (e.g., turning rate, escape velocity and distance) (Domenici et al., 2007), that are activated by fish neural circuits, particularly Mauthner neurons, and allow fish to avoid threats (Domenici and Hale, 2019). In juveniles, recent studies with rainbow trout (*Oncorhynchus mykiss*) also linked dietary n-3 LC-PUFA and feeding behaviour, as well as inflammatory, oxidant and stress status of fish brain (Roy et al., 2020). Although some work has been carried out on larvae of farmed fish species, very few studies have been conducted to understand the impact of n-3 LC-PUFA in brain and behaviour responses of fish in later stages of development and, to our knowledge, no studies were carried out in gilthead sea bream (*Sparus aurata*) juveniles. This topic deserves further attention on more species for a few reasons: (1) With the increasing replacement of fish oil (FO) in aquafeeds, mostly with terrestrial plant ingredients, n-3 LC-PUFA dietary contents could be significantly reduced. Dietary fatty acid composition is reflected in fish tissues and could affect fish cell responses (Turchini et al., 2009). Therefore, fish nutrition research has been recently focusing in finding new n-3 LC-PUFA sources for fish feeds, in which microalgae are some of the most promising alternatives (Shah et al., 2018). (2) Fish behaviour and neural function are good indicators of fish health and development (Martins et al., 2012), thus being of pivotal importance their study and monitoring for an effective production of fish (Benitez-Santana, 2012, 2014; Lund et al., 2014). (3) Fish models are gaining interest in biomedical and behavioural research as alternatives to mammals, in which DHA and EPA were shown to ameliorate neuropathological disorders, such as depression, schizophrenia, Alzheimer disease or autism (Sanchez-Villegas et al., 2018; Shahidi and Ambigaipalan, 2018). Thus, studying the effect of novel oils as lipid sources for aquafeeds and alternative to FO, on fish neural function could contribute to increasing our knowledge in these fields.

Therefore, using a long-term feeding protocol for sea bream juveniles, the aim of the present study was to investigate the effect of three different dietary formulations with conventional or novel lipid sources with distinct dietary fatty acid composition on (1) brain fatty acid incorporation, (2) fish behavioural response to an external mechano-sensory stimulus and (3) expression of neurogenesis and neural activity-related markers in the telencephalon.

7.3 MATERIALS AND METHODS

7.3.1 EXPERIMENTAL DIETS AND NUTRITIONAL TRIAL

Three experimental diets were formulated containing similar amounts of digestible protein, lipid, and energy (Table 7.1) but differing in their dietary fatty acid composition (Table 7.2). A control diet was formulated based on FO (FO diet), whereas the two other dies were based on either a combination of poultry oil and a DHA and n-6 docosapentaenoic acid (DPA, 22:5n-6)-rich microalgae oil (DD diet), or only poultry oil (PO diet) (Table 7.1). All diets had similar contents in saturated fatty acids (Table 7.2). The FO and DD diets were similar in MUFA, n-9, n-3, n-3 PUFA and EPA+DHA. However, FO diet was the highest in EPA, EPA/ARA, and EPA/DHA, whereas the DD diet was the highest on DHA, DPA and n-6 LC-PUFA, containing 5 times more DHA than EPA (Table 7.2). On the contrary, the PO diet contained low levels of n-3, n-3 PUFA, EPA and DHA. Thus, the n-3 LC-PUFA contents in PO diet were below the requirements estimated for gilthead sea bream juveniles (1.3-1.6% of dry weight of the diet; Izquierdo, 2005) and could cause a marginal EFA-deficiency. The diets were manufactured by Skretting ARC at their Feed Technology Plant (Stavanger, Norway).

A nutritional trial of 2.5 months was conducted in triplicated groups of 55 gilthead sea bream (*Sparus aurata*) fingerlings (initial body weight of 2.50 ± 0.01 g), whose results concerning fish growth performance and hepatic lipid metabolism are described elsewhere (Carvalho et al., 2020, 2021) and were not the aim of the present study. After this feeding period, 12 fish per tank (36 per treatment) were transferred to 500 L tanks kept under natural photoperiod and continuously supplied with filtered sea water (salinity: 37g/L, water temperature: 23.1 ± 0.3 ºC) and aeration (dissolved oxygen: 6.4 ± 0.2 mg/L). Fish were fed with the corresponding experimental diet for another 2.5 months.

Table 7.1. Ingredients and proximate composition of the experimental diets

	Diets		
Ingredients (%)	FO	DD	PO
Fish meal[a]	15.00	15.00	15.00
Wheat [a]	12.30	12.13	11.43
Corn gluten [a]	6.58	6.20	10.00
Hi-pro soya [a]	5.00	5.00	5.00
Wheat gluten [a]	17.71	17.92	15.38
Soya protein concentrate [a]	25.00	25.00	25.00
Faba beans [a]	5.00	5.00	5.00
Fish oil [a]	5.28	0.00	0.00
Rapseed oil [a]	7.92	5.69	7.29
Veramaris algal oil[b]	0.00	0.00	0.00
DHA Natur oil [c]	0.00	3.67	0.00
Poultry oil [f]	0.00	3.92	5.70
Vitamin premix[d]	0.10	0.10	0.10
Mineral premix[e]	0.10	0.10	0.10
Proximate analysis (%)			
Crude protein	49.31	48.06	50.18
Crude lipids	18.19	18.23	17.50
Neutral lipids	89.32	90.15	91.96
Polar lipids	10.68	9.85	8.04
Moisture	6.60	7.85	8.28
Ash	4.66	4.70	4.64
Starch (theorical value)	10.03	10.18	9.87
Fiber (theorical value)	2.53	2.53	2.54

[a] Skretting AS (Norway)

[b] Veramaris algal oil (Veramaris, The Netherlands)

[c] DHA Natur oil (ADM Animal Nutrition, USA)

[d, e] Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition Skretting ARC,

[f] Poultry oil: Sonac. B.V. The Netherlands

Table 7.2. Fatty acid composition (% total identified FA) of the experimental diets

	Diets		
Fatty acid	FO	DD	PO
14:0	2.05	1.33	0.72
14:1n-7	0.40	0.34	0.08
14:1n-5	0.37	0.35	0.03
15:0	0.45	0.35	0.10
15:1n-5	0.29	0.36	0.03
16:0ISO	0.37	0.40	0.03
16:0	9.72	10.51	11.59
16:1n-7	2.51	1.42	1.90
16:1n-5	0.31	0.38	0.06
16:2n-4	0.44	0.52	0.07
17:0	0.49	0.41	0.06
16:3n-4	0.28	0.35	0.11
16:3n-3	0.31	0.49	0.04
16:3n-1	0.28	0.46	0.05
16:4n-3	0.54	0.44	0.13
18:0	2.72	2.70	3.40
18:1n-9	30.01	29.26	39.78
18:1n-7	2.68	1.73	2.38
18:1n-5	0.37	0.47	0.07
18:2n-9	0.41	0.39	0.00
18:2n-6	15.74	16.36	23.16
18:2n-4	0.32	0.41	0.03
18:3n-6	0.00	0.52	0.08
18:3n-4	0.34	0.49	0.10
18:3n-3	5.02	3.61	5.29
18:3n-1	0.29	0.46	0.00
18:4n-3	0.94	0.53	0.39
18:4n-1	0.27	0.47	0.03
20:0	0.68	0.50	0.52
20:1n-9	0.37	0.43	0.18
20:1n-7	2.33	1.91	1.96
20:1n-5	0.47	0.41	0.17
20:2n-9	0.30	0.35	0.05
20:2n-6	0.32	0.56	0.27
20:3n-6	0.32	0.58	0.11
20:4n-6	0.59	0.48	0.32
20:3n-3	0.29	0.46	0.10
20:4n-3	0.44	0.64	0.11
20:5n-3	5.30	1.54	1.24
22:1n-11	2.37	1.56	1.94
22:1n-9	0.56	0.58	0.39
22:4n-6	0.36	1.32	0.15
22:5n-6	0.47	3.05	0.13
22:5n-3	1.31	1.09	0.31
22:6n-3	5.36	9.04	2.11
∑ SFA	16.11	15.80	16.39
∑ MUFA	43.02	39.20	48.96
Σn-9	31.93	31.01	40.58
∑ n-6	17.79	22.88	24.29
∑ n-6 LC-PUFA	2.06	5.99	0.98
∑ n-3	19.51	17.84	9.72
∑ n-3 LC-PUFA	12.70	12.77	3.87
EPA+DHA	10.66	10.58	3.35
EPA/ARA	8.91	3.22	3.88
DHA/EPA	1.0	5.9	1.7

7.3.2 BEHAVIOURAL RESPONSE TO AN EXTERNAL MECHANO-SENSORY STIMULUS

After the long-term feeding period (5 months), 9 fish per treatment were randomly selected for testing behaviour response to an external mechano-sensory stimulus. Gilthead sea bream is a schooling species and presents social hierarchies between individuals (Goldan et al., 2003; Montero et al., 2009; Arechavala-Lopez et al., 2019, 2020). Previous observations noticed a freezing-like behaviour (no movement) in gilthead sea bream under isolation in a tank (author's own observations), as also observed in other fish species under fear/anxious situations (Midttun et al., 2020). Therefore, to avoid the stress of isolation, for the present trial fish were tested in three groups per treatment, of three fish each, using a white bottom-tank of 100 L, indirectly illuminated, and covered by a dark plastic canvas to avoid disturbance during acclimation of 1 hour and testing. Each fish was gently tagged with a colour pearl attached to the base of the first ray of the dorsal fin, under anaesthesia with clove oil (4 ml/100 L). Preliminary observations showed that tagging does not compromise fish normal behaviour regarding swimming or response to the stimulus, and this type of tagging was previously used in fish behaviour trials (Alfonso et al., 2019; Cerqueira et al., 2020). For that, prior to tagging fish, preliminary test trials were conducted to observe if the typical pattern of escape response and swimming activity were influenced by tagging and acclimation time. Although these trials were only observational, non-tagged fish showed the regular swimming activity, like tagged fish after 1h of acclimation. Furthermore, all tagged fish responded to the stimulus as well as non-tagged fish, indicating that tagging and acclimation protocol did not affect the responsiveness and type of response of the fish to the stimulus. Even if clove oil and tagging may have influenced fish behaviour, the protocol was strictly maintained in all fish to accurately compare behavioural parameters among the different experimental groups. Then, fish were let to recovered and acclimated in the testing tank for 1h, according to the acclimation time used in most of locomotor activity- related studies in several fish species (Melvin et al., 2017). After this acclimation period, fish were exposed to a mechano-sensorial vibrational stimulus, which consisted in an iron pendulum of 600 g that was dropped without any additional force against the tank wall from 71 cm. Fish behaviour was monitored by a video-camera (Xiaomi Mijia 4k, China) placed above the tank and recording at 200 fps. The first 60 seconds of recording prior to stimulation were used to measure fish basal activity level (cruise speed), while the first 300 ms immediately after applying the stimulus were used for measuring the activity level (measured through swimming speed) in fish after stimulation. Furthermore, fish motion during the escape response (Domenici and Hale, 2019) was analysed at the moment of applying the stimulus using the Kinovea® software (Kinovea Project, 2016; da Silva Souza et al., 2020) software for measuring specific points on the fish, i.e. the centre of mass of the

fish (at 0.35 lengths, based on other fish species with similar body shape) (Webb, 1978; Dadda et al., 2010) and the tip of the head, as well as turning angles. The following variables were analysed:

(1) Responsiveness: responsiveness to the stimulation was defined as the number of fish that responded to the sensory-mechanical stimulus with an escape response (Domenici and Hale 2019); non-responders were considered individuals that did not display any movement in the moment of the stimulation.

(2) Escape type: Escape responses consists of a fast body muscular contraction, usually in C-shape form (stage 1), where a second contralateral contraction (stage 2) may or may not following (Domenici and Blake, 1997). Thus, escape type was defined as either single if escape response consisted of stage 1 alone (SB) or double bend (DB) if stage 2 followed stage 1 (Domenici and Blake 1997).

(3) Escape latency: Escape latency was measured as the time (in ms) from the frame F0 in which the stimulus started disappearing below the upper edge of the tank seen from the camera above until the first detectable movement by the fish. Because F0 presumably occurred a few milliseconds before the stimulus hit the side of the tank, these latency values are likely to be longer than those typically found in teleosts. They are, however, a proxy of the readiness to react since the stimulus was delivered to the side of the tank in a consistent manner.

(4) Turning rate: Turning rate was measured as the ratio between the turning angle observed in stage 1 and the duration of the stage 1 (degrees /s) (Domenici et al., 2008). The duration of this stage was measured between the frame 0 (prior to the first visible reaction) and the moment when a change in the direction of rotation of the head occurred (Domenici and Blake, 1997). Stage 1 turning angle was measured (in degrees) as the rotation of the line passing through the centre of mass and the tip of the head of each individual, during the whole duration of stage 1.

(5) Distance covered: Distance covered was defined as the distance (in cm) between the centre of mass of the fish at the frame before the first visible response and 70 ms later. The 70ms time-period was chosen as a shortest fixed time through which all fish had completed stage 2 (if DB) or stage 1 (if SB) (Meager et al., 2006; Turesson et al., 2009). This fixed time was chosen because stage 1 and 2 correspond to the period considered crucial for survival when avoiding stressors, for instance ambush predator attacks (Walker et al., 2005).

(6) Basal activity level: Basal activity level (in cm/s) was defined as the cruise speed (distance/ time) during the first 60 seconds prior to stimulation.

(7) Post-stimulation activity level: Post-stimulation activity level (in cm/s) was defined as the swimming speed (distance/ time) during the 300 ms immediately after the stimulation.

7.3.3 SAMPLES COLLECTION

For sampling procedures, fish were fasted 24h and euthanasia was carried out by an overdose of clove oil (6 ml/10 L). Fish brains were carefully removed from skull and collected at short (2.5 months of feeding) from 15 fish per treatment (n=5 per triplicate) that were chosen randomly, for analysing their fatty acid composition. Brains were also weighed to calculate cephalic index (%) as following: Brain weight (g)/ Body weight (g) *100. Additionally, telencephalons of 9 fish per treatment were micro-dissected and conserved in RNA later (Sigma-Aldrich, Madrid, Spain) for molecular analysis. The remaining brain parts were used for analysing brain fatty acid composition after long-term feeding (5 months). All samples were frozen at -80ºC until analysis.

7.3.4 DIETS AND BRAIN BIOCHEMICAL COMPOSITION

All samples were homogenized prior to analysis. Dietary protein, ash and moisture contents were additionally analysed according A.O.A.C. (2000). Lipid content of diets and brains was determined by extraction with chloroform/methanol (2:1 v/v) (Folch et al., 1957) and then transmethylated to obtain fatty acid methyl esters (FAMES) (Christie, 1989). FAMES were then separated by gas liquid chromatography (Izquierdo et al., 1990), quantified by a flame ionization detector (Finnigan Focus SG, Thermo electron Corporation, Milan, Italy) and identified comparing with previously characterized standards.

7.3.5 BRAIN GENE EXPRESSION

7.3.5.1 RNA extraction, quantification, and complementary DNA synthesis

Total RNA was obtained from fish telencephalons, using the RNeasy Mini Kit extraction (Qiagen, Hilden, Germany). Briefly, samples were homogenized using TissueLyzer-II (Qiagen) and adding TRI Reagent (Sigma-Aldrich, Sant Louis, MO, USA). Then, samples were centrifuged with chloroform at 12000g for 15 min, at 4 °C, and RNA phase was mixed with 75% ethanol and transferred into a RNeasy spin column, using RW1 and RPE buffers

(Qiagen) to purify RNA. RNA was posteriorly eluted with RNase-free water. The NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Gel Red™ staining (Biotium Inc., Hayward, CA) on 1.4% agarose electrophoresis gel were used to determine the quantity and integrity of RNA, respectively.

From the extracted RNA, complementary DNA (*cDNA*) was synthetized using iScriptcDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad).

7.3.5.2 RT-qPCR analysis

Brain (telencephalon) *mRNA* levels of the following genes were analysed from each individual: *neurogenic differentiation factor 6* (*neurod6*) and *early growth response gene 1* (egr1), related with neurogenesis (Alfonso et al., 2019); and *cerebral nitric oxide synthase 1* (*nos1*), *c-fos* and *brain-derived neurotrophic factor* (*bdnf*), markers of neural activity and plasticity (Alfonso et al., 2019), and *fatty acyl desaturase 2* (*fads2*), involved in PUFA biosynthesis (Izquierdo, 2005). Primer sequences were designed from gilthead sea bream genome using Primer3 software (Rozen and Skaletsky, 2000) (Table 7.3) and respective relative gene expressions were determined by RT-PCR in an iQ5 Multicolour Real-Time PCR detection system (Bio-Rad). *ef1*α and tubulin were used as reference gene (RG) and best RG to normalize the data was chosen by comparison of the two RGs with BestKeeper software. According to BestKeeper, both genes showed a very small variation (SD= 0.45 and 0.50 for *ef1a* and *tubulin*, respectively, below the maximum SD acceptable in the literature (Zhou et al., 2018), as well as r values close to 1 (r= 0.95 and 0.93, respectively). Therefore, although both genes were suitable for be used as RG, *ef1a* was chosen for presenting a minor slight lower SD and higher r value than tubulin. RT-PCR conditions used were the following: a first step of 3 min 30 s at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at annealing temperature for each primer (61ºC for *neurod6, egr1, nos1, c-fos* and *bdnf* and 60ºC for *fads2*, *ef1*α and *tubulin*), 30 s at 72 °C, 1 min at 95 °C, and a final denaturing step from 58 °C to 95 °C for 10 s. All PCR reactions were carried out in in a final volume of 15 µl, with 7.5 µl of Brillant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA), 0.6 µl of each primer (10 mM), 5 µl of cDNA (1:10 dilution) and 1.3 µl of MiliQ water. MiliQ water also replaced cDNA in blank control reactions. In each run, an analysis of melting curve leading to a melting peak specific for the amplified target DNA was carried out.

Table 7.3 Primers sequence of genes measured in telencephalon of gilthead sea bream fed the experimental diets

Genes	Forward	Reverse	Gene Bank Access nº
neurod6	TCGGCAGGAAAAGAAAAAGA	CACAATATCGGCTCCATGTG	XM_030401584.1
egr1	GACGAGAGGAAGAGGCACAC	ACGGGAGAGGGGTAAGAAGA	XM_030396900.1
nos1	GGTCAACAAAGAGCCTCAGC	ATTCCTCTGGCCTTCTCCAT	XM_030416914.1
c-fos	TGACCTGTCCAACTCCCTCT	GTTGCTGTTGCTTCCTCTCC	XM_030405977.1
bdnf	ATCAGCAACCAAGTGCCTTT	GCCGTCTTTTTATCCACAGC	XM_030405977.1
fads2	GCAGGCGGAGAGCGACGGTCTGTTCC	AGCAGGATGTGACCCAGGTGGAGGCAGAAG	AY055749
ef1α	CATGGTTGTGGAGCCCTTCT	TCCTGCACGACCATTCATTTC	AF184170
tubulin	ATCACCAATGCCTGCTTCGA	CTGTGGGAGGCTGGTAGTTG	AY326430,1

7.3.6 Statistical analysis

All data are presented as mean ± standard deviation (SD). Shapiro–Wilk and Levene's tests were used to test data normality and homogeneity of variances, respectively. A one-way ANOVA was applied to growth, biochemical and gene expression, using diet as factor. When necessary, transformations to normalize data were applied (Sokal and Rohlf, 1981). If normalization was not possible, for instance in some minor FA, the data were analysed using the non-parametric Kruskall-Wallis test. Relative gene expression data were normalized according to Livak method ($2^{-\Delta\Delta ct}$). Tukey (Tukey, 1949) was used as post-hoc test to compare means when significant differences were detected. When applicable, data were also subjected to the best fit regressions (linear, exponential, or logarithmic), which were also checked for significance. Behaviour response such as responsiveness and type of escape response were analysed applying a Chi-2 square test (critical CHI values =5.99 for two degrees of freedom), while numeric variables including activity levels, latency, distance, and turning rates were analysed using a two-way ANOVA, where diet was used as fixed factor and group within each diet was used as random factor. Total length of each fish (cm) was used as co-variable for all numeric behavioural variables. A significance level of less than 5% was used for all statistical analysis ($P<0.05$). Statistical treatment of the data was carried out using SPSS 21.0 for macOS 10.15 (IBM, 2020).

7.3.7 Ethical approval

All the protocols involving animals in this experiment were strictly conducted according to the European Union Directive (2010 / 63 / EU) and Spanish legislation (RD 1201 / 2005) on the protection of animals for scientific purposes, at ECOAQUA-UI from University of Las Palmas de Gran Canaria (Canary Islands, Spain). The procedures used were approved by the Bioethical Committee of the University of Las Palmas de Gran Canaria (Ref OEBA-ULPGC-21/ 2018 & OEBA-ULPGC-07/2019).

7.3 RESULTS

7.4.1 GROWTH PERFORMANCE AND CEPHALIC INDICES

After 5 months of feeding the experimental diets, body weight of sea bream fed PO diet was significantly lower ($P<0.05$) than those fed FO or DD diets (Figure 7.1A). No significant differences were observed in cephalic index after 2.5 months of feeding (data not shown), whereas after 5 months of feeding, cephalic index was higher in fish fed DD and PO diets compared to those fed the FO ($P<0.05$; Figure 7.1B).

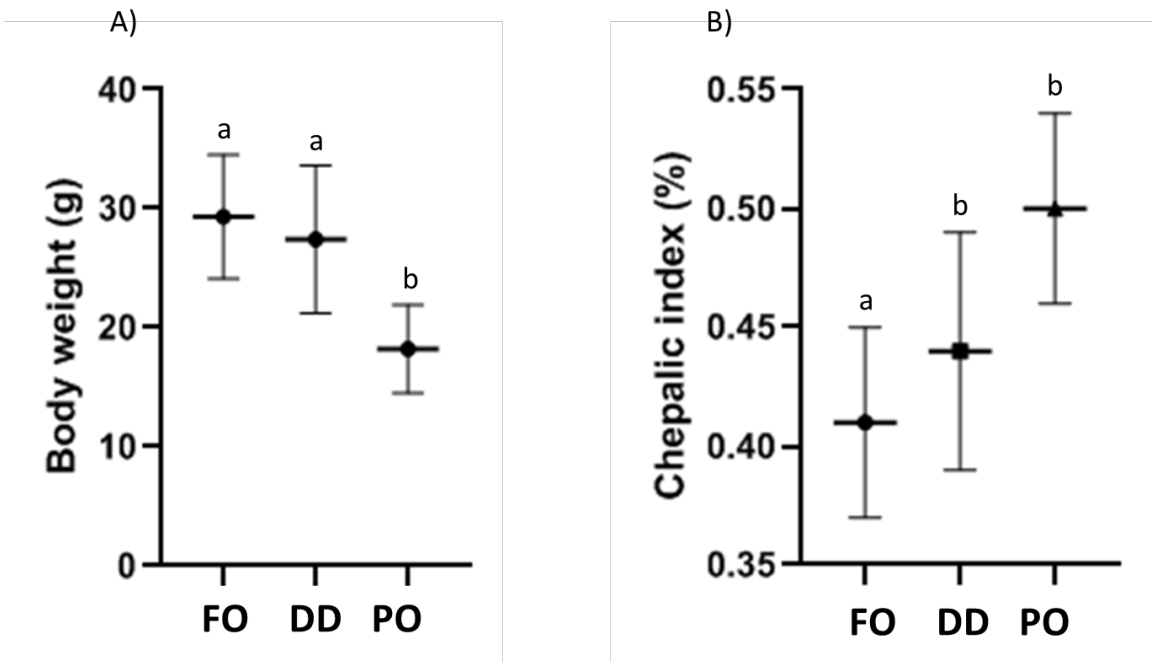

Figure 7.1. Growth (A) and cephalic index (B) of gilthead sea bream fed the different experimental diets for 5 months. Different letters above bar errors denote significant differences among the experimental groups ($P<0.05$).

7.4.2 BEHAVIOURAL RESPONSE TO AN EXTERNAL MECHANO-SENSORY STIMULUS

All individuals (9/9) responded to stimulus with a typical escape response in fish fed FO diet, while 7/9 and 8/9 responded in those fed DD and PO, respectively (Chi^2= 2.25; P>0.1; Figure 7.2A). From the responding fish in each treatment, 33% presented a SB escape response while 67% displayed a DB escape response in those fed FO; 29% and 71%, respectively in those fed DD; and 13% and 87%, respectively in those fed PO (Chi^2= 1.05; P>0.05; Figure 7.2B). However, these differences were not significant (P>0.05) among treatments or among groups of fish. Similarly, no significant differences (P>0.05) were recorded in distance covered (P=0.3; Figure 7.2C), turning rate during escape response (P=0.9; Figure 7.2D) and escape timing (P=0.1; Figure 7.2E). However, there was a tendency for escape latency to be the longest in fish fed PO and DD diets (P=0.1; Figure 7.2E), which is in line with the significant correlation found between escape latency and individual brain n-6 DPA contents, although with a low r^2 value (r^2=0.18; P=0.03; Figure 7.3). In addition, a total of two of the fish (as mentioned above) showed no response and were not included in the original correlation test. Therefore, an additional correlation was performed by considering these fish conservatively, i.e., assigning them the highest latency we recorded (i.e., 210 ms), which lead to a more significant correlation value (P=0.01; r^2 =0.23). No significant differences were observed for activity basal (P=0.8) and after stimulation (P=0.6) levels (Figure 7.4).

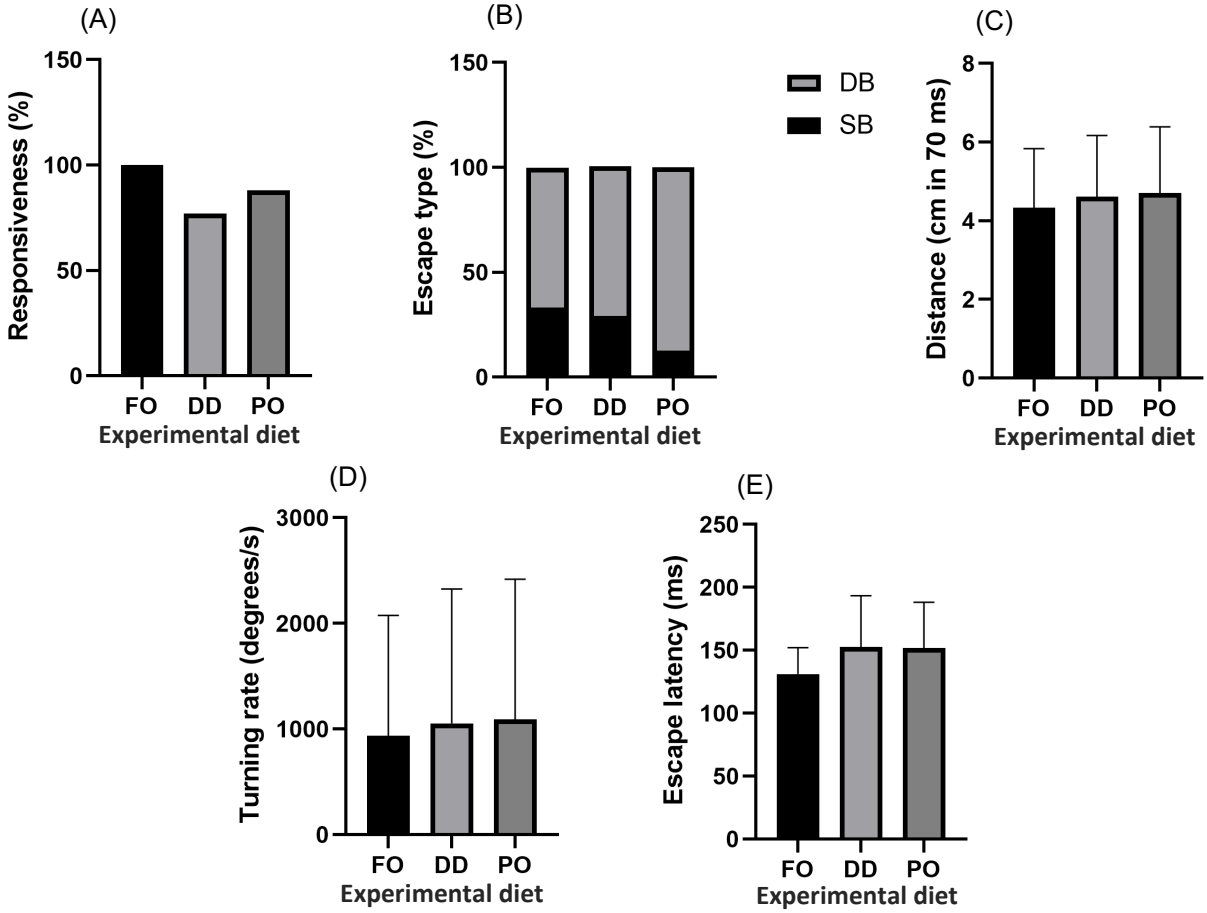

Figure 7.2. Behavioural response to an external mechano-sensory stimulus of gilthead sea bream fed the different experimental diets for 5 months. (A) responsiveness; (B) escape response type; (C) distance covered after stimulus; (D) turning rate during escape; (E) escape latency.

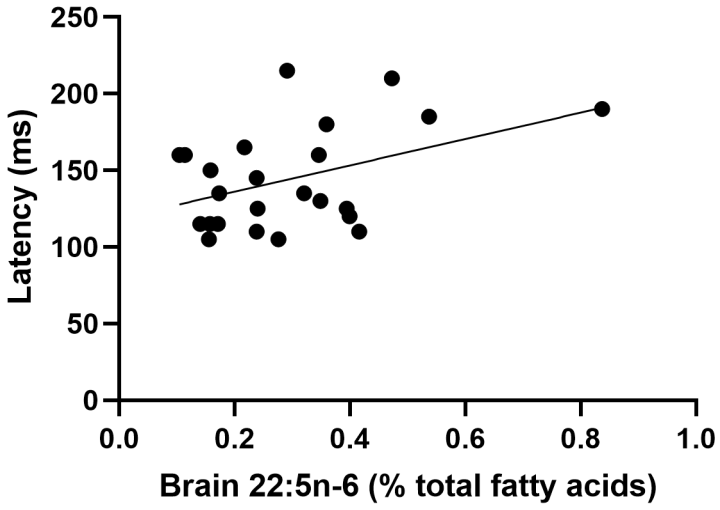

Figure 7.3. Linear correlation between brain n-6 docosapentaenoic acid and escape latency (P=0.03).

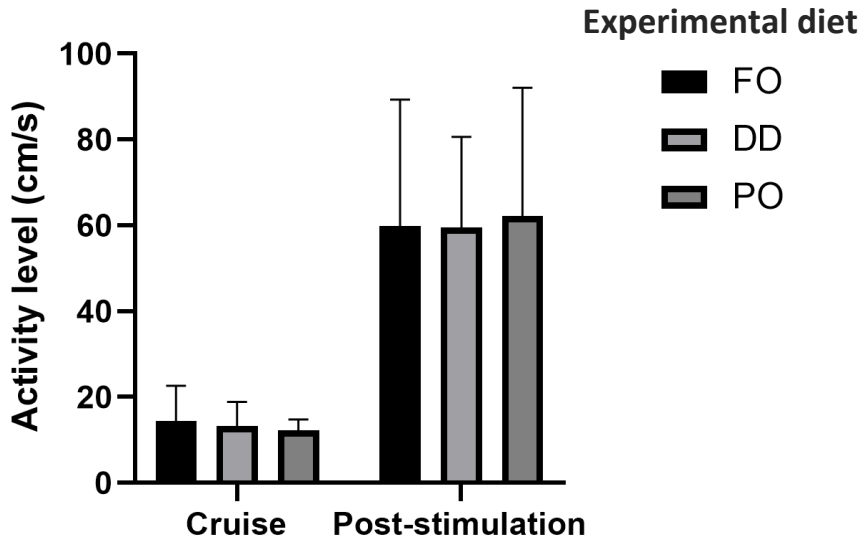

Figure 7.4. Activity basal and after stimulation level in gilthead sea bream fed the different experimental diets for 5 months.

7.4.3 BRAIN FATTY ACID COMPOSITION

No differences were observed in brain lipid content, either at 2.5 or 5 months of feeding with the experimental diets (Tables 7.4 and 7.5).

After 2.5 months of feeding, brain fatty acid profiles of sea bream were similar among fish fed the different diets, regardless the dietary differences (Table 7.4). As in diet, FO fish had more EPA, EPA/ARA and EPA/DHA and DD fish had a higher content in n-6 DPA. However, PO fish was not lower in n-3, n-3 LC-PUFA or EPA+DHA as it occurred in the diet. Moreover, 18:2n-9, 18:3n-6 and 20:3n-6, fatty acids that are products from FADS2 activity, were higher in PO fish, despite their low dietary values ($P<0.05$; Tables 7.4 and 7.5). These tendencies in brain fatty acid profiles were even stronger after 5 months of feeding the experimental diets (Table 7.5). Thus, products of FADS2, as well as products of elongases, were high in brain of fish fed the PO diet (18:2n-9, 18:3n-6, 20:3n-6, 20:4n-6). Besides, the FADS2 substrates 18:1n-9 and 18:2n-6 were low, contrary to their high dietary content ($P<0.05$; Tables 7.4 and 7.5). Furthermore, PO fish also had the highest DHA content in brain, despite the lowest contents of this fatty acid in PO diet (Table 7.5). Accordingly, the EPA level in brain of PO fish was similar to that of sea bream fed the other two diets, regardless the lowest EPA content in PO diet (Table 7.5). Interestingly, brain of sea bream fed the DD diet, not only was the highest in n-6 DPA, in agreement with its high dietary levels, but it was also highest in ARA after 2.5 months of feeding (Table 7.4) and higher than in fish fed the FO diet after 5 months (Table 7.5), regardless the low ARA contents in DD diet.

Table 7.4. Brain lipid content (% wet weight) and fatty acid composition (% total fatty acids) of gilthead sea bream fed the different experimental diets after 2.5 months

	Diets		
	FO	DD	PO
Lipids	7.88±0.59	8.62±0.50	8.65±1.04
Fatty acid			
14:0	0.48±0.02	0.21±0.11	0.31±0.05
14:1n-7	0.01±0.00	0.07±0.06	0.02±0.00
14:1n-5	0.02±0.00	0.01±0.00	0.01±0.00
15:0	0.08±0.01	0.08±0.01	0.0±0.01
15:1n-5	0.01±0.00	0.02±0.01	0.01±0.00
16:0ISO	0.02±0.00	0.02±0.01	0.01±0.00
16:0	14.56±0.81^{ab}	16.49±2.54^{a}	12.55±0.21^{b}
16:1n-7	1.82±0.10	1.77±0.24	1.47±0.04
16:1n-5	0.05±0.00	0.04±0.01	0.03±0.00
16:2n-4	0.04±0.00^{a}	0.04±0.01^{ab}	0.02±0.00^{b}
17:0	0.07±0.00^{a}	0.05±0.01^{ab}	0.04±0.00^{b}
16:3n-4	0.13±0.01^{a}	0.11±0.01^{ab}	0.10±0.00^{b}
16:3n-3	0.03±0.00	0.02±0.01	0.02±0.00
16:3n-1	4.77±0.34	4.11±0.59	4.18±0.27
16:4n-3	0.69±0.04	0.61±0.06	0.67±0.05

Table 7.4. Brain lipid content (% wet weight) and fatty acid composition (% total fatty acids) of gilthead sea bream fed the different experimental diets after 2.5 months (continued)

	Diets		
	FO	DD	PO
16:4n-1	0.40±0.03	0.36±0.03	0.37±0.03
18:0	11.22±0.63	12.01±0.98	9.92±0.38
18:1n-9	22.73±1.24	22.42±0.64	22.18±1.09
18:1n-7	1.63±0.11	1.54±0.07	1.51±0.07
18:1n-5	0.05±0.00	0.07±0.01	0.04±0.00
18:2n-9	0.47±0.03^{b}	0.47±0.00^{b}	0.63±0.02^{a}
18:2n-6	3.89±0.27	3.82±0.30	5.70±1.02
18:2n-4	0.04±0.00^{a}	0.02±0.01^{b}	0.02±0.00^{b}
18:3n-6	0.23±0.03^{b}	0.23±0.04^{b}	0.45±0.03^{a}
18:3n-4	0.08±0.02	0.04±0.01	0.05±0.01
18:3n-3	0.76±0.07	0.54±0.06	0.85±0.23
18:3n-1	0.01±0.00	0.01±0.01	0.00±0.00
18:4n-3	0.43±0.01	0.31±0.05	0.37±0.02
18:4n-1	0.13±0.01	0.11±0.02	0.13±0.01
20:0	0.31±0.03	0.29±0.04	0.29±0.01
20:1n-9	0.13±0.01	0.11±0.01	0.13±0.02
20:1n-7	0.76±0.05	0.62±0.06	0.69±0.07
20:1n-5	0.14±0.01	0.12±0.00	0.10±0.01
20:2n-9	0.26±0.01	0.26±0.01	0.35±0.02
20:2n-6	0.15±0.01	0.16±0.01	0.21±0.02
20:3n-9	0.05±0.02	0.07±0.05	0.07±0.02
20:3n-6	0.28±0.02^{b}	0.34±0.06^{b}	0.61±0.01^{a}
20:4n-6	1.31±0.07^{b}	1.92±0.02^{a}	1.48±0.09^{b}
20:3n-3	0.06±0.01	0.07±0.01	0.07±0.01
20:4n-3	0.19±0.01	0.10±0.05	0.15±0.01
20:5n-3	3.25±0.33^{a}	1.98±0.17^{b}	2.43±0.13^{ab}
22:1n-11	0.27±0.04	0.17±0.03	0.21±0.04
22:1n-9	0.46±0.09	0.41±0.05	0.41±0.03
22:4n-6	0.14±0.01	0.22±0.04	0.16±0.01
22:5n-6	0.10±0.02^{b}	1.01±0.20^{a}	0.27±0.14^{b}
22:5n-3	1.22±0.11^{a}	0.63±0.09^{b}	0.98±0.05^{ab}
22:6n-3	26.07±2.72	25.93±2.80	29.70±1.71
ΣSFA	26.75±1.46	29.14±3.19	23.17±0.51
ΣMUFA	28.07±1.59	27.36±0.53	26.79±1.34
Σn-9	23.97±2.25	23.63±1.20	23.65±1.93
Σn-6	6.11±0.47	7.70±0.91	8.88±1.88
n-6 LC-PUFA	1.83±0.08	3.43±0.38	2.57±0.11
Σn-3	32.70±5.53	30.19±5.47	35.25±2.89
n-3 LC-PUFA	30.79±5.46	28.71±5.25	33.34±3.22
EPA+DHA	29.32±3.04	27.91±2.91	32.13±1.84
EPA/ARA	2.48±0.21^{a}	1.03±0.08^{c}	1.64±0.03^{bc}
EPA/DHA	0.12±0.00^{a}	0.08±0.01^{b}	0.08±0.00^{b}

Different letters denote significant differences among the experimental groups at the end of the trial ($P<0.05$)

Table 7.5. Brain lipid content (% wet weight) and fatty acid composition (% total fatty acids) of gilthead sea bream fed the different experimental diets after 5 months

	Diets		
	FO	DD	PO
Lipids	11.30±2.70	12.18±2.45	11.65±3.18
Fatty acid			
14:0	0.90±0.22	0.86±0.24	0.74±0.30
14:1n-7	0.02±0.00	0.02±0.01	0.03±0.01
14:1n-5	0.04±0.01^{b}	0.03±0.00^{b}	0.10±0.03^{a}
15:0	0.15±0.03^{a}	0.15±0.02^{a}	0.09±0.02^{b}
15:1n-5	0.02±0.03	0.01±0.00	0.02±0.01
16:0ISO	0.02±0.01^{a}	0.02±0.01^{a}	0.01±0.01^{b}
16:0	17.28±2.31	15.97±1.05	18.00±1.63
16:1n-7	1.98±0.27^{a}	1.93±0.41^{a}	1.22±0.33^{b}
16:1n-5	0.07±0.01	0.07±0.01	0.07±0.02
16:2n-6	0.01±0.01^{ab}	0.00±0.00^{b}	0.01±0.01^{ab}
16:2n-4	0.10±0.02^{a}	0.10±0.04^{a}	0.03±0.02^{b}
17:0	0.09±0.02^{a}	0.09±0.02^{a}	0.04±0.01^{b}
16:3n-4	0.15±0.02^{a}	0.14±0.03^{a}	0.07±0.01^{b}
16:3n-3	0.05±0.01	0.05±0.01	0.06±0.02
16:3n-1	0.69±0.22^{b}	0.59±0.26^{b}	1.22±0.17^{a}
16:4n-3	0.32±0.12^{b}	0.27±0.14^{b}	0.69±0.30^{a}
16:4n-1	0.05±0.03^{b}	0.07±0.04 ^{ab}	0.10±0.02^{a}
18:0	9.85±2.55^{b}	8.77±2.49^{b}	14.76±1.23^{a}
18:1n-9	27.64±3.21^{a}	27.68±4.89^{a}	16.11±2.91^{b}
18:1n-7	2.01±0.20^{a}	2.00±0.25^{a}	1.58±0.27^{b}
18:1n-5	0.07±0.01	0.07±0.01	0.07±0.01
18:2n-9	0.53±0.07^{b}	0.47±0.08^{b}	0.66±0.04^{a}
18:2n-6	8.64±1.73^{a}	9.50±2.69^{a}	3.80±1.91^{b}
18:2n-4	0.07±0.01^{a}	0.07±0.02^{a}	0.02±0.00^{b}
18:3n-6	0.18±0.03^{b}	0.17±0.04^{b}	0.21±0.09^{a}
18:3n-4	0.06±0.01^{a}	0.07±0.03^{a}	0.03±0.01^{b}
18:3n-3	2.02±0.50^{a}	2.19±0.71^{a}	0.64±0.41^{b}
18:3n-1	0.01±0.00	0.01±0.00	0.01±0.01
18:4n-3	0.29±0.07^{ab}	0.31±0.10^{a}	0.18±0.11^{b}
18:4n-1	0.05±0.01	0.05±0.01	0.24±0.61
20:0	0.20±0.03^{a}	0.21±0.04^{a}	0.12±0.02^{b}
20:1n-9	0.12±0.03^{a}	0.14±0.03^{a}	0.06±0.03^{b}
20:1n-7	1.29±0.29^{a}	1.42±0.38^{a}	0.48±0.16^{b}
20:1n-5	0.08±0.01	0.08±0.01	0.07±0.04
20:2n-9	0.09±0.01	0.08±0.01	0.10±0.02
20:2n-6	0.34±0.08	0.39±0.11	0.33±0.64
20:3n-9	0.02±0.01^{a}	0.01±0.00^{a}	0.01±0.01^{b}
20:3n-6	0.11±0.01^{b}	0.11±0.01^{b}	0.18±0.02^{a}
20:4n-6	0.94±0.11^{c}	1.26±0.36^{b}	1.64±0.25^{a}
20:3n-3	0.11±0.01^{a}	0.11±0.03^{a}	0.06±0.01^{b}
20:4n-3	0.19±0.05^{ab}	0.21±0.04^{a}	0.09±0.02^{c}
20:5n-3	3.42±0.61	3.19±0.47	2.93±0.57
22:1n-11	0.39±0.07^{a}	0.43±0.11^{a}	0.14±0.07^{b}
22:1n-9	0.21±0.03	0.25±0.04	0.21±0.06
22:4n-6	0.06±0.02^{b}	0.07±0.01^{ab}	0.08±0.03^{ab}
22:5n-6	0.16±0.04^{c}	0.49±0.16^{a}	0.29±0.05^{b}
22:5n-3	0.72±0.18^{a}	0.65±0.15^{a}	0.41±0.06^{b}
22:6n-3	18.19±3.50^{b}	19.16±6.19^{b}	32.02±5.54^{a}
ΣSFA	28.47±4.57^{b}	26.04±3.19^{b}	33.75±1.98^{a}
ΣMUFA	33.95±3.84^{a}	34.13±5.99^{a}	20.14±3.57^{b}
Σn-9	28.49±3.18^{a}	28.50±4.85^{a}	17.08±2.95^{b}
Σn-6	10.09±1.70^{a}	11.61±2.24^{a}	6.20±1.84^{b}
n-6 LC-PUFA	1.60±0.14^{b}	2.33±0.41^{a}	2.51±0.61^{a}

Table 7.5. Brain lipid content (% wet weight) and fatty acid composition (% total fatty acids) of gilthead sea bream fed the different experimental diets after 5 months (continued)

	Diets		
	FO	DD	PO
Σn-3	23.28±3.95^{b}	23.95±5.74^{b}	36.44±5.70^{a}
n-3 LC-PUFA	22.63±3.85^{b}	23.32±6.00^{b}	35.51±3.57^{a}
EPA+DHA	21.61±3.77^{b}	22.35±5.86^{b}	34.95±5.69^{a}
EPA/ARA	3.66±0.65^{a}	2.74±0.88^{b}	1.81±0.40^{c}
EPA/DHA	0.19±0.03^{a}	0.18±0.06^{a}	0.09±0.02^{b}

Different letters denote significant differences among the experimental groups at the end of the trial (P<0.05)

7.4.4 TELENCEPHALON GENE EXPRESSION

No significant differences were observed for the relative expressions of *egr1* or *c-fos* among fish fed the experimental diets (Figure 7.5). However, sea bream fed the PO diet showed an upregulation of telencephalon *mRNA* levels of *neurod6*, *bdnf* and *nos1* (P<0.05) compared with those fed FO, but not with those fed DD diet (Figure 7.5). Additionally, a tendency to present higher relative expression of *fads2* was also noted in fish fed PO (P=0.1), particularly when compared with those fish fed DD (with the highest dietary DHA) (Figure 7.5). Indeed, significant correlations were found between individual telencephalon DHA content and the respective *mRNA* levels of these genes (*neurod6*: r^2= 0.23, P=0.01; *bdnf*: r^2= 0.27, P= 0.01; *nos1*: r^2= 0.21, P= 0.02; *fads2*: r^2= 0.24, P= 0.01; Figure 7.6).

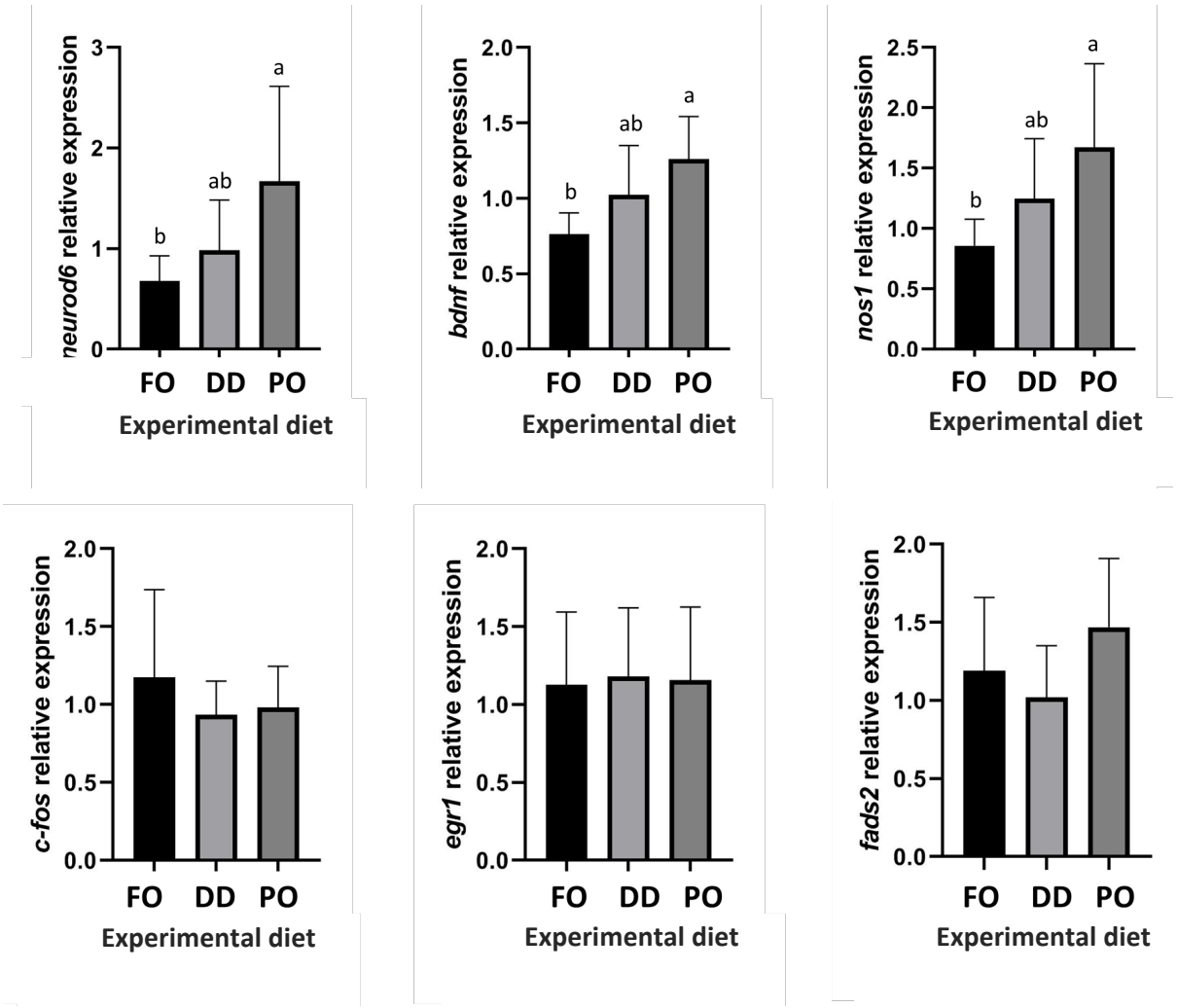

Figure 7.5. Relative gene expression ($2^{-\Delta\Delta ct}$) of neural markers in brain of gilthead sea bream fed the different experimental diets after 5 months; *neurod6, neurogenic differentiation factor 6; bdnf, brain-derived neurotrophic factor; nos1, nitric oxide synthase 1 (neuronal); egr1, early growth response gene; fads2, fatty acyl desaturase 2.*

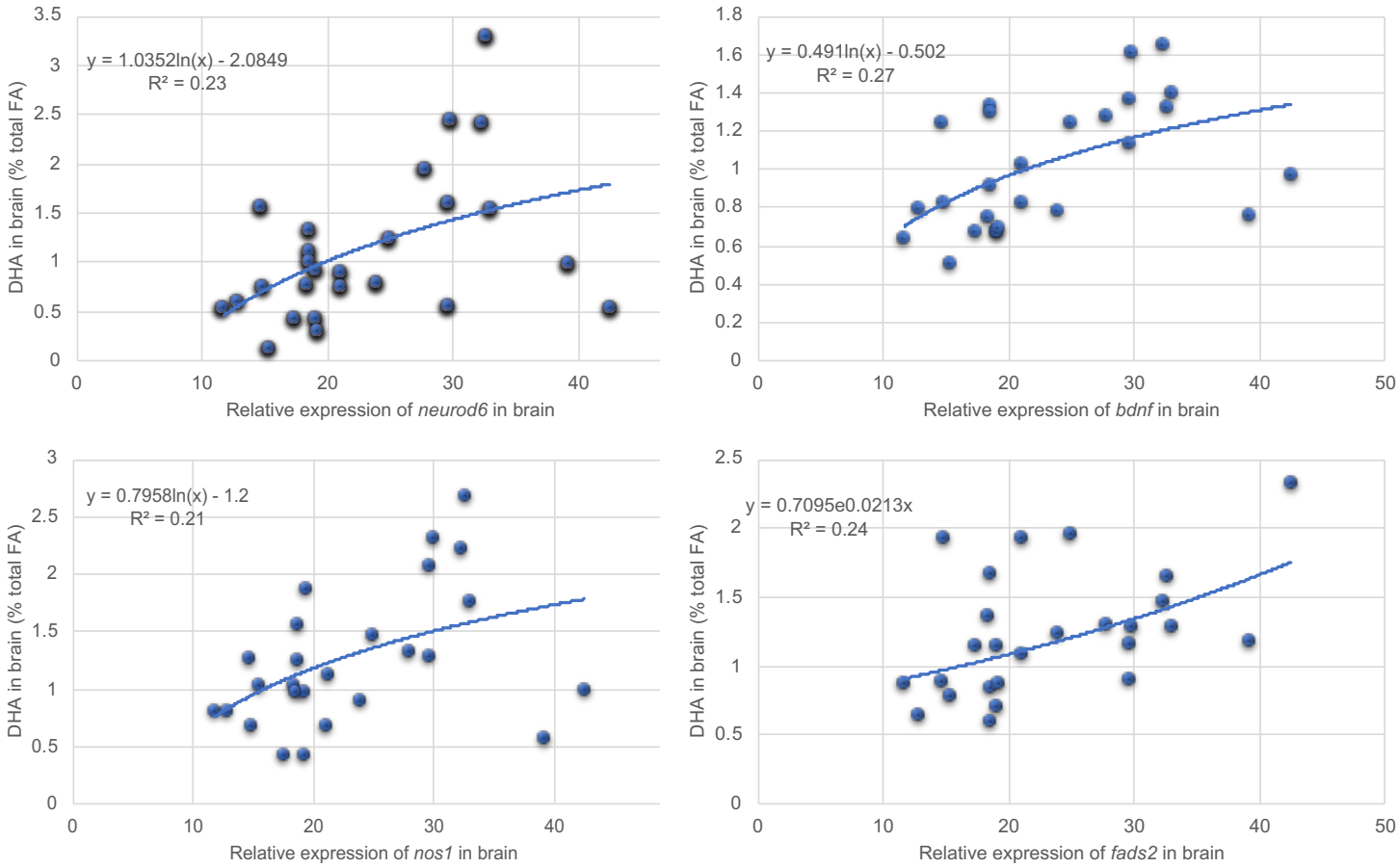

Figure 7.6. Significant correlations between individual telencephalon DHA content and the *mRNA* levels of neural markers.

7.4 DISCUSSION

Essential fatty acids, in particular the n-3 LC-PUFA DHA and EPA, are of key importance in fish metabolism, health, and development. After a long period of feeding of 5 months, gilthead sea bream fed the PO diet, with low EPA and DHA levels, showed reduced growth compared to those fed FO or DD diets, with high DHA. In fact, only 2.5 months of feeding was sufficient to reduce growth in sea bream fed PO (Carvalho et al., 2020), suggesting a marginal deficiency in these fish, in agreement with other marine species, for which these fatty acids are essential (reviewed in Izquierdo, 2005). Furthermore, this EFA-deficiency was likely to be more related to a dietary DHA deficiency, since the low inclusion of EPA in DD diet did not cause a decrease in growth performance in fish juveniles, when compared to those fed FO (with high EPA and DHA). Although both DHA and EPA compete for the incorporation into cell membranes phospholipids, the former has a higher function as growth promoter and stress resistance than the latter (Watanabe et al., 1989; Watanabe and Kiron, 1994), explaining the effect in growth performance associated to dietary DHA rather than EPA.

Indeed, the present results suggest a selective incorporation and retention of DHA over EPA into brain lipids of sea bream juveniles. Accordingly, in mammals, DHA and ARA are activated and incorporated into phospholipid cell membranes, while EPA is catabolised (Chen and Bazinet, 2015). This could explain the lower efficiency of EPA compared to DHA in regulating membrane phospholipids fluidity, important for neural cells growth, health, and normal function (Bowel and Clandinin, 2002). The results provided in the literature suggest a similar mode of action in fish and highlight the higher biological importance of DHA and, possibly ARA, compared to EPA as modulator of neural function (Mourente, 2003). Indeed, contrary to the lowest dietary levels, brain contents of these two FA were the highest in brains of fish fed PO diets, which was evidenced more at 5 months of feeding than 2.5, suggesting a very high retention capacity of sea bream brains when fed EFA-deficient diets for prolonged time. This, added to the highest transcriptional levels of telencephalon *fads2*, as well as with the increased contents of other products of FADS2 activity (18:2n-9, 20:2n-9, 18:3n-6 and 20:3n-6) suggests an activation of the biosynthetic pathways in response to a DHA deficiency, which seem to be stronger over the time. This increase in desaturation products is also consistent with the decrease of the desaturation substrates in these fish, particularly of 18:1n-9 and 18:2n-6. It is well recognised that marine species have lower ability to endogenously synthesize LC-PUFA from their C_{18} precursors due to a limited activity of desaturases and elongases, for instance FADS2 (Izquierdo and Koven, 2011). Many studies reported an upregulation of *fads2* expression or synthesis of its products in many tissues of fish fed low EFA diets, for instance in alternative diets where FO was replaced by EFA-devoid ingredients (Houston et al., 2017; Carvalho et al., 2018, 2020b), but few studies have focused on the

expression of this gene in brain. Indeed, brain is often the organ with the highest *fads2* transcription in many marine fish (Monroig et al., 2013). In gilthead sea bream, FADS2 have both Δ6 and Δ8 desaturase activities, but weak Δ5 activity (Monroig et al., 2011). This is the main reason why in most of the studies there is an increased in Δ6 products but the failure in their further desaturation to ARA, EPA, or DHA to meet the requirements. In agreement, in the freshwater striped snakehead (*Channa striata*), juveniles fed a diet low in FO and high in linseed oil, brain *fads2* was upregulated (Kuah et al., 2015). In contrast, expression of *fads2* was increased in liver but not in brain in meagre fed diets where FO was replaced by VO (Silva-Brito et al., 2016). These differences suggest the species and tissue-specificity of FADS2 expression and activity in fish in response to dietary FA composition but could possibly be also influenced by the duration of the deficiency period (being 2 months in the latter study compared to a prolonged period of 5 months in our study). Interestingly, the high brain ARA contents observed in fish fed DD diet, contrary to the lower dietary levels of this FA might also indicate a retro-conversion of the high dietary n-6 DPA to ARA (Ganuza et al., 2008; Carvalho et al., 2020).

Furthermore, this high retention capacity of DHA to compensate dietary deficiency could, at least partially, explain the lack of effects in behavioural response among sea bream fed the different diets. For instance, in sea bream larvae, high dietary DHA boosted escape behaviour, which was partly related with the increase in the neural activity of the Mauthner cells (Benítez-Santana et al., 2014). Besides, escape swimming speed was slower in fish fed low EPA and DHA levels, in response to a similar stimulus to that of the present study (Benítez-Santana et al., 2014). Essential fatty acid deficiency was also related to an impaired visual ability in larvae of this species (Benítez-Santana et al., 2007) and herring (Bell et al., 1995), and to a delayed development of normal behaviour in yellowtail (Masuda et al., 1998). These results suggest that fish larvae are more susceptible to an EFA-deficiency than juveniles, probably due to their higher requirement for the faster growth rates and the incomplete neuronal development without selective retention mechanisms for dealing with nutritional deficiency. Brains of fish juveniles are fully developed, being a very conservative tissue and, consequently, less sensitive to a nutritional EFA-deficiency, which could explain that neural circuits are not primarily affected and most of vital brain function is preserved. Indeed, in the above-mentioned studies with sea bream larvae, clear reductions in brain EPA and, particularly, DHA contents were noticed (Benítez-Santana et al., 2007; 2014), whereas the opposite was noticed in the present study. In agreement with this finding, in rainbow trout and European sea bass (*Dicentrarchus labrax*) juveniles, brain tissue showed the highest DHA concentration, despite the low dietary concentration of this FA, displaying the greatest capacity to regulate and preserve brain functionality (Skalli et al., 2006; Benedito-Palos et al., 2010; Kuah et al., 2015).

This may allow higher percentages of replacement of FO with alternative lipid sources with lower n-3 LC-PUFA contents in juveniles than in larvae diets, without affecting fish behaviour and brain function.

Indeed, in the present study, an upregulation of genes involved in neurogenesis and neural activity, including *neurod6*, *bdnf* and *nos1* was demonstrated in telencephalons of fish fed PO diet that was positively correlated with brain DHA content (highly retained in these fish). These results suggest an apparent increase in neurogenesis and neural activity in those fish. Despite the important roles that EFA play in nervous system in vertebrates, studies on the effect of EFA on modulation of neurogenesis and neural plasticity in fish juveniles are scarce. In agreement, Turkmen et al. (2017) demonstrated for the first time in fish an increased proliferative activity of retinal ganglion cells (neural cells) associated to an increment of dietary LC-PUFA, particularly DHA, by increasing BrdU positive cells, in gilthead sea bream larvae. Also, DHA is known to promote neurogenesis and neural differentiation in mammals (Kawakita et al. 2006; Katakura et al., 2009).

Despite the selective incorporation and retention of DHA in fish brains, there is non-significant tendency for an increase in fish escape latency in response to the external stimulus in sea bream fed DD or PO diets. These results suggest a slower reactivity to the stimulus in these fish, which might be related to a lower speed in the translation of the neural signal to activate escape response. Furthermore, this activation response may be controlled by alternative neural commands (i.e., non-Mauthner cells), since it is known that non-Mauthner cell responses show longer latencies than Mauthner cell responses (Domenici and Hale 2019; Hecker et al., 2020). In line with this tendency, a significant (P=0.03) correlation was observed between escape and n-6 DPA in brain, which showed the highest concentration in fish fed with PO diet and, particularly DD diet, reflecting the dietary composition. The effect of excessive n-6 DPA in fish brain is poorly studied because this FA is not found in high concentrations in fish tissues or farmed fish fed FO or VO diets. However, with the increase of new emergent ingredients, for instance microalgae, often with high n-6 DPA contents in some species, this concern might arise in the future. Therefore, the present study suggests, for the first time in fish, a possible influence of high neural n-6 DPA on the behavioural response of fish. However, this effect needs further corroboration and deserves more investigation in the future. In mammals, n-6 DPA competes with DHA for the incorporation in neural phospholipids and, therefore, an increase in n-6 DPA is observed in DHA-deficient individuals, which is correlated with poorer cognitive function and mental health in general (García-Calatayud et al., 2005). This is because DPA and DHA present different biochemical structures, with DPA lacking a double bond at the end of the chain methyl, affecting fluidity of

cell membranes, and inducing alterations in neurotransmission which may lead to behavioural deficits (Wainwright, 2002; Eldho et al., 2003; Aid et al., 2003; García-Calatayud et al., 2005).

7.6 CONCLUSIONS

The present study showed that FO replacement by PO in diets for juvenile gilthead sea bream, namely the reduction in EPA and DHA, stimulates neurogenesis and neural activity in sea bream upregulating *neurod6*, *bdnf* and *nos1*, possibly in relation to the increased DHA content in brain. These results also corroborate the importance of DHA for neural function. In addition, the low dietary EPA and DHA in PO diet activated LC-PUFA synthesis pathways in brain, as denoted by an increased telencephalic *fads2* expression and the presence of desaturation and elongation products in brain fatty acid profiles. Although this increased LC-PUFA synthesis was not sufficient to maintain fish growth, it protected neural circuits and locomotor behavioural response, which was not primarily affected by EFA-deficiency, as shown by the lack of effects of PO in fish when performed a behaviour test. Moreover, a slight but significant positive linear relation between n-6 DPA in brain on non-locomotor behaviour (escape latency) of fish was observed for the first time. Therefore, more investigation is needed to corroborate this possible effect of n-6 DPA on fish behaviour as alternative dietary lipid sources arise in the industry, in agreement with the negative effect of n-6 DPA on behavioural aspects that has been previously recorded in mammals.

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CHAPTER 8. GENERAL DISCUSSION AND FUTURE QUESTIONS OPENED

8.1 WHAT ARE THE MAIN BOTTLENECKS FOR SUSTAINABLE AQUACULTURE DEVELOPMENT CONCERNING FISH NUTRITION?

Global population is expected to increase up to 9.5 billion people by 2050 and fish consumption is the major source of n-3 LC-PUFA for human nutrition and health (FAO, 2020). With wild fisheries stagnated in the last decades, without expectation of increasing in the near future, aquaculture is the best and, possibly, only option to meet the global demand of n-3 LC-PUFA. Therefore, aquaculture sustainable expansion is of foremost importance.

In an aquaculture farm, fish feeds are, if not the most, one of the most important components behind a high productivity, economic viability, environmental sustainability, and a safe and high-quality product for delivering to consumers. On one hand, aquafeeds need to have sufficient quality to optimize fish performance, feed utilization and health. Besides, high-quality feeds are necessary to avoid environmental pollution through feed and fish wastes, while supporting final product nutritional (and organoleptic) characteristics to meet consumer demand and expectations. On the other hand, since feed constitutes one of the major costs in aquaculture farming, representing more than 40% of the global costs, a cost-effective feed is mandatory to achieve economic viability of the farm. Among all the nutrients that need to be present in fish diets to meet fish nutritional requirements, n-3 LC-PUFA are one of the most important, both for fish and humans, due to the important functions they play in all vertebrates (Tocher, 2010). They are also particularly important in marine species since their limited capacity for produce these FA endogenously (due to the low or absence activity of desaturases and elongases). Therefore, aquafeeds ingredients should supply the needed levels of n-3 LC-PUFA for meeting each species requirement, as well as for maintaining the levels of these FA in fish tissues, including in flesh, to guarantee the health benefits in humans associated to fish consumption. The unsustainable use of finite wild fish resources (the main origin of FM and FO) led to the decrease in the supply and an increase in the price of these traditional feed ingredients (FAO, 2020). To maintain economic viability of fish farms and to increase sustainability parameters of the sector, the industry was forced to decrease the contents of FM and FO in aquafeeds and to replace them by alternative ingredients (FAO, 2020). However, whereas great improvements in reducing and replacing FM in marine fish diets were accomplished in the last decades, the total replacement of FO is still the major

challenge (Colombo and Turchini, 2021). Consequently, the most recent data showed that aquaculture feed industry is still responsible for 75% of the use of the total FO production (Colombo and Turchini, 2021).

This is because few ingredients are natural rich sources of n-3 LC-PUFA as they are the marine organisms, including fish. Even tough, during more than 2 decades, research and industry have joined efforts for finding alternative lipid sources that could be combined with FO, decreasing the dependency on this ingredient, and, consequently, increasing environmental and economical sustainability of the feeds. Plant oils and terrestrial animal oils, such as poultry oil, were intensively studied because they are readily and constantly available in the market, are cheaper than FO, and represent a good energy source for fish, rich in in SFA and MUFA (Turchini et al., 2009). However, both lack n-3 LC-PUFA (Turchini et al., 2009; Nasopoulpou and Zabetakis, 2012). Therefore, compiling the available information in the literature, the main conclusion is that the amount of FO that could be replaced by these conventional, already used, alternative lipid ingredients, should consider several factors, including species environment, feed habits and nutritional requirements. In which concerns marine species, when EFA requirements of the species are met, through FM or other n-3 LC-PUFA source, high percentages of FO (60-100%) can be replaced by VO or PO, without affecting fish productivity in almost all species (Turchini et al., 2009; Nasopoulpou and Zabetakis, 2012). However, the lower the dietary FM content, the more challenging the FO replacement, particularly in carnivorous marine fish (Drew et al., 2007; Torrecillas et al., 2007a,b). Furthermore, although some conventional ingredients may have not a negative impact on fish performance, what remained to be unsolved when using n-3 LC-PUFA-devoid ingredients, is the marked reduction of n-3 LC-PUFA in fish fillets and the consequent decrease of the nutritional value for the consumer, irrespective of the species (Turchini et al., 2009). To solve all these issues, a new era of research was opened in aquaculture nutrition in the recent years, for finding novel n-3 LC-PUFA-rich sources for aquaculture. Fish by-products, lower trophic marine organisms, including zooplankton, microalgae, and genetic modified plants, were possible options due to their high contents in EPA and/or DHA. Microalgae appear to be the most promising alternative to the traditional marine derived ingredients since they are the primary producers of EPA and DHA in the marine ecosystems and can become a cost competitive product alternative to fish oil over the next years. Therefore, they are attracting considerable attention and several commercial products are available or emerging in the market, particularly as a source of n-3 LC-PUFA for larval and juveniles' nutrition. However, their accurate evaluation on fish performance is mandatory for validating their potential (Tibbetts, 2018; Shah et al., 2018). Furthermore, some microalgae species often present high contents of other FA, in particular the n-6 DPA, that are not

commonly found on FO or VO and, therefore, its study in fish physiology, metabolism and health is necessary to validate the real potential of microalgae as lipid source and total FO replacer.

8.2 ARE MICROALGAL PRODUCTS EQUALLY EFFECTIVE IN LARVAL AND JUVENILE PERFORMANCE?

Fatty acid requirements vary qualitatively and quantitatively among teleosts, mainly determined by the presence and activity of the desaturases (FADS) and elongases (ELOVL) in each species, capable of producing fatty acids *de novo*. The functionality of FADS2 is hypothesised to be driven by several factors, including the environment, trophic level, and phylogeny (Garrido et al., 2019). Despite the aspects driven PUFA biosynthesis capacity and functionality are wide and still not fully understood, it is well recognised that marine carnivorous species, such as gilthead sea bream and meagre, have low activity of desaturases and elongases, including the Δ6, but particularly the Δ4 and Δ5 (Izquierdo, 2005). Consequently, these species have a qualitative essential requirement for n-3 and n-6 LC-PUFA, including EPA, DHA, and ARA, respectively (Izquierdo, 2005; Tocher, 2010). Furthermore, larval stages, which are characterised for a higher metabolism for the faster growth and development of the tissues, require higher levels of these FA compared with juveniles (Izquierdo, 2005). In gilthead sea bream and meagre larvae, 3% or more of n-3 LC-PUFA in dry weight is required for maintaining an optimal growth performance during the whole cycle (Izquierdo, 2005). For juveniles of the same species, 1.5-1.8% dry weight is required for optimize growth (Ibeas et al., 1994) and stress resistance (Montero et al., 1998), respectively, but 2.5% is recommended to optimize growth during the on-growing period with high lipid diets (Izquierdo, 2005). Similarly, in meagre juveniles an optimal requirement of 2.1% was necessary to maximize growth for only 1 month, whereas a higher requirement for the whole on-growing period is foreseen. Therefore, modern marine aquafeeds, require ingredients highly rich in n-3 LC-PUFA to meet species optimal requirements for a high growth and good health during the production cycle.

Evaluating the potential of microalgal products rich in n-3 LC-PUFA, three commercial microalgal products (ALL G RICH™- Alltech Inc., DHA GOLD- DSM, and a development product MO060- Adisseo) were effective total replacers of FO in sea bream larvae (Chapter 3) and led to similar larval performance than a FO diet by providing the necessary dietary n-3 LC-PUFA for optimal growth and survival. Furthermore, higher dietary n-6 DPA (as in DHG and M60) seemed to improve growth performance and stress resistance, in agreement with

the few studies regarding the role of this FA in fish larvae (Garcia et al., 2008; Basford et al., 2020). This finding also highlights the need to further study the role of n-6 DPA, that, as mentioned above is not commonly found on the traditional lipid sources used in aquafeeds, but it is present high quantities in some microalgal products.

Similarly, it was possible to completely replace FO by algal oils, in combination with poultry oil and rapeseed oil in diets with 15% FM, both in gilthead sea bream (Chapter 4; Carvalho et al., 2020) and in meagre (Chapter 5) young juveniles. These replacement combinations show high growth rates, survival, feed utilization and dietary protein efficiency for both species, in a similar way of FO. The microalgal products used were commercially available oils, both extracted from *Schizochytrium sp.* microalga, where one contained both EPA and DHA (ED; Veramaris algal oil, Veramaris) and the other algal oil contained mainly DHA and n-6 DPA (DD; DHA Natur Oil, ADM). Both microalgal products resulted in dietary n-3 LC-PUFA contents of 2.8 and 2.4% in dry weight, respectively, which were above the optimal requirement for juveniles of both species: 1.5-1.8% for sea bream (Izquierdo, 2005; Houston et al., 2017) and 2.1% for meagre (Carvalho et al., 2018) and, thus, explain the high growth rates obtained. Furthermore, DD oil also supported sea bream growth after 5 months of feeding (Chapter 7). Although ED oil was not study in long-term feeding, a similar response is foreseen due to its high DHA and EPA contents and the demonstrated good results for a shorter period (Chapter 4).

It is also worthy to note that in the three mentioned studies of the present thesis, despite the efforts of some products such as the ED algal oil or the M60 to increase dietary EPA, none of the microalgal products attained the same EPA levels as FO. However, the good results of all the microalgal products indicates that, despite the lower EPA levels of the microalgal diets, these were sufficient to cover EPA requirements of fish larvae and juveniles since performance was not correlated with the dietary EPA. Additionally, contrary to what happened in larvae (Chapter 3), no boosting effect of n-6 DPA in growth and survival was observed for juveniles (Chapters 4 and 5), suggesting that this FA seemed to have a more biological importance for early stages of life in fish than for latter stages.

Furthermore, the reduction of the dietary FM (from 15% to 7.5%) seems to markedly reduce dietary nutrients utilization and, consequently, sea bream juveniles' growth performance (Chapter 4). Besides, an antagonistic interaction between protein and lipid concomitant replacement was found (Chapter 4), suggesting an effect of the protein quality on lipid metabolism (Dias et al., 2005; 2009), which deserves further attention in the near future of fish nutrition.

8.3 ARE FEEDS USING MICROALGAL PRODUCTS ALTERING FISH PHYSIOLOGY AND METABOLISM?

The replacement of FO in modern aquafeeds is known to alter fish metabolism, including different lipid pathways. Lipid metabolism homeostasis is achieved by an equilibrium between anabolic and catabolic pathways, which are regulated by different enzymes, and in which liver plays a central role (Bradbury, 2006). The use of microalgal products, unaffected hepatic lipogenesis and/or lipolytic pathways in sea bream larvae and juveniles (Chapters 3 and 6). This was indicated by the similar gene expression of lipogenic (*srebp1, srebp, fas*), as well as lipolytic (*lpl, cpt1* and *ppar-α*) enzymes genes, between fish fed microalgal diets and FO, possibly because of the high dietary n-3 LC-PUFA, particularly of DHA, found in larval whole-body and juveniles' livers of microalgae-fed fish. Additionally, the high n-3 LC-PUFA contents of microalgal products, down-regulated *fads2* and/or *scd-1* expressions in sea bream larvae (Chapter 3) and juveniles (Chapter 4), as well as in meagre juveniles (Chapter 5). These genes participate in PUFA and palmitoleoyl-CoA (16:1) and oleyol-CoA (18:1) synthesis, respectively, and its suppression by microalgal diets (like is observed in FO-based diets) indicates that EFA-requirements are well met by these novel sources for both species and different stages of development.

Interestingly, the ED microalgal oil (Veramaris algal oil, Veramaris), rich in EPA and DHA, activated the conversion of 16:3n-3 to 18:3n-3 in meagre juveniles (Chapter 5), a species that can perform this elongation at a higher rate than other marine species (Monroig et al., 2013). This was supported by the increased content of 18:3n-3 in fish tissues, contrary to the dietary level, added to the slight highest hepatic expression of *elovl5.* This highlights the potential of this microalgal oil, rich in 16:3n-3, as an alternative sources for aquafeeds to increase 18:3n-3 contents in fish tissues. In sea bream juveniles fed the same oil, no such result was observed (Chapter 4), where *elovl5* expression and their substrates and consequent products were unaffected by the microalgal diets, indicating the species-specific capacity to perform these conversions.

Additionally, both species retro-converted the high dietary n-6 DPA to ARA when fed the microalgal DD oil (DHA Natur Oil, ADM) (Chapters 4, 5 and 6).

It is however important to consider that all the mentioned effects (or lack of them) of microalgal products in fish lipidic metabolism are dependent on the experimental conditions of the different trials, including dietary inclusion levels, feeding period, species, and size of the fish, for instance. Therefore, the long-term effect of these sources on fish metabolism would of interest for further studies.

8.4 ARE THE INCLUSION OF MICROALGAL PRODUCTS IN FEEDS AFFECTING FISH HEALTH OR WELFARE?

N-3 LC-PUFA have been related to a reduced hepatic lipid infiltration by maintaining lipid metabolism homeostasis, since they reduce triacylglycerides synthesis (Berge et al., 1999). Besides, n-3 LC-PUFA increase the expression of microsomal transfer protein or apolipoprotein A1 (Kjaer et al., 2008), whereas down-regulate adipophilin expression, which is a molecular marker of intracellular lipid accumulation (Leaver et al., 2008). Therefore, the high n-3 LC-PUFA of the microalgal oils seemed to be the main reason for the unaltered expression of lipid synthesis-related genes, as mentioned above (Section 8.3), resulting in the smallest alterations in fish hepatic histology (Chapters 3 and 6). This low lipid infiltration and low steatotic alterations in liver indicates the good hepatic health of fish fed these microalgal oils. The use of microalgae oils could even improve liver health status when comparing with FO diet by slightly reducing the hepatic lipid accumulation and preserving thus hepatocyte morphology (Chapter 6). Even tough, further studies are needed to determine the potential positive long-term effects of microalgae sources.

Additionally, alternative lipid sources can directly affect fish health by altering EPA/ARA ratios of fish cells, since both fatty acids compete for the COX and LOX enzymes, to produce metabolites involved in fish immune responses. Thus, inadequate ARA/EPA/DHA ratios can lead to imbalanced eicosanoids production (Ganga et al., 2005). In general terms, although with some exceptions, ARA-derived eicosanoids have pro-inflammatory action but can have also anti-inflammatory. In contrast, EPA and DHA-derived eicosanoids are highly anti-inflammatory. In this context, the replacement of FO by the microalgal meals resulted in dietary EPA/ARA ratios that were 7 times lower than a FO diet. This was reflected in EPA/ARA ratios of phospholipids, particularly in those fed AGR and DHG, in agreement with negative relation observed between this ratio and *cox-2* expression. A positive relation was also found between DHA contents in PL and *5-lox* expression. These results indicate the importance of these fatty acids for COX and LOX metabolism and suggest a possible down-regulation of *cox-2* by the increase in EPA/ARA ratios in fish membranes. However, none of the genes were significantly altered in larvae by the feeding with microalgal meals (Chapter 3). These results suggest that eicosanoids metabolism and larval health seems to be not compromised by the replacement of FO with microalgae products.

Similarly, replacing FO by the microalgal oils did not alter health in meagre juveniles (Chapter 5), given the similar expressions of genes related with oxidative stress (*gpx* and *cox*) and heat shock protein metabolism (*hsp70* and *hsp90*). It is important to bear in mind that the effect of diets including microalgae was evaluated in fish basal health. The further study of

these ingredients on health and immune function under different stressful conditions would be of importance for increasing confidence in microalgal products.

Indeed, a retarded effect of brain n-6 DPA in non-locomotor behaviour (escape latency) in sea bream juveniles was suggested for the first time in fish, resulting from the high dietary concentration on this FA, which was highly incorporated in fish brain (Chapter 7). This possible negative effect of n-6 DPA on behavioural aspects has been previously recorded in mammals and deserves further attention in fish as some microalgae species presents often high dietary concentrations of this FA.

8.5 CAN CONSUMERS BENEFIT FROM EATING FARMED FISH THAT WERE FED WITH MICROALGAE-SUPPLEMENTED FEEDS?

Dietary composition, particularly in terms of the fatty acid profile, is of pivotal importance for meeting consumer's needs and expectations, since fish tissues, including the flesh, reflect the dietary pattern (Cowey and Sargent, 1972). Therefore, n-3 LC-PUFA-rich ingredients are necessary to improve the deposition of these important fatty acids for human health in fish tissues and guarantee that fish is a safe and a healthy food.

Despite fish did not reach commercial size in any of the trials of the present thesis, the composition of fish tissues, particularly flesh, and some indices related with lipid quality were calculated to have an estimation of the potential impact of the diets on the fillet nutritional quality for the consumer. In this regard, microalgae products were effective in increasing the n-3 LC-PUFA levels of the diets, which were reflected in fish body and fillets, both in sea bream and meagre (Chapters 3, 4 and 5). All microalgal products, irrespective their origin, improved the deposition of DHA in fish (Chapters 3, 4 and 5), demonstrated by the high DHA contents of whole-body or fillets, as well as the high retention of this FA (measured in Chapter 4). However, in which concerns EPA, microalgae did not present equal results. Therefore, in sea bream larvae, all the microalgal meals reduced around 50% the content of EPA in larval total and polar lipids, but DHG presented similar EPA levels as FO in larval neutral lipids (Chapter 3). In of sea bream fillets, microalgal oils were not able to deposit EPA contents as FO due to the lower dietary EPA, although ED oil (Veramaris algal oil, Veramaris) presented higher EPA than DD oil (DHA Natur Oil, ADM) (Chapter 4). Indeed, the latter microalgal oil reduced EPA by 59% and 70% in sea bream whole-body and fillet, respectively, in comparison to FO, whereas the former reduced only by a 37%. Similar reductions in EPA were observed in meagre juveniles' fillets using the same microalgal oils (Chapter 5). However, these oils were used not as a sole lipid source but rather in combination with other lipid mixtures as poultry

oil, under a cost-effective approach, which may explain such reduction in EPA levels. Higher dietary levels of these microalgal oils would probably increase EPA contents in fish flesh, increasing the health benefit for the consumer. Furthermore, analysing the concentration of the sum of both fatty acids (EPA+DHA), in mg/100 g fish fillet for instance, would be of interest to validate if the present levels are above or below the recommended by World Health Organizations (250-500 mg per day).

Atherogenic index reflects the risk of lipid deposition in blood vessels by considering the ratio between proatherogenic fatty acids (SFA) and antiatherogenic fatty acids (PUFA). Thrombogenic index estimate the probability of forming clots in blood vessels, which is favoured by the prothrombogenic fatty acids (SFA) and prevented by the antithrombogenic fatty acids (MUFA or PUFA). Both indices are common indicators of the potential value of fish nutritional composition for the coronary health of the consumer (Ulbricht and Southgate, 1991). The fish oil replacement by the microalgal ingredients allowed to maintain very good values (similar as FO) of the atherogenic and thrombogenic indices, thus indicating that fish fed those microalgal oils are a healthy food for consumers, concerning, at least, lipid nutrition (Chapters 4 and 5).

Additionally, microalgae diets, particularly the meals DHG and M60 as well as the oil DD (DHA Natur Oil, ADM) lead to an increase in the contents of n-6 DPA in fish body (Chapter 3) and fillets (Chapters 4 and 5), in concordance with the high dietary levels of this FA in the products. This FA was associated with neural and behaviour alterations in mammals and fish at early stages of life due to the competition with DHA for the incorporation in brain phospholipids membrane (García-Calatayud et al., 2005; Carvalho et al. 2021). However, some studies reported that intake of this FA does not impact DHA accumulation and does not constitute a problem for adult human health (Nauroth et al. 2010; Sprague at al., 2017).

8.6 CAN MICROALGAL DIETS IMPROVE FISH PRODUCTION COMPARED TO SOME TRADITIONAL ALTERNATIVE LIPID SOURCES USED IN AQUAFEED INDUSTRY (VEGETABLE OIL AND POULTRY OIL)?

All microalgal products tested can significantly improve the growth and feed utilization of both fish larvae and juveniles, compared to a diet based on traditional alternative lipid sources, for instance VO (oleic acid was used in larval trial to mimic VO) or PO (Chapters 3, 4 and 5).

Additionally, the total replacement of FO by PO altered lipid metabolism in liver of sea bream juveniles, inducing lipogenic processes (up-regulation of hepatic *sbrep1* and *fas*),

possibly due to a low dietary DHA (Houston et al., 2017; Jin et al., 2017; Morais et al, 2011). Consequently, PO led to a severe hepatic steatosis in sea bream juveniles, denoting poorer hepatic health when compared to microalgal feeds (Chapter 4).

In addition, microalgal feeds also increased survival in sea bream juveniles compared to PO (Chapter 4) as well as stress resistance in sea bream larvae compared to OA (Chapter 3). Furthermore, feeds with microalgal products might favoured the animal under stressful conditions compared to PO by compensation with a higher basal expression of antioxidant enzymes (Carvalho et al., 2019) and heat shock proteins (Chapter 5). Consequently, microalgae feeds could suppose an increased fish welfare, but studying the response of these genes under stress needs to be further studied.

In contrast, concerning fish behaviour, feeding with microalgal products rich in DHA and n-6 DPA did not suppose an improvement in sea bream juveniles' behaviour or brain function (Chapter 7). Unexpectedly, fish fed PO, with the lowest dietary n-3 LC-PUFA, showed the highest DHA content in brain, denoting a high retention of this FA as compensatory mechanism for a long-term n-3 LC-PUFA deficiency. Furthermore, FO replacement by PO, stimulated neurogenesis, and neural activity in sea bream (up-regulating of *neurod6, bdnf* and *nos1*), in relation to this increased DHA content in brain and highlighting the importance of this FA for neural function.

One constrains of the FO replacement by VO or PO is the reduction in EPA+DHA levels of fish fillets. In this context, all microalgal products were able to restore n-3 LC-PUFA levels of fish bodies and/or fillets compared to VO or PO (Chapters 3, 4 and 5). Furthermore, PO diet increased fillet n-6/n-3 ratio and TI of fish fillets due to the higher SFA and MUFA, and low n-3 LC-PUFA. Therefore, feeding PO as the sole lipid source negatively affects nutritional quality of fish products to the consumer, in relation with the low EPA and DHA levels and the higher risk of thrombogenicity (Chapters 4 and 5). In contrast, microalgae were able to improve these indices and contribute for the human health benefits associated to fish consumption. However, microalgal feeds led to a higher peroxidation index, indicator of fillet shelf life, compared to PO, indicating a higher risk of lipid oxidation (Chapter 4), due to the higher contents in PUFA, known to be more sensible to oxidation than MUFA (Hulbert et al., 2007).

Besides, using FEEDNETICS^{TM} to predict the long-term effects (12 months) in real farming conditions of the different diets used in sea bream juveniles, microalgal diets were shown to reduce the fish oxygen consumption, carbon dioxide and ammonia production compared to PO (results not included in this PhD thesis). Therefore, microalgal-supplemented feeds would be expected to improve water quality in relation the other traditional sources (PO).

8.7 WHICH TYPE OF MICROALGAL PRODUCT (BIOMASS OR OIL) IS THE BEST OPTION FOR THE INDUSTRY?

Microalgal products, either as whole-cell biomasses (Chapter 3) or extracted oils (Chapters 4 and 5) were shown to be effective replacers of FO in diets for fish at different developmental stages in the marine species sea bream and meagre. Microalgae as different type of products (meals and oils) maintained or improved production as the traditional FO diet, concerning fish performance, dietary utilization, DHA levels and nutritional quality of fish fillets. Besides, microalgal products were able to significantly improve these parameters compared with modern alternative aquafeeds, which include VO or PO as replacers of FO. Furthermore, no major alterations were observed related with fish health and welfare, but further studies are needed to better understand if the high n-6 DPA contents of some microalgae species, consistent with the most produced species (*Schizochytrium sp*. for instance), can potentially affect fish production and welfare in a long-term.

Additionally, it is known that the low digestibility of whole-cell biomasses is one constrains that industry needs to overcome (Sprague et al., 2015). Microalgae cells walls contain cellulose, which is poorly degraded by marine species (Chen et al., 2021). Furthermore, recent studies have also reported the presence of anti-nutritional factors in algal meals, like those present in plant meals, which can suppose a negative effect in digestibility and digestive health of fish (Chen et al., 2021). All microalgal products tested seemed to be well digested by the two different species since growth and dietary utilization was not affected. Although the present thesis did not aim to specifically study the effect of the different type of microalgal products in fish digestibility and digestive health, this would be an interesting question to look at in the future. Pre-treatment of microalgae whole cells, for instance by enzymatic digestion or physical treatments such as sonication or freezing, can damage cell walls and cellulose and other ANFs are released, improving digestibility (Chen et al., 2021). Technical treatment of algal biomasses further implies an additional cost. In this context, the use of extracted oils from microalgae (Chapters 4-7) could be a better alternative to meals for avoiding cellulose and other ANFs and, consequently, digestibility and other health problems related. Oils extraction also requires an additional cost comparing to the use of non-treated algal meals, but they allow more easily the combination with other oils to optimize the dietary fatty acid profile, thus providing higher flexibility in formulation and production of fish feeds. Therefore, evaluation of the feasibility of the different options of products for the industry should be based in cost-effectiveness of the products.

8.8 CAN MICROALGAE BE PART OF THE SOLUTION FOR THE COST-EFFECTIVE AND SUSTAINABLE DEVELOPMENT OF INTENSIVE MARINE AQUACULTURE?

Despite the demonstrated potential of microalgal products, particularly as n-3 LC-PUFA sources and replacers of FO in marine fish, microalgae field intended for aquaculture feed industry still needs to face some challenges (Sprague et al., 2015). Apart from the nutritional limitations of some microalgal meals related with digestibility as mentioned above, the current high costs of production of microalgae still limits their large-scale production and their extensive use in aquafeeds. In fact, microalgae production involved several technical expensive steps, from harvesting (which supposes 30% of the total production costs) to pre-treatment for inclusion in fish pellets (Nagappan et al., 2021). For instance, the type and size of the reactor used for microalgae production, growth media and other products, all influence the biomass cost, which could be around 13 $/kg in a small-scale raceway (Sui et al., 2020), but can be reduced to 5 $/kg increasing the size of the raceway for more biomass (Fernández et al., 2019). Furthermore, new technologies for the different steps of the production, including the harvesting, are being developed to reduce production costs and allow the large-scale production. They include, among others, microalgae immobilization to form biofilms and facilitate harvesting processes, for instance (Nagappan et al., 2021). The extraction of the microalgal oils for producing high quality, n-3 LC-PUFA-rich products, implies an additional cost, but allows the combination with other cheaper conventional lipid sources, such as vegetable and poultry oils (Chapters 4-7). Thus, this reduce the microalgae inclusion level needed in the diet and could be an efficient cost-effective strategy to produce high quality fish oil-free feeds. An economical study of the dietary combinations of microalgal oils with poultry and rapeseed oils (Chapters 4-7) would be of interest in the future. Indeed, similar costs were estimated between a microalgae-based feed (free of FO and FM) and a control FO/FM feed, for tilapia (Sarker et al., 2020). Therefore, microalgal products, either delivered as meals or oils, will predictably become cost-competitive alternatives to FO (and FM), reducing the dependency on wild biological resources and maintaining or improving fish productivity and nutritional quality of the product for the consumers. This will contribute for the cost-effective and product-safe development of marine aquaculture worldwide.

Additionally, microalgae were positioned as sustainable ingredients for aquaculture. Regarding the environmental aspect, life-cycle assessment studies have been used for investigating if inclusion of microalgae in fish feeds is an eco-friendly option for the future. In which concerns water footprint, microalgae production uses less freshwater than the production of other traditional alternatives, such as plant ingredients (Nagappan et al., 2021). It is true that FM and FO have a zero-water footprint, but these ingredients are not comparable because they do not meet sustainability requirements in many other aspects in a long-term.

However, recent studies have pointed out that microalgae feeds seem to currently represent a much higher environmental impact than other ingredients (Nagappan et al., 2021). Microalgal systems have been associated to higher global warming potential, acidification, energy demand, and sometimes also water use because they represent higher emissions of CO_2 to the environment (Maiolo et al., 2020). However, efforts have been done to find out ways of reducing environmental impact of producing microalgal feeds, for instance by improving reactors, reducing the energy spend on harvesting, using recycled water or using wastes as carbon source (Nagappan et al., 2021). Furthermore, producing microalgal feeds in large-scale has also been shown to greatly reduce (by 20 times) carbon footprint (Taelman et al., 2013). Therefore, it is probably that these microorganisms become eco-friendly ingredients for aquafeeds, with the technological improvement and the increasing study and knowledge on microalgae for large-scale production, favouring the further aquaculture development.

CHAPTER 9. GENERAL CONCLUSIONS

1. Commercial microalgal meals (ALL G RICH™- Alltech Inc., DHA GOLD- DSM, and the development product MO060- Adisseo) are effective FO replacers in sea bream larvae diets, leading to similar larval performance than a FO based diet by providing the necessary dietary n-3 LC-PUFA for optimal growth and survival.

2. Blends of commercial microalgal oils (Veramaris algal oil, Veramaris and DHA Natur Oil, ADM) and the less costly poultry oil allow total replacement of FO and meeting of marine fish EFA requirements in practical diets for gilthead sea bream and meagre.

3. The reduction of the dietary FM (from 15% to 7.5%) and its replacement by the plant protein sources used in this thesis markedly reduces dietary nutrients utilization and, consequently, sea bream growth performance, but the concomitant replacement of both FM and FO by poultry oil alone has a further negative and synergic effect on lipid metabolism.

4. Compared to a FO based diet, the use of microalgal products does not affect hepatic lipogenesis and/or lipolytic processes in sea bream larvae and juveniles.

5. The replacement of FO by microalgae oils downregulates the expression of lipogenesis and FA desaturation-related genes, reducing the hepatic lipid accumulation compared to PO.

6. Microalgal products, either as meals or oils, can be effective total replacers of FO in fish larvae and juveniles, without negative consequences in general aspects of fish health, including in oxidative stress status, heat shock proteins metabolism and eicosanoids metabolism.

7. Incorporation of n-6 DPA in brain of sea bream juveniles negatively affects non-locomotor behaviour (escape latency), as it was observed in these studies for the first time in fish.

8. The total replacement of dietary FO by PO reduces n-3 LC-PUFA levels but increases DHA content in brain of juvenile gilthead seabream, denoting the high retention of this FA as a compensatory mechanism for a prolonged n-3 LC-PUFA deficiency.

9. The total replacement of dietary FO by PO, leading to an increased DHA content in brain, stimulates neurogenesis and neural activity in sea bream, highlighting the importance of this FA for neural function in marine fish.

10. Microalgal products can be one of the solutions for increasing the n-3 LC-PUFA levels, especially DHA, in fish fillets, in the current context of low dietary FO/FM of aquaculture feed industry. However, the microalgal products used (Veramaris algal oil, Veramaris and DHA Natur Oil, ADM) reduced EPA contents in fillets compared to FO.

11. Microalgae-supplemented diets improve the nutritional quality of fish fillets for the consumers, reducing atherogenicity and thrombogenicity risk indicators. Nevertheless, these products increase the peroxidation index, an indicator of the fillet shelf-life.

CHAPTER 10. RESUMEN EN ESPAÑOL

10.1 BENEFICIOS DEL CONSUMO DE PESCADO PARA LA SALUD HUMANA: LA CONTRIBUCIÓN DEL PESCADO DE PISCIFACTORÍA

Los peces representan la fuente principal de ácidos grasos poliinsaturados de cadena larga de la serie n-3 ("omega-3"; LC-PUFA) en la dieta humana, ya que los ecosistemas marinos son los más ricos en estos nutrientes (Tocher, 2010). Los beneficios de los n-3 LC-PUFA en la salud y bien estar humano están estrechamente asociados a sus importantes funciones en la función celular. En los seres humanos, el consumo de n-3 LC-PUFA es necesario para el desarrollo de la visión en la infancia y el correcto desarrollo neural. Además, variados estudios reportaron un efecto positivo del consumo de n-3 LC-PUFA, en particular del ácido docosahexanóico (DHA), en el tratamiento de varios trastornos neurológicos o comportamentales, incluyendo la depresión y la hiperactividad por déficit de atención (Ortega et al., 2012), bien como en la prevención de las patologías neuronales asociadas al envejecimiento, como por ejemplo la enfermedad de Alzheimer, al reducir la pérdida neuronal y mejorar la función cognitiva (Hoojimans et al., 2012). Además, debido a su efecto en la disminución del colesterol sanguíneo, la disminución de la síntesis de triglicéridos en el hígado y en la función plaquetaria, los n-3 LC-PUFA son beneficiosos para la prevención de enfermedades cardiovasculares (ECV) (Calder, 2006). Por lo tanto, muchas organizaciones mundiales de salud recomiendan hasta 500 mg diarios de EPA + DHA para reducir el riesgo de ECV, y hasta 2 g diarios en pacientes con ECV (ISSFAL, 2004; EFSA, 2012), lo que equivale al consumo de 2 comidas de pescado a la semana. También hay evidencia de que los n-3 LC-PUFA pueden ser beneficiosos en enfermedades inflamatorias, como la enfermedad de Crohn, aunque las dosis diarias requeridas son mucho más altas (3 g por día). Por último, muchos estudios reportaron un efecto protector de estos ácidos grasos en varios tipos de cáncer (Gerber, 2012), así como en la reducción de los efectos secundarios y la pérdida muscular asociada a la quimioterapia, ayudando a los pacientes a manejar más ciclos (Murphy et al., 2011). Los mecanismos subyacentes a todos estos efectos positivos son todavía poco conocidos, pero posiblemente también están relacionados con el papel de n-3 LC-PUFA en las funciones inflamatorias e inmunes.

10.2 FUNCIONES Y METABOLISMO DE LOS N-3 LC-PUFA EN LOS PECES

10.2.1. PRODUCCIÓN DE ENERGÍA

Los lípidos son uno de los principales nutrientes presentes en los peces, así como en sus dietas, ya que juegan un papel importante como sustrato energético, teniendo la mayor energía total por unidad de peso (9,5 Kcal / g). Además, también son fuente de ácidos grasos esenciales (EFA), así como portadores de vitaminas y ciertos micronutrientes de características liposolubles (Tocher, 2003, 2010; Glencross, 2009). En particular, los ácidos grasos son la principal fuente de energía metabólica para el crecimiento, la actividad natatoria y la reproducción en peces, proporcionando ATP a través de su oxidación en las células (Frøyland et al., 2000).

10.2.2. ESTRUCTURA DE LAS MEMBRANAS Y FUNCIÓN CELULAR

Los ácidos grasos en los fosfolípidos (PL) juegan un papel importante en la función celular, manteniendo la integridad y fluidez de la membrana, la función proteica y controlando las señales químicas de la membrana (Sargent et al., 1995; Izquierdo, 2005). Además, los PL también son sustratos para la formación de segundos mensajeros, como diacilgliceroles, lisofosfosfolípidos y otras moléculas bioactivas (Calder, 2016). El DHA es el componente principal de las membranas celulares en todos los vertebrados, incluso en los peces teleósteos (Izquierdo, 2005), evidenciando su importancia estructural y funcional en las células.

10.2.3. DESARROLLO Y FUNCIÓN NEURAL Y EFECTO EN EL COMPORTAMIENTO

El papel de los n-3 LC-PUFA para la salud cerebral y el desarrollo y función del tejido nervioso de los vertebrados es ampliamente reconocido, particularmente en las primeras etapas de la vida (Izquierdo, 2005). De hecho, el DHA es el ácido graso con más importancia fisiológica para la función cerebral, tanto en mamíferos como en peces (Bourre, 2004; Marszalek y Lodish, 2005). Las membranas de las células neuronales contienen aproximadamente 30-40% de DHA (Innis, 1991; Lauritzen et al., 2001) y este ácido graso también está ampliamente presente en las membranas de las células de la retina, estando por ello asociada con el desarrollo de la visión y el sistema nervioso central. A pesar de ser

menos estudiado, el EPA también puede jugar un papel importante en la función neuronal al mejorar el desarrollo cerebral (Furuita et al., 1998; Izquierdo et al., 2001) y también producir metabolitos neuroprotectores (Kidd, 2007). De hecho, el cerebro es el órgano primario para anticipar un estímulo o un factor estresante y traducirlo en una acción comportamental. El comportamiento de los animales es, por tanto, un fuerte indicador de su salud y su bienestar. Por lo tanto, los cambios en la composición de ácidos grasos de las células del sistema nervioso pueden afectar directamente a la percepción de los estímulos y el, en consecuencia, al comportamiento. En peces, estudios previos vincularon el efecto de los n-3 LC-PUFA de la dieta, particularmente el DHA y el EPA, con el comportamiento de los peces. Benítez-Santana et al. (2012, 2014) proporcionaron la primera evidencia sobre los efectos de estos ácidos grasos en las neuronas y la respuesta de escape en larvas de peces (Benítez-Santana et al., 2012; 2014). Lo que concluyeron fue que las dietas deficientes en DHA reducen en gran medida la incorporación de DHA en el tejido cerebral de las larvas y, en consecuencia, retrasan la respuesta de escape a un estímulo sonoro o lumínico (Benítez-Santana et al., 2012; 2014). Los datos recopilados, tanto en mamíferos como en peces, sugieren que el cerebro es sensible a la falta de PUFA, particularmente en las primeras etapas de la vida, y que una deficiencia crónica en n-3 LC-PUFA, podría cambiar la función cerebral y la salud de los peces (Lauritzen et al., 2001).

10.2.4. FUNCIÓN INMUNE, PRODUCCIÓN DE EICOSANOIDES Y RESISTENCIA AL ESTRÉS

Uno de los papeles principales de los LC-PUFA en las membranas es su oxidación controlada para la producción de eicosanoides, que son moléculas bioactivas, compuestos similares a hormonas con una vida media corta, que desempeñan acciones importantes en la coagulación de la sangre, la respuesta inmune, la respuesta inflamatoria, la función cardiovascular, la función renal, la función neuronal y la reproducción (Calder, 2006; Tocher, 2010). Estos incluyen derivados cíclicos como las prostaglandinas (PG) y los tromboxanos (TX), que se forman por la acción de las enzimas ciclooxigenasas (COX) a través de la oxidación de los PUFA de 20 carbonos, particularmente el ARA y EPA, así como derivados lineales producidos por la acción de lipoxigenasas (LOX), incluyendo los ácidos grasos hidroxi y hidroperoxi, los leucotrienos (LT) y lipoxinas (LX) (Yaqoo, 2004; Calder, 2006). Los eicosanoides se producen en todos los tejidos de los peces durante situaciones estresantes y este proceso es parte de un mecanismo natural para volver a la homeostasis. Además de los eicosanoides pro-inflamatorios, derivados del ARA y el EPA, este ultimo junto con el DHA, también son sustratos para la producción de otros derivados anti-inflamatorios, en particular

los resolvinas (E-resolvinas y D-resolvinas, respectivamente), las protectinas y las maresinas (Marcheselli et al., 2003; Serhan, 2006, 2014).

10.2.5. CONTROL DE LA HOMEOSTASIS LIPÍDICA

La homeostasis del metabolismo lipídico se logra mediante un equilibrio entre las vías anabólicas y catabólicas. Para ello, intervienen varios genes, que son regulados a nivel transcripcional por diversos ácidos grasos, en particular por los PUFA, ya sea por efectos directos o indirectos a través de la modificación de la composición de membrana, producción de eicosanoides, expresión génica o factores de transcripción (Tocher, 2010). En cuanto a este último, se reconoce la implicación de n-3 LC-PUFA en la lipogénesis y la lipólisis, que tienen lugar principalmente en el hígado, al estimular o deprimir varias enzimas clave para la síntesis de lípidos o su catabolismo (Pierron et al., 2007; Zheng et al., 2013).

10.3 REQUERIMIENTOS EN ÁCIDOS GRASOS ESENCIALES PARA LOS PECES

10.3.1. REQUIRIEMENTOS QUALITATIVOS

Todos los vertebrados tienen un requerimiento esencial de ácidos grasos poliinsaturados (PUFA), incluyendo el ácido linolénico (18:3n-3; LNA) y el ácido linoleico (18:2n-6; LA). Sin embargo, las formas más biológicamente activas de estos ácidos grasos son generalmente sus productos de desaturación, particularmente el 20:4n-6 (ARA), 20:5n-3 (EPA) y 22:6n-3 (DHA), que desempeñan funciones importantes en el desarrollo, el metabolismo y la salud en general, como se describió anteriormente. Las diferentes especies de peces tienen diferentes habilidades para realizar la conversión de 18:2n-6 y 18:3n-3 a ácidos grasos de cadena más larga e insaturada. Estas conversiones involucran un paquete enzimático que incluye Δ4, Δ5, Δ6 y Δ8 desaturasas, reguladas a nivel transcripcional por genes de desaturasa de ácidos grasos (FADS) y elongasas (ELOVL). Debido a la alta disponibilidad de los n-3 LC-PUFA en los ecosistemas marinos, las especies de peces marinos no tuvieron presión evolutiva para producir endógenamente estos ácidos grasos. Esta incapacidad para sintetizar estos ácidos grasos *de novo* en cantidades suficientes para satisfacer sus requerimientos se debe principalmente a un deterioro, deficiencia o baja actividad de las desaturasas, en particular las Δ4, Δ5 y Δ6, bien como de las elongasas. Por esta razón, los LC-PUFA, tanto de la serie n-3 como de la n-6, se consideran esenciales para

las especies marinas y debe suministrarse a través de la dieta. En contraste, los ambientes de agua dulce tienen baja abundancia de n-3 LC-PUFA porque el fitoplancton de agua dulce es principalmente productor de 18:2n-6 y 18:3n-3, a pesar de tener cantidades moderadas de EPA, pero bajo contenido de DHA. En consecuencia, las especies de peces de agua dulce mantuvieron la presión evolutiva para producir los LC-PUFA a partir de estos precursores y, por lo tanto, tienen una mayor actividad de las desaturasas y elongasas que los peces marinos para producirlos (Tocher, 2010). En suma, mientras que, en los peces de agua dulce, los requerimientos de ácidos grasos esenciales (EFA) pueden ser proporcionados principalmente por el LA y el LNA, en los peces marinos los requerimientos son proporcionados por los n-6 y n-3 LC-PUFA, particularmente el ARA, EPA y DHA (Watanabe, 1993; Bell y Sargent, 2003; Tocher, 2003).

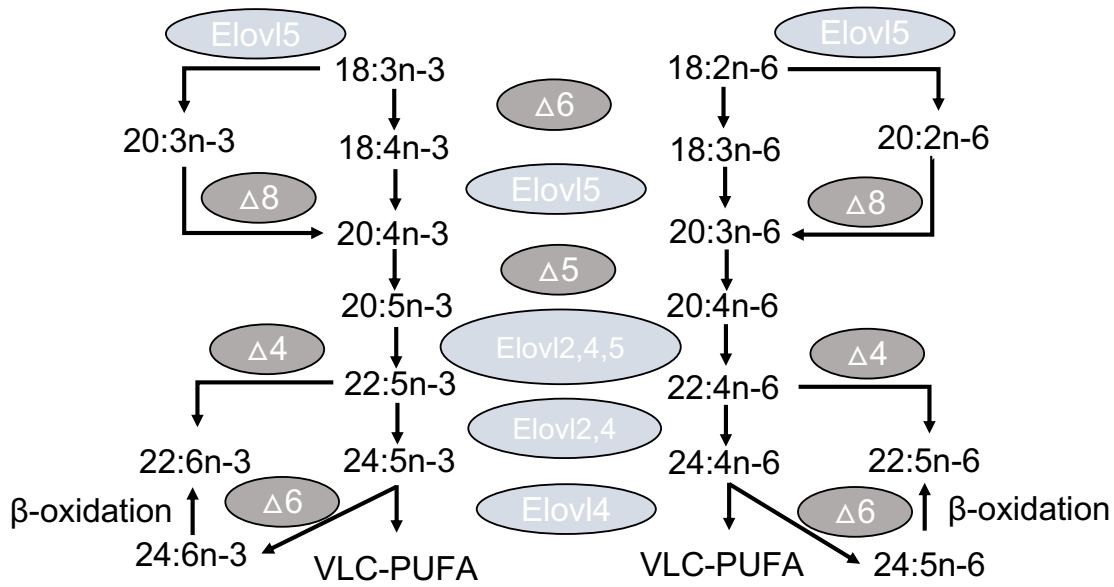

Figura 10.1. Vías de desaturación y elongación en la biosíntesis de ácidos grasos.
Δ, delta desaturasas; Elovl, elongasas; VLC-PUFA, ácidos grasos poliinsaturados de cadena muy larga (más de 24 carbonos).

10.3.2. REQUIREMIENTOS QUANTITATIVOS

Es difícil cuantificar un requisito absoluto de EFA en peces, ya que este requisito varía con el desarrollo, la etapa fisiológica, el estado nutricional, el contenido de lípidos en la dieta y las condiciones ambientales, como la temperatura, la salinidad y la luz (Izquierdo, 2005). Sin embargo, los requisitos cuantitativos de EFA están determinados principalmente por la fase de vida y las condiciones fisiológicas de los peces. En general, los peces en las primeras etapas de la vida, es decir, en las larvas, tienen mayores requisitos fisiológicos en comparación con los peces juveniles y sub-adultos (Tabla 10.1; Izquierdo, 2005). Esto es particularmente cierto para el DHA en larvas de organismos marinos, por ejemplo, debido a la alta tasa de crecimiento de los primeros estadios de desarrollo y a su función en el desarrollo de órganos neuronales y visuales, como se ha descrito anteriormente.

Tabla 10.1. Requerimientos de ácidos grasos poliinsaturados de cadena larga de la serie n-3 (%) de las principales especies de peces marinos de la acuicultura Mediterránea

	Larvas	Juveniles	Reproductores
Sparus aurata	>3	2.5	2.2
Psetta maxima	3.2[2]	0.8[3]	n.d.
Dicentrarchus labrax	2.3[12]	>3.5[4]	2.7[13]
Seriola dumerili	4[1]	2.5[1]	1.7[14]
Pagrus pagrus	<3.4[5]	<5[1]	n.d.
Dentex dentex	4[9]	n.d.	n.d.
Argyrosomus regius	>3[10]	2.1[11]	n.d.

Adapted and updated from Izquierdo (2005). [1]Izquierdo (own data); [2]Le Milinaire, 1984; [3]Gatesoupe et al., 1977; [4]Lanari et al., 1999; [5]Hernández-Cruz et al., 1999; [6]Radunz-Neto et al., 1993; [7]Corraze et al., 1933; [8]Takeuchi and Watanabe, 1976; [9]Mourente et al., 1999; [10] El-Kertaoui et al., 2015; [11] Carvalho et al., 2018; [12]Villeneuve et al., 2005; [13]Bruce tet al., 1999; [14]Sarih et al., 2020; n.d., not determined.

10.4 EL PROBLEMA ASOCIADO AL USO EXCLUSIVO DE LA HARINA Y EL ACEITE DE PESCADO EN PIENSOS PARA ACUICULTURA Y SOLUCIONES PARA EL DESARROLLO DE LA ACUICULTURA INTENSIVA

10.4.1 HARINA Y ACEITES DE PESCADO- EL PROBLEMA ASOCIADO

FO (y FM) fueron los principales ingredientes tradicionales utilizados en los alimentos de acuicultura desde el comienzo de los sistemas intensivos de producción. Esto se debió principalmente a su alta digestibilidad y palatabilidad por parte de los peces y su composición equilibrada de nutrientes, cumpliendo con los requisitos nutricionales de las especies de peces, básicamente porque son el alimento natural para los peces en entornos naturales (NRC, 2011). En particular, FO (y la parte lipídica derivada de FM), son la principal fuente de n-3 LC-PUFA en los alimentos acuáticos. La producción de FO y FM fluctúa con varios factores, en particular los cambios en las poblaciones silvestres de especies pelágicas. Además, el uso insostenible de recursos finitos y la dramática disminución de las poblaciones de peces silvestres llevaron al estancamiento de la disponibilidad de estos ingredientes marinos procedentes de la pesca (FAO, 2020). Esta disminución de la oferta se acompañó de un aumento de sus precios de mercado. Estos problemas de sostenibilidad ambiental y económica llevaron a la disminución del uso de FO y FM en las dietas de pescado y su sustitución por fuentes alternativas en las últimas décadas. Sin embargo, mientras que en los últimos años se han logrado grandes mejoras en la reducción y sustitución de la FM en la dieta de los peces marinos, la sustitución total de la FO sigue siendo el principal reto (Colombo and Turchini, 2021). En consecuencia, los datos más recientes mostraron que la industria de piensos para la acuicultura sigue siendo responsable del 75% del uso de la producción total de FO (Colombo and Turchini, 2021).

10.4.2 FUENTES TRADICIONALES ALTERNATIVAS AL ACEITE DE PESCADO

Una de las primeras y más estudiadas alternativas a la FO en los piensos para peces son los aceites derivados de plantas (VO) o animales terrestres, como las aves (PO). A diferencia de FO, la producción de los VO y PO mostró una tendencia creciente durante las últimas décadas. Una consecuencia de su amplia disponibilidad es la disminución de sus precios de mercado, siendo significativamente más baratos que el FO. Por lo tanto, estos aceites son considerados alternativas atractivas al FO, y durante las dos últimas décadas se

han realizado intensas investigaciones para estudiar su inclusión en los alimentos para peces. Tanto los VO como los PO son fuentes ricas en ácidos grasos saturados (SFA) y monoinsaturados (MUFA), y se utilizan de manera similar al FO como fuente de energía por los peces, siendo igualmente catabolizados para el crecimiento (Bell et al., 2001; Turchini et al., 2009). Sin embargo, a pesar de que seren una fuente de lípidos, carecen de los n-3 LC-PUFA y, por consiguiente, los niveles de EPA y DHA en los peces se reducen cuando se alimentan con una dieta basada en estos aceites (Turchini et al., 2009). Esto impacta no sólo el producto final para el consumidor, disminuyendo los beneficios asociados al consumo de pescado para la salud humana, sino que también podría afectar a la producción acuícola en sí, en particular las especies carnívoras marinas, para las cuales estos ácidos grasos son esenciales, como mencionado anteriormente. Por lo tanto, sus contenidos nulos de n-3 LC-PUFA limita su uso como sustitutos totales del FO en alimentos para peces carnívoros marinos, posiblemente causando una deficiencia de EFA, particularmente cuando la FM, que también contribuye para un contenido residual n-3 LC-PUFA, se sustituye en la dieta simultáneamente con el FO. Además, los VO y PO a menudo contiene altos ácidos grasos n-9 y n-6, principalmente ácido oleico (18:1n-9) y linoleico (18:2n-6), respectivamente, que podrían imponer un desequilibrio y proporciones inadecuadas n-3/n-6 y reducir la salud de los peces, alterando la síntesis de eicosanoides (Fracalossi et al., 1994). Además, altos porcentajes de reemplazo del FO por VO o PO causan una regresión en el crecimiento, inmunosupresión y menor resistencia al estrés debido a una deficiencia de EFA (Montero et al., 2003).

10.4.3 FUENTES NOVEDOSAS ALTERNATIVAS AL ACEITE DE PESCADO

En un intento por resolver todos estos problemas, se abrió una nueva era de investigación en la nutrición acuícola en los últimos años, para encontrar nuevas fuentes ricas en n-3 LC-PUFA para los alimentos de acuicultura, sin comprometer el potencial rentable y sostenible de otras fuentes convencionales, aumentando la productividad de los peces y el valor nutricional del producto final para el consumidor. Varias fuentes de lípidos han sido probadas en los últimos años, incluyendo microorganismos, krill y plantas genéticamente modificadas.

Sin embargo, las microalgas son probablemente la fuente de n-3 LC-PUFA más prometedora para la industria de alimentación acuícola. Estas son los principales productores naturales de n-3 LC-PUFA en ambientes acuáticos y comprenden un grupo diverso de organismos, desde los procarióticos unicelulares, como las cianobacterias (pertenecientes a

bacterias, pero a menudo incluidas en la taxonomía de microalgas), hasta los microorganismos fotosintéticos eucarióticos (dinoflagelados, diatomeas y otras algas marrones y verdes). Al ser autótrofos o heterotróficos muy simples, estos microorganismos se consideran más sostenibles que otras fuentes de lípidos por su baja huella y su menor uso de la tierra y el agua no reciclada (Tibbetts, 2018; Shah et al., 2018). Las biomasas de algas son una rica fuente de nutrientes como aminoácidos, EFA, vitaminas y antioxidantes naturales, posicionando estos productos como ingredientes prometedores en la formulación de piensos para peces. Varias especies de microalgas producidas autótrofamente o heterotróficamente han sido probadas en dietas de peces marinos por su alto contenido de EPA y/o DHA, incluyendo aquellas del género *Crypthecodinium* (Eryalcyn et al., 2015; Schafberg et al., 2018, 2020), *Nannochloropsis* (Eryalcyn et al., 2015; Metsoviti et al., 2018), *Phaeodactylum* (Ruyter et al., 2016; Sørensen et al., 2016) y, en particular, *Schizochytrium sp.* (Metsoviti et al., 2018; Perez-Velazquez et al., 2018; Kosoulaki et al., 2020). El contenido de lípidos y los perfiles de ácidos grasos dietéticos de estos microorganismos dependen de la especie y la etapa de crecimiento, pero también de los diferentes aspectos de la producción y la tecnología de procesamiento empleada. Por lo tanto, los perfiles de ácidos grasos de estas fuentes de lípidos o sus combinaciones pueden adaptarse para cubrir los requerimientos de EFA de las diferentes especies de peces y crustáceos y sus diferentes etapas de desarrollo.

A pesar de su alto potencial, en particular como fuentes de omega-3 en los alimentos para la acuicultura, los productores de piensos todavía tienen que hacer frente a algunos desafíos, principalmente debido a sus altos costos de producción, la gran variación en la composición bioquímica de las algas y la baja digestibilidad de algunas especies debido a la pared celular si se utiliza como biomasa de células (Sprague et al., 2015). Por lo tanto, la producción comercial actual de microalgas no es capaz de satisfacer las demandas de n-3 LC-PUFA para la acuicultura, que tiene como objetivo no solo cubrir los requerimientos de los peces, sino también para aumentar la deposición n-3 LC-PUFA en el pescado. De hecho, a pesar de que la extracción de aceite a partir de biomasa de algas podría resolver potencialmente los problemas de digestibilidad asociados con las paredes celulares en algunas especies, es necesario emplear más tecnología para extraer aceites. Por esta razón, la combinación de microalgas con otras fuentes de lípidos convencionales más baratas, incluso carentes de n-3 LC-PUFA, como pueden ser los VO y/ PO, podría ser una estrategia eficiente y rentable para lograr la sustitución total del FO y, posiblemente, también de la FM, en las dietas de los peces.

10.5 OBJETIVOS

Los ingredientes marinos tradicionales utilizados en los piensos acuícolas (FM y FO) ya no son rentables ni sostenibles para satisfacer la demanda de n-3 LC-PUFA de la acuicultura, que está en continua expansión. Además, los productos de la acuicultura son la principal fuente de n-3 LC-PUFA para la nutrición humana. Teniendo en cuenta las posibles consecuencias negativas del uso de aceites vegetales como alternativa al aceite de pescado en los piensos modernos (con un bajo contenido de harina de pescado) para peces marinos, nuevas fuentes de lípidos ricos en n-3 LC-PUFA son necesarias para garantizar una producción eficiente, así como para asegurar que se mantiene la calidad nutricional del producto que llega a los consumidores. Dentro de los potenciales ingredientes estudiados en los últimos años, las microalgas son una de las alternativas más prometedoras para todos los estadios de desarrollo de los peces. Así, los principales objetivos generales de esta tesis fueron:

1. Investigar el potencial de la sustitución completa del FO por productos comerciales de biomasa de microalgas en dietas para larvas de dorada (*Sparus aurata*) (Capítulo 3).

2. Validar el potencial de mezcla de aceites comerciales de microalgas con fuentes alternativas convencionales más baratas (PO y VO) para la sustitución completa del FO en dietas prácticas para juveniles de dos especies marinas importantes para la acuicultura mediterránea: dorada y corvina (*Argyrosomus regius*) (Capítulos 4 y 5).

3. Comprender los efectos de los productos derivados de microalgas en algunos indicadores de salud y bienestar de los peces (Capítulos 3, 5, 6 y 7).

4. Comprender los efectos de los productos de microalgas en las vías del metabolismo de los lípidos de los peces (Capítulos 3, 5 y 6).

5. Evaluar los efectos de los productos derivados de microalgas después de un periodo de alimentación a largo plazo sobre el comportamiento y la función neuronal de juveniles de peces (Capítulo 7).

10.6 RESUMEN DE LOS CAPÍTULOS

10.6.1 CAPÍTULO 3- REEMPLAZO COMPLETO DE ACEITE DE PESCADO POR TRES PRODUCTOS DE MICROALGAS RICOS EN ÁCIDOS GRASOS POLIINSATURADOS DE CADENA LARGA DE LA SERIE N-3 EN MICRODIETAS PARA DORADA (SPARUS AURATA)

El objetivo del presente estudio fue evaluar el efecto de diferentes microalgas ricas en n-3 LC-PUFA en sustitución al aceite de pescado (FO) en dietas para larvas de la dorada (*Sparus aurata*), estudiando su crecimiento, la composición bioquímica, salud y resistencia al estrés. El FO (FO) fue reemplazado por ácido oleico (OA) o 3 productos de microalgas ricas en LC-PUFA (2 productos comerciales: All G Rich y DHA Gold, y un producto en desarrollo: MO060). Las cinco microdietas tempranas de destete fueron alimentadas con larvas de 20-dah durante 21 días. Se analizó la composición proximal y los perfiles de ácidos grasos de los lípidos totales, neutros y polares de las larvas, así como la morfología de los hepatocitos y la expresión de genes relacionados con el metabolismo de los lípidos. La dieta de OA inhibió significativamente el crecimiento y la supervivencia de las larvas después de una prueba de estrés, mientras que la sustitución del FO por DHA Gold y MO060 condujo a un mayor peso corporal, longitud total y resistencia al estrés. Las larvas alimentadas con dietas que contenían microalgas presentarón un mayor contenido de DHA y mostraron una baja expresión de la *fads2*, lo que indica una inhibición de la biosíntesis de LC-PUFA. La expresión relativa de genes relacionados con el metabolismo de los lípidos (*ppar-α*) o eicosanoides (*cox-2* y *5-lox*) no se vio afectada por la sustitución del FO por cualquiera de los tres productos de microalgas o el OA. En conclusión, todos los productos de microalgas probados fueron sustitutos totales efectivos del FO en microdietas de destete para larvas de dorada, proporcionando la cantidad necesaria de n-3 LC-PUFA en la dieta para un crecimiento larvario y supervivencia óptimos.

10.6.2 CAPÍTULO 4- REEMPLAZO COMPLETO Y EFECTIVO DEL ACEITE DE PESCADO POR LA COMBINACIÓN DE ACEITE DE MICROALGAS Y ACEITE DE POLLO EN DIETAS PRÁCTICAS PARA JUVENILES DE DORADA (SPARUS AURATA)

Pocos ingredientes permiten la sustitución completa de las harinas (FM) y aceites (FO) de pescado en los piensos de acuicultura, sin afectar el rendimiento de los peces o el valor nutritivo del filete. Esto se debe al contenido adecuado de nutrientes esenciales, incluidos los ácidos grasos poliinsaturados de cadena larga de la serie n-3 (PUFA LC n-3), y la alta palatabilidad y digestibilidad de la FM y del FO. Algunas microalgas presentan cantidades abundantes de estos ácidos grasos, en particular de ácido docosahexaenoico (DHA). Por lo tanto, el objetivo del presente estudio fue evaluar el efecto de dos aceites de microalgas, uno rico en DHA y en ácido eicosapentaenoico (EPA; dieta ED) y el otro en DHA y en ácido n-6 docosapentaenoico (DPA; dieta DD), en combinación con un aceite de pollo (PO) y un aceite de colza, como sustitutos totales del aceite de pescado, y bajo dos contenidos diferentes de harina de pescado en la dieta (15 y 7,5 %). Se estudiaron los efectos de estas combinaciones de aceites sobre el crecimiento, la composición y los índices de calidad nutritiva en juveniles de dorada (*Sparus aurata*) y se compararon con una dieta control positivo (basada en FO) y dos dietas controles negativos (basada en PO), uno por cada contenido de harina de pescado probado. Ambos productos de microalgas, en combinación con los aceites de pollo y de colza, fueron capaces de reemplazar completamente el aceite de pescado en dietas prácticas con 15% FM, sin afectar el crecimiento, la utilización de ácidos grasos de la dieta o la calidad nutricional de filetes de pescado para el consumidor. Por el contrario, el PO por sí solo no fue capaz de reemplazar completamente el aceite de pescado y afectó negativamente el crecimiento de los peces debido a un contenido insuficiente de n-3 LC-PUFA en la dieta. También se observó una disminución similar del crecimiento con la reducción del contenido de la FM en la dieta al 7,5%. En conclusión, ambos aceites de microalgas, que proporcionaban DHA y EPA o DHA y n-6 DPA, son fuentes eficaces de n-3 LC-PUFA para juveniles de dorada y permiten la sustitución completa del aceite de pescado en combinación con fuentes de lípidos más rentables económicamente, como los aceites de pollo y de colza.

10.6.3 CAPÍTULO 5- MEZCLA DE ACEITES CON PRODUCTOS DERIVADOS DE MICROALGAS COMO UNA HERRAMIENTA PARA EL INCREMENTO DE N-3 LC-PUFA EN DIETAS SIN ACEITE DE PESCADO PARA LA CORVINA (ARGYROROMUS REGIUS)

La diversificación de la acuicultura es una herramienta eficaz para satisfacer la demanda de la industria y el mercado en el futuro, tanto aumentando el número de especies cultivadas como los ingredientes utilizados en los alimentos para peces. El objetivo del presente estudio era validar el potencial de una mezcla de aceites de microalgas con aceite de pollo como sustitutos totales del aceite de pescado en los piensos para corvina, una prometedora especie para la diversificación de la acuicultura mediterránea con un alto requerimiento de ácidos grasos poliinsaturados de cadena larga de la serie n-3. A tal efecto, se evaluaron cuatro dietas experimentales en juveniles de esta especie (2,7 g de peso corporal inicial). Una dieta control (dieta FO) se basó en un 5% de aceite de pescado y un 7% de aceite de colza y en las otras tres dietas experimentales, el aceite de pescado fue sustituido totalmente por aceite de pollo exclusivamente (dieta PO) o por la mezcla de aceite de pollo con uno de los dos aceites de microalgas (dietas ED y DD) que diferían en su composición de ácidos grasos. Los peces fueron alimentados con dietas experimentales durante 30 días cuando se analizó el crecimiento, la composición de los tejidos y el perfil de ácidos grasos. Además, también se determinó la expresión de genes relacionados con la biosíntesis de ácidos grasos, el sistema antioxidante de defensa y la respuesta inmune. Las dietas de microalgas fueron capaces de soportar el crecimiento y la utilización del alimento de los peces, manteniendo los niveles de DHA y los índices de calidad lipídica de sus filetes similares a los de los peces que alimentaron con la dieta de control de aceite de pescado. Además, la dietas con ED mostró la expresión más alta de *gpx* relacionada con el ambiente pro-oxidante más alto. Las dietas de microalgas también condujeron a una expresión más alta de la *elovl5* debido a la conversión más alta del 16:3n-3 de la dieta en 18:3n-3. Una expresión hepática ligeramente más alta de *hsp* también se relacionó con el aumento de los niveles de ARA dietéticos, siendo ligeramente más alta en los peces alimentados con las dietas FO, ED y DD. En contraste, el PO no fue capaz de mantener el crecimiento de los peces ni el contenido de ácidos grasos poliinsaturados de cadena larga de la serie n-3, mientras que aumentó el índice de trombosidad en los filetes de los peces. Además, el PO indujo un aumento en la expresión de la *fads2* y una reducción de la expresión de *gpx y hsp*, lo que podría sugerir una salud menos optimizada.

10.6.4 CAPÍTULO 6- EFECTOS BIOQUÍMICOS, MORFOLÓGICOS Y MOLECULARES DE LA ALIMENTACIÓN CON MICROALGAS Y ACEITE DE POLLO EN EL HÍGADO DE JUVENILES DE DORADA (SPARUS AURATA)

El presente trabajo pretendió investigar cómo la combinación de un aceite de pollo con dos aceites de microalgas, uno rico en ácido eicosapentaenoico y ácido docosahexaenoico (dietas ED) y otro en ácido docosahexaenoico y ácido n-6 docosapentaenoico (dietas DD), modulan el metabolismo lipídico hepático en juveniles de dorada. Las dietas se probaron utilizando dos contenidos diferentes de harina de pescado (15% y 7,5%) y se compararon con una dieta basada en aceite de pescado (CTRL) y dos dietas controles negativo basadas en aceite de pollo (dietas PO). Después de 74 días de alimentación, las doradas alimentadas con 15% FM ED o DD mostraron un índice de crecimiento diario similar a las alimentadas con la dieta CTRL, mientras que los peces alimentados con las dietas PO causaron un crecimiento reducido. Los hígados de los peces reflejaron los contenidos más altos en ácidos grasos poliinsaturados de cadena larga de la serie n-3 cuando alimentados con las dietas CTRL, ED o DD, que redujeron la expresión de la *fas*, *scd-1a*, *fads2*, *lpl* y *cpt1*, y reduciendo así la acumulación de lípidos en el hígado bien como el tamaño de los hepatocitos. Por el contrario, las dietas de PO indujeron al contenido depósito más bajo de ácidos grasos poliinsaturados de cadena larga de la serie n-3 y más alto de ácido oleico en el hígado, lo que llevó a un mayor índice hepatosomático debido al aumento de grasa en los hepatocitos. Por lo tanto, estos peces revelaron una esteatosis hepática severa asociada con una mayor expresión de genes relacionados con la lipogénesis, especialmente la *fas*, *lpl* y *sbrep1*. Además, las dietas de PO activaron las vías de desaturación de ácidos grasos, reflejadas por una mayor acumulación de ácidos grasos que son productos de las desaturasas bien como una mayor expresión de *fads2* y *scd-1a*. Por otra parte, la reducción del contenido de harina de pescado en la dieta al 7,5% redujo el crecimiento de los peces, aunque el metabolismo de los lípidos hepáticos parecía estar más afectado por el reemplazo del FO que por el reemplazo de la FM. En conclusión, se corrobora que la combinación de microalgas con aceite de pollo puede ser una fuente alternativa de lípidos y ácidos grasos esenciales al aceite de pescado en las dietas de los peces marinos.

10.6.5 CAPÍTULO 7- ACEITES INNOVADORES MODULAN LA FUNCIÓN NEURONAL Y PRESERVAN LA RESPUESTA LOCOMOTORA EN JUVENILES DE DORADA (SPARUS AURATA) REGULANDO LA SÍNTESIS Y EL CONTENIDO DE ÁCIDOS GRASOS EN EL CEREBRO

Los ácidos grasos poliinsaturados de cadena larga de la serie n-3 (LC-PUFA), particularmente el ácido docosahexaenoico (22:6n-3, DHA), son los ácidos grasos más importantes en la función cerebral de todos los vertebrados. Recientemente, nuevas fuentes de lípidos están disponibles para la industria, incluyendo productos obtenidos de microorganismos. Su estudio es importante ya que los perfiles de ácidos grasos de la dieta pueden afectar la composición de los tejidos y el funcionamiento celular de los peces. Para determinar el efecto de las nuevas fuentes de lípidos sobre el comportamiento y la función neural de la dorada (*Sparus aur*ata), a los juveniles se les administraron tres dietas que contenían o bien (FO), o bien un aceite de microalgas combinado con aceite pollo (DD) o bien solo aceite de pollo (PO) como sustituto al aceite de pescado. La respuesta de comportamiento a un estimulo externo de los peces, bien como el perfil de ácidos grasos de sus cerebros y la expresión relativa de genes relacionados con la neurogénesis y la actividad neuronal se evaluaron tras 5 meses de alimentación con las dietas experimentales. El cerebro de las doradas alimentadas con la dieta PO mostró el mayor contenido de DHA, así como un aumento de los productos de desaturación y alargamiento, contrariamente a lo que se observaba en el perfil la dieta. Esto, junto con un aumento de la expresión de la *fads2* en cerebro, sugiere la activación efectiva de la síntesis y retención de LC-PUFA como mecanismo compensatorio para una deficiencia de estos ácidos grasos, esenciales para peces marinos, en la dieta. Además, la expresión de *neurod6, bdnf* y *nos1*, marcadores cerebrales de la actividad neuronal, se relacionaron proporcionalmente con el contenido de DHA en cerebro. La respuesta locomotora de los peces al estimulo no se vio afectado por las dietas experimentales. Sin embargo, hubo una tendencia a que la latencia de escape fuera más larga en las doradas alimentadas con las dietas de PO y DD, además de una relación significativa entre la latencia de escape y el contenido de ácido docosapentaenoico n-6 (DPA) del cerebro. Esta aparente relación del n-6 DPA con déficits de comportamiento se mostró anteriormente en mamíferos y el presente estudio sugiere que el efecto de este acido graso presente en grandes cantidades en algunas microalgas, merece más atención y corroboración en los peces en el futuro.

10.7 CONCLUSIONES GENERALES

1. Las harinas comerciales de microalgas (ALL G RICH™- Alltech Inc., DHA GOLD-DSM, y un producto de desarrollo MO060- Adisseo) son sustitutos totales efectivos del FO en microdietas para larvas de dorada, llevando a un rendimiento larvario similar al de una dieta de FO al proporcionar la cantidad necesaria de n-3 LC-PUFA para un crecimiento y supervivencia óptimos de las larvas.

2. La mezcla de aceites de microalgas comerciales (aceite de algas de Veramaris, Veramaris y DHA Natur, ADM) con aceite de pollo menos dispendioso, es posible cubrir los requerimientos de EFA de los peces marinos y sustituir totalmente el FO, en dietas prácticas para la dorada y la corvina.

3. La reducción de la FM en la dieta (del 15% al 7,5%) y su sustitución por las fuentes de proteína vegetal usadas en este tesis, reduce notablemente la utilización de los nutrientes de la dieta y, en consecuencia, el crecimiento de la dorada. Además, la sustitución simultanea de la FM y del FO, este ultimo por PO, tiene un efecto aún más negativo en el metabolismo de los lípidos de los peces.

4. En comparación con una dieta FO, el uso de productos de microalgas no afecta a los procesos de lipogénesis y/o lipolíticos en el hígado, bien de las larvas como de los juveniles de dorada.

5. La sustitución del FO por aceites de microalgas reduce la lipogénesis en el hígado de los peces bien como los procesos de desaturación de ácidos grasos, reduciendo la acumulación de lípidos hepáticos en comparación con el PO.

6. Los productos de microalgas, ya sea en forma de harinas/biomasa o de aceites, pueden ser sustitutos totales y efectivos del FO en larvas y juveniles de peces, sin consecuencias negativas en al menos algunos aspectos de la salud de los peces, incluido el estrés oxidativo, el metabolismo de las proteínas de choque térmico y el metabolismo de los eicosanoides.

7. La incorporación del n-6 DPA en el cerebro de los juveniles de dorada afecta negativamente al comportamiento no locomotor (latencia de escape), como se observó en estos estudios por primera vez en peces.

8. La sustitución total del FO por el PO reduce los contenidos de n-3 LC-PUFA, pero conduce al mayor contenido de DHA en el cerebro de los peces, denotando una alta retención de este ácido graso como mecanismo compensatorio a una deficiencia prolongada de n-3 LC-PUFA.

9. La sustitución total del FO por el PO que resulta en un incremento en el contenido de DHA en el cerebro de los peces, estimula la neurogénesis y la actividad neuronal en la dorada, y pone de relieve la importancia de este ácido graso para la función neuronal de los peces.

10. Los productos de microalgas pueden ser una de las soluciones para aumentar el contenido de n-3 LC-PUFA, especialmente de DHA, en el cuerpo y los filetes de peces marinos, en el contexto actual de la baja cantidad de FO/FM de la industria de piensos para la acuicultura. Sin embargo, los productos de microalgas utilizados (Veramaris algal oil, Veramaris and DHA Natur Oil, ADM) reducen el contenido de EPA en filetes en comparación con el FO.

11. Las dietas suplementadas con microalgas mejoran la calidad nutricional de los filetes de pescado para el consumidor, reduciendo el riesgo de aterogénesis y trombogénesis. Sin embargo, estos productos incrementan el índice de peroxidación, un indicador de vida útil del filete.

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ANNEX

Publications of the PhD project

- **Carvalho, M.**, Montero, D., Rosenlund, G., Fontanillas, R., Ginés, R., Izquierdo, M., 2020. Effective complete replacement of fish oil by combining poultry and microalgae oils in practical diets for gilthead sea bream (*Sparus aurata*) fingerlings. *Aquaculture*, *529*, 735696.
- **Carvalho, M**., Montero, D., Torrecillas, S., Castro, P., Zamorano, M. J., Izquierdo, M., 2021. Hepatic biochemical, morphological, and molecular effects of feeding microalgae and poultry oils to gilthead sea bream (*Sparus aurata*). *Aquaculture*, *532*, 736073.
- **Carvalho, M**., Montero, D., Domenici, P., Afonso, J. M., Izquierdo, M., 2021. Dietary novel oils modulate neural function and preserve locomotor response in gilthead sea bream (*Sparus aurata*) juveniles by regulating synthesis and contents of fatty acids in brain. Aquaculture, 737873.
- **Carvalho, M**., Marotta, B., Xu, H., Geraert, P., Kaushik, S., Montero, D., Izquierdo, M., 2022. Complete replacement of fish oil by three microalgal products rich in n-3 long-chain polyunsaturated fatty acids in early weaning microdiets for gilthead sea bream (*Sparus aurata*)- submitted to Aquaculture (AQUACULTURE-D-21-03381).
- **Carvalho, M.**, Izquierdo, M., Valdés, M., Montero, D., Farías, A., 2022. Oils combination with microalgal products as a strategy for increasing the n-3 long-chain polyunsaturated fatty acids content in fish oil-free diets for meagre (*Argyrosomus regius*)- submitted to Aquaculture (AQUACULTURE-S-22-00339).

International oral and poster presentations of the PhD project

- **Carvalho, M. (presenting author; oral presentation)**, Montero, D., Rosenlund, G., Fontanillas, R., Ginés, R., Izquierdo, M. Effective complete replacement of fish oil by combining poultry and microalgae oils in practical diets for gilthead sea bream (*Sparus aurata*) fingerlings. Aquaculture Europe 2019, Berlin, Germany.
- **Carvalho, M. (presenting author; oral presentation)**, Montero, D., Domenici, P., Afonso, J. M., Izquierdo, M. The effect of conventional and novel oils on brain fatty acid incorporation, behaviour, and neural function of gilthead sea bream (*Sparus aurata*) juveniles. Aquaculture Europe 2021, Funchal, Madeira.
- **Carvalho, M. (presenting author; oral presentation)**, Montero, D., Izquierdo, M. Microalgal products as innovative sources of omega-3 fatty acids in aquafeeds: implications for fish production, consumers needs and sustainability of the industry. International Symposium of Fish Nutrition and Feeding 2021. Busan, South Korea.

Collaborative works (other publications, other presentations, teaching)

Other publications

- **Carvalho, M.**, Peres, H., Saleh, R., Fontanillas, R., Rosenlund, G., Oliva-Teles, A., Izquierdo, M. 2018. Dietary requirement for n-3 long-chain polyunsaturated fatty acids for fast growth of meagre (*Argyrosomus regius*, Asso 1801) fingerlings. Aquaculture, 488, 105- 113.
- **Carvalho, M.**, Castro, P., Montero, D., Peres, H., Acosta, F., Fontanillas, R., Rosenlund, G., Robaina, L., Izquierdo, M. 2019. Essential fatty acid deficiency increases hepatic steatosis and granulomatosis in meagre (*Argyrosomus regius*, Asso 1801) fingerlings. Aquaculture 505, 393-404.
- **Carvalho, M.**, Montero, D., Gesto, M., Lencina, A., Lund, I., Izquierdo, M. 2019. The effect of dietary n-3 LC PUFA on response to an acute and prolonged stress of meagre (*Argyrosomus regius*, Asso 1801) juveniles. Aquaculture 506, 112-118.
- Fernández-Montero, Á., Torrecillas, S., Acosta, F., Kalinowski, T., Bravo, J., Sweetman, J., Roo, J., Makol, A., Docando, J., Izquierdo, M., **Carvalho, M**., Montero, D. 2020. Improving greater amberjack (*Seriola dumerili*) defenses against monogenean parasite *Neobenedenia girellae* infection through functional dietary additives. Aquaculture, 736317.

- Torrecillas, S., Montero, D., **Carvalho, M.**, Benítez-Santana, T., Izquierdo, M., 2021. Replacement of fish meal by Antarctic krill meal in diets for European sea bass *Dicentrarchus labrax*: Growth performance, feed utilization and liver lipid metabolism. Aquaculture, *545*, 737166.
- **Carvalho, M.,** Sanmartín, A., Montero, D. Fontanillas, R., Farías, A., Hernández Velásquez, J., Izquierdo, M., 2022. Insect meal and single-cell protein as replacers of fish meal in diets for gilthead sea bream (*Sparus aurata*).

Other presentations

- **Carvalho, M. (presenting author; oral presentation)**, Castro, P., Montero, D., Peres, H., Acosta, F., Fontanillas, R., Rosenlund, G., Robaina, L., Izquierdo, M. Essential fatty acid deficiency increases hepatic steatosis and granulomatosis in meagre (*Argyrosomus regius*, Asso 1801) fingerlings. International Symposium of Fish Nutrition and Feeding 2018, Las Palmas de Gran Canaria, Spain.
- **Carvalho, M. (presenting author; poster presentation)**, Montero, D., Gesto, M., Lencina, A., Lund, I., Izquierdo, M. The effect of dietary n-3 LC PUFA on response to an acute and prolonged stress of meagre (*Argyrosomus regius*, Asso 1801) juveniles. International Symposium of Fish Nutrition and Feeding 2018, Las Palmas de Gran Canaria, Spain.
- **Carvalho, M. (presenting author; poster presentation),** Sanmartín, A., Montero, D. Fontanillas, R., Izquierdo, M. Insect meal and single-cell protein as replacers of fish meal in diets for gilthead sea bream (*Sparus aurata*). Aquaculture Europe 2021, Funchal, Madeira.

Teaching

- Venia docendi 2018/2019 of 24 practical hours in Aquaculture (Bsc Veterinary Sciences at University of Las Palmas de Gran Canaria)
- Venia docendi 2019/2020 of 40 practical hours in Aquaculture (Bsc Veterinary Sciences at University of Las Palmas de Gran Canaria)
- Venia docendi 2020/2021 of 40 practical hours in Nutrition of Aquatic animals (Msc Marine Resources at University of Las Palmas de Gran Canaria)
- Co-supervisor of the master thesis “Growth and lipid digestive capacity of gilthead sea bream (*Sparus aurata*) juveniles in response to novel dietary lipid sources”- Paula Sarmiento Mendonza- 2019/2020

- ◊ Co-supervisor of the master thesis “Insect meal and single cell protein as alternative sources to fish meal in diets for gilthead sea bream (*Sparus aurata*) juveniles” – Antonio Sanmartín Almeida- 2019/2020
- ◊ Invited Speaker (Fat content) at Advanced Course of New Feeds and Feeding Technologies in Aquaculture, organized by CIHEAM Zaragoza and medAID EU funded project, with the collaboration of PerformFISH EU funded project and NewTechAqua EU funded project; June 2021.
- ◊ Invited Speaker at BlueFest (Lisbon, Portugal); Aquaculture as the solution for a sustainable and healthy diet; November 2021.

Awards and scholarships during the PhD

- ❖ 4-year grant for PhD studies funded by Agencia Canaria de Investigación, Innovación y Sociedad de la Información del Gobierno de Canarias.
- ❖ Best student oral presentation in XVIII Symposium of Fish Nutrition and Feeding (June 2018, Las Palmas de Gran Canaria).
- ❖ Erasmus+ Internship Scholarship 2020/2021 to do an international internship in NUTRIMU, at the University of Porto, Portugal, for 3 months where I have learnt *in vitro* techniques and trials with fish cell cultures.
- ❖ Global Women in Aquaculture 2021 (international award funded by Kvaroy Arctic and SAGE- 10.000 USD)
- ❖ 2nd Prize V Concurso Cátedra Telefónica de la Universidad de Las Palmas de Gran Canaria- Premios Cátedra Telefónica de Investigación, Innovación y Cultura Científica 2021- Categoría Proyectos de Grado, Posgrado o Tesis (717,64€, and certificate Cátedra Telefónica ULPGC).
- ❖ Winner of the 2nd edition SPAROS CONTEST for aquaculture nutrition research- Awarded 6 months free license of FEEDNETICS and grant to participate in XX International Symposium on Fish Nutrition and Feeding (June 5-9, 2022, Sorrento, Italy).
- ❖ Best oral presentation in XIX International Symposium of Fish Nutrition and Feeding 2021. Busan, South Korea.