
UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Instituto Universitario de Sanidad Animal
y Seguridad Alimentaria
Alex Makol
PhD Thesis 2011
Effects of dietary Conjugated Linoleic Acid (CLA) on European sea bass (Dicentrarchus labrax) juvenile culture
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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario de Sanidad Animal Seguridad Alimentaria

Anexo I

Dª MARÍA SORAYA DÉNIZ SUÁREZ, SECRETARIA DEL INSTITUTO UNIVERSITARIO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

CERTIFICA

Que el Consejo de Doctores del Departamento en su sesión de fecha 18 de octubre de 2011 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada: **"EFFECT OF DIETARY CONJUGATED LINOLEIC ACID (CLA) ON EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) CULTURE ". ("EFECTO DE LA SUPLEMENTACIÓN DIETÉTICA DEL ÁCIDO LINOILÉICO CONJUGADO (CLA) EN EL CULTIVO DE LUBINA (*DICENTRARCHUS LABRAX*)")**, presentada por el doctorando D. Alex Makol, dirigida por la Dra. Dª. Marisol Izquierdo López.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a diecinueve de octubre de dos mil once.

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Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Programa de Doctorado: Acuicultura

Título de la Tesis

"EFFECTS OF DIETARY CONJUGATED LINOLEIC ACID (CLA) ON EUROPEAN SEA BASS (*DICENTRARCHUS LABRAX*) CULTURE". ("EFECTO DE LA SUPLEMENTACIÓN DIETÉTICA DEL ÁCIDO LINOLÉICO CONJUGADO (CLA) EN EL CULTIVO DE LUBINA (*DICENTRARCHUS LABRAX*)").

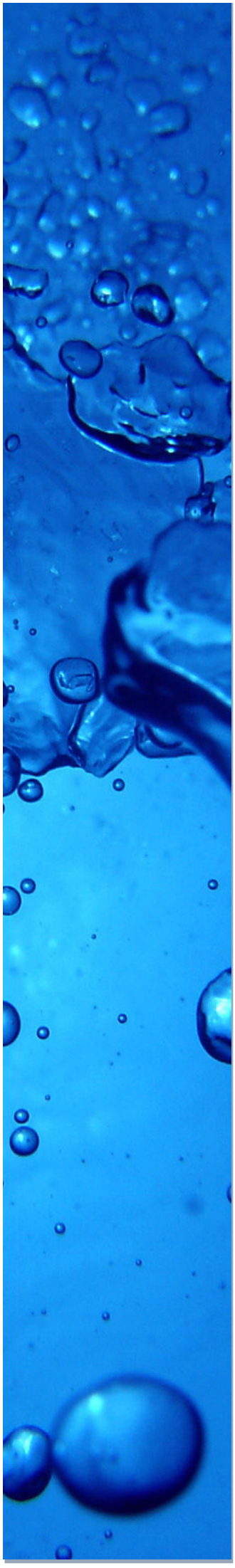
Tesis Doctoral presentada por Don Alejandro Makol Arenas

Dirigida por la Dra. Marisol Izquierdo López

El Director, **El Doctorando,**

Marisol Izquierdo López **Alejandro Makol Arenas**

Las Palmas de Gran Canaria, a 3 de Octubre de 2011



UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
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Effect of dietary Conjugated Linoleic Acid (CLA) on European seabass (*Dicentrarchus labrax*) culture

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Doctorado en Acuicultura: Producción controlada de animales acuáticos
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Director:
Prof. Marisol Izquierdo

A mi familia,

Contents

Abbreviations

ACO, Acyl-CoA oxidase

ACP, Alternative complement pathway

ADC, Apparent digestibility coefficient

ARA, Arachidonic acid

BSA, Bovine serum albumin

CLA, Conjugated linoleic acid

COX-2, Cyclooxigenase-2

CPT-1, Carnitine palmitoyl transferase-1

DHA, Docosahexaenoic acid

EFA, Essential fatty acids

ELISA, Enzyme linked immuno sorbent assay

EPA, Eicosapentaenoic acid

EU, European Union

FA, Fatty acids

FAS, Fatty acid synthase

FCR, Feed conversion ratio

FID, Flame ionization detector

G6PD, Glucose-6-phosphate dehydrogenase

H&E, Hematoxylin and eosin

HBSS, Hank´s balanced salt solution

HSI, Hepatosomatic index

HUFA, Highly unsaturated fatty acids

IFN-γ, Interferon-γ

Ig, Immunoglobulin

IU, Enzyme activity units

K, Condition factor

L3HOAD, L-3-hydroxyacyl-CoA dehydrogenase

LC-PUFA, Long chain polyunsaturated fatty acids

LDCF, 2',7'-dichlorofluorescein diacetate

LNA, α-linolenic acid

LT, Leukotrienes

LX, Lipoxins

ME, Malic enzyme

MEM, Minimal essential medium

MMT, Million metric tons

MUFA, Monounsaturated fatty acids

NADPH, Reduced nicotinamide adenine dinucleotide phosphate

NBT, Nitroblue tetrazolium

PFI, Perivisceral fat index

PG, Prostaglandin

PGE_2, Prostaglandin E_2

PLs, Phospholipids

PMA, Phorbol 12-myristate 13-acetate

PPARs, Peroxisome proliferator-activated receptors

PUFA, Polyunsaturated fatty acids

SCD, Steaoryl-CoA desaturase

SD, Standard deviation

SFA, Saturated fatty acids

SGR, Specific growth rate

TAG, Triacylglycerol

TNF, Tumor necrosis factor

TPA, Texture profile analysis

TX, Tromboxanes

VSI, Viscerosomatic index

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Alex

Chapter 1

Introduction

1.1. European sea bass (*Dicentrarchus labrax*) aquaculture production

Based on the last APROMAR report (2011), over the 50% of the total aquatic food consumed worldwide comes from aquaculture and is predicted to reach the 65% in 2030. In 2009, total aquaculture production for human consumption reached 73 MMT in contrast with the 65 MMT from fisheries sector. Aquaculture production has grown amazingly from the 1950 where produced 0.6 MMT in contrast with the 73 MMT in 2009 and representing approximately a market value of € 88120 million. Even though, is perceptible that the exponential growth of the sector has been minimized in the last decade decreasing from an annual growth rate of 9% in the 80´s and 90´s to a 6% in the first decade of the XXI century, fact that is especially accentuated in the European region. Despite this data, in 2009, total fish aquaculture production in the European Union (EU) reached 629401 tonnes, increasing a 0.9% from 2008, which signified approximately € 2398 million. Spanish fish aquaculture production represented the 10.2% of the total EU production, achieving a commercial market value of € 286 million.

Regarding marine fish production in the south of Europe and Mediterranean regions, gilthead sea bream (*Sparus aurata*), European sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maxima*) are the main cultivated species. Nevertheless, nowadays the culture of meagre (*Argyrosomus regius*) is a common culture in some of the Mediterranean countries. European sea bass production in 2010 reached 118931 tonnes where main producers were Greece (39.6%), Turkey (29.5%) and

Spain (10.5%). The production of European sea bass in Spain during 2010 reached 12495 tonnes decreasing a 9.7% from 2009, mainly derived from the marked 40.7% increase experienced during the 2008-2009 period. Despite this data, the average annual growth of European sea bass Spanish production remains in a 10% in the last 4 periods and it is pointed to continue growing. Inside the Spanish region, Canary Islands are the main European sea bass producer with the 30% of the total country production; the rest of the production is divided between Andalucía, Murcia, Valencia and Cataluña.

Unfortunately, the current trend to the use of increased energy contents in fish feeds and the inclusion of vegetable protein or lipid sources in substitution of fish meal and oil in fish diets (Caballero *et al*., 1999, 2004) has led to an enhanced perivisceral, hepatic and subcutaneous fat deposition which may affect product quality and restrains optimization of fish production concerning fish farmers (Izquierdo *et al*., 2005). One of the marine fish species more susceptible to excess fat deposition is European sea bass, where this lipid accumulation may be a physiological response to the diet rather than a pathological situation (Izquierdo *et al*., 2003). So, attending to this fact and that conjugated linoleic acid (CLA) has been demonstrated to reduce fat deposition in rodents and other mammals (Park *et al*., 1999, 2005; Park and Pariza, 2007), European sea bass is considered a good target species for this thesis.

1.2. Lipids and fatty acids

Lipids constitute a large and diverse group of oils and fats that are organic compounds in nature, soluble in organic solvents (hydrocarbons, chloroform, benzene, ethers and alcohols) and sparingly soluble in water. They include a varied range of compounds such as fatty acids and their derivatives, carotenoids, terpenes, steroids and bile acids.

Fatty acids are the basic constituents of lipids, which have different physiological and structural functions such as membrane structure, enzymatic, energetic and hormonal (McMurchie, 1988), among others. A fatty acid is referred to as saturated (SFA) when it contains no carbon to carbon double bonds. Conversely, monounsaturated fatty acids (MUFA) contain one double bond, whereas fatty acids with two or more double bonds are named polyunsaturated fatty acids (PUFA). Highly

unsaturated fatty acids (HUFA) are those PUFA of 20 or more carbons with 3 or more double bonds. The geometry of double bonds in PUFA could be *cis* and/or *trans* depending if the hydrogen atoms are on the same side or in the opposite side of the double bond, respectively.

In fish, the main present PUFA are arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (22:6n-3, DHA) and α-linolenic acid (LNA, 18:3n-3) being fish the major dietary source of n-3 HUFA (Sargent and Tacon, 1999). Freshwater and marine fish have different essential fatty acids (EFA) requirements due to they differ in the elongation and desaturation capabilities of fatty acids. EFA are those fatty acids that must be supplied in the fish diet because they are not capable to synthetize them *de novo* from other fatty acids. Freshwater species have enough Δ5 and Δ6 desaturase and elongase enzyme activities to produce ARA, EPA and DHA from their precursors linoleic (LA; 18:2n-6) and LNA fatty acids when supplied in the diet, whereas in marine fish these enzymatic activities are very restricted, and as a consequence, they must be supplied in the diet (Fig.1.1). Therefore, the EFA in freshwater species are LN and LNA, whereas in marine species are ARA, EPA and DHA (Sargent *et al*., 1995; Izquierdo, 1996, 2005; Bell, 1998).

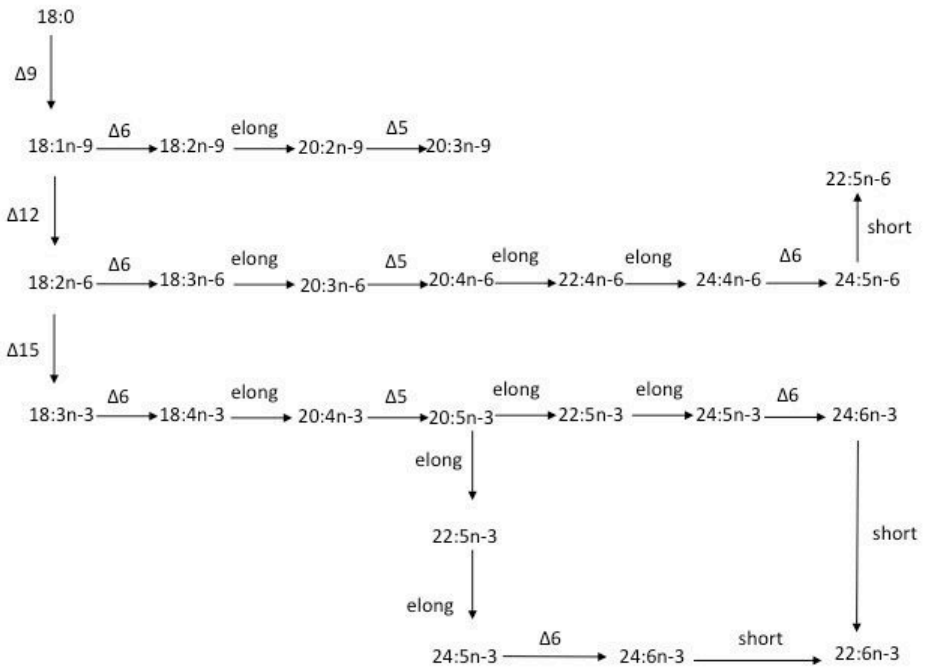


Figure 1.1. Fatty acids elongation and desaturation pathway. Adapted from Vagner and Santigosa (2011)

1.3. Lipids functions in fish

Main lipids functions are energy storage molecules, cell membrane structural components, precursors of eicosanoids and ligands for transcription factors. Lipids are mainly stored as triacylglycerol (TAG), which consist in one glycerol molecule and three esterified fatty acids. Fatty acids are released from TAG to deliver energy through β-oxidation processes when energy is required (Sargent *et al*., 1989; Frøyland *et al*., 2000) or as a source of structural components for cell membranes. PUFA are important phospholipid (PL) constituents conferring different properties to membranes such as decreasing their fluidity, whereas SFA and MUFA give additional rigidity (Tocher, 2003).

Lipids, and specifically fatty acids, are the preferred source of energy for growth, reproduction and swimming in marine fish, being SFA acids and MUFA the first ones used to obtain energy, keeping PUFA for structural functions. This specificity of fatty acid oxidation in fish determines the fatty acid composition of TAG in fish adipose tissue and it is important for the well-being of fish and for the consumer.

Fatty acids stored as PL have structural functions in cell membranes, being more stable than fatty acids stored as TAG. As state above, membrane fluidity and permeability, depends on its fatty acid composition. PUFA are incorporated into PL or TAG based on the degree of unsaturation, so LNA and LN are usually more esterified into TAG, whereas ARA, EPA and DHA are mainly esterified into PL (Henderson and Tocher, 1987). In fish, the high levels of n-3 HUFA found in cellular membranes are vital for maintaining its structure and function (Tocher, 2003).

Long chain PUFA act as eicosanoid precursors, being the dihomo-γ-linolenic acid (DHGLA, 20:3n-6), ARA and EPA the primarily precursors of eicosanoids such as prostaglandins (PG), leukotrienes (LT), thromboxanes (TX) and lipoxins (LX), which are bioactive fatty acids metabolites that could modulate immune functions (Gershwin *et al*., 1985; Uhing *et al*., 1990; Tocher, 2003). Whereas ARA is the major eicosanoid precursor, particularly of the 2-series PG (Horrobin, 1983), DHGLA and EPA are of the 1- and 3-series PG, respectively (Crawford, 1983). Besides, the ratio ARA/EPA could determine eicosanoid action in cellular membranes, so the dietary intake of n-3 and n-6 PUFA could affect these actions (Tocher, 2003).

Fatty acids, mainly PUFA and their derivatives are also involved in the regulation of the activities of a variety of transcription factors that control genes responsible for fatty acid metabolism (Jump, 2004). One of these transcription factors are the peroxisome proliferator-activated receptors (PPARs), which are nuclear hormone receptors that regulates the expression of genes involved in lipid homeostasis in mammals (Desvergne and Wahli, 1999; Hihi *et al*., 2002). There are three known PPARs genes in mammals (PPARα, PPARβ/δ and PPARγ), each one expressed in different tissues, whereas in fish there are scarce studies, data point out to the same PPARs genes expressed in fish tissues (Boukouvala *et al*., 2004; Leaver *et al*., 2005).

1.4. Fish immune system and lipids

Cooperation between different cells of the immune system via membrane-associated events and through different lipid mediators is essential in mounting a successful immune system response (FAO, 1994). Studies with animals, tissue cultures, as well as humans indicate that both the level and degree of saturation of dietary lipids influence inflammatory and immunological responses, even thought, the nature of the effect markedly depends on the type of fatty acid, age, antioxidant included in the diet (Waagbø *et al*., 2003; Puangkaew *et al*., 2004), health status of the subject (FAO, 1994) and species EFA requirements. For example, in laboratory animals, a high dietary fat content suppresses lymphocyte function, according to the level and type of fat (Calder *et al*., 2002; Montero and Izquierdo, 2010), whereas SFA seem to have little effect on lymphocyte proliferation, cytokine production or natural killer (NK) cell activity (Yaqoob *et al*., 1994a, b; Montero and Izquierdo, 2010). The n-3 PUFA family is able to reduce lymphocyte proliferation, NK cell activity through eicosanoid production, neutrophil chemotaxis and secretion of several cytokines, such as interleukin (IL)-1, IL-2 and tumor necrosis factor (TNF) (Endres *et al*., 1989, 1993; Yaqoob and Calder, 1995, 2007; De Pablo and Álvarez, 2000; Wallace *et al*., 2001). In fact, the cytokine modulation by these fatty acids seems to be responsible for the reduction of lymphocyte proliferation in both animals and humans based in the inhibitory effects of n-3 PUFA on the expression of the CD25 molecule that constitutes an IL-2 receptor (Soyland *et al*., 1994; De Pablo and Álvarez, 2000). The mechanisms involved in the modification of cytokine synthesis

remain unclear, but one possible explanation could remain in the reduction of the cytokine mRNA production by PUFA (Robinson *et al*., 1996; De Pablo and Álvarez, 2000). Instead, the n-6 series are able to stimulate the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, and consequently increasing vasodilatation or vascular permeability (Calder, 2002; Montero and Izquierdo, 2010).

In fish, generally, three main mechanisms have been studied by which dietary fatty acids may affect immune system are membrane fatty acid composition. The first one regards their influence on cell membrane lipid composition through changes in the PL membrane fatty acid composition (Balfry and Higgs, 2001), influencing many immune responses that are based on leucocyte cell membrane interactions, such as phagocytosis, antigen-antibody or cytokine production. The second mechanism altered by dietary fatty acids involves changes of signal transduction, possibly due to effects on protein kinase C (Balfry and Higgs, 2001) and based on the effect of fish oil supplementation on increased sensitivity to interferon-γ (IFN-γ) on mammalian macrophages (Erickson and Hubbard, 1993). The third way whereby dietary fatty acids could affect immune system is through the production of eicosanoids from non-esterified ARA, EPA and DHA. Even thought, some studies in mammalian cellular cultures have highlighted the effects of dietary lipids on apoptosis, lipid peroxidation and regulation of gene expression (For review see De Pablo and Álvarez, 2000) and its involvement on immunemodulation.

1.5. Lipids and flesh quality

The quality of seafood can be characterized by both; physical aspects like product freshness and appearance and nutritional and sensory properties (Rosenlund *et al*., 2010).

In cultured fish, feeding with artificial diets provides a wide range of nutrients and this fact, not only determines fish growth rate but also the fillet composition, and in particular the lipid content, which may be quantitatively and qualitatively modified (Izquierdo *et al*., 2003). Qualitatively, providing high contents of n-3 long-chain (LC)-PUFA that are normally associated to the seafood health benefits when consumed (Kris-Etherton *et al*., 2002; Calder, 2004; Wang *et al*., 2006; Rosenlund *et al*., 2010) or by the

inclusion of some other lipids with beneficial aspects to the human health as could be CLA, resulting in new functional foods. Quantitatively, an increase in the dietary fat composition could result in higher lipid concentration of the fillet produced, that in turn will influence physical and sensory fillet quality (Lopparelli *et al*., 2004). Particularly, intramuscular fat deposition seems to limit collagen and muscle fibers crosslinks, reducing their mechanical strength (Fasolato *et al*., 2005) and decreasing texture, which is correlated by consumer as fatter fillet, with a reduction of acceptance and market value (Torrissen *et al.*, 2001).

1.6. Fatty acid metabolism

1.6.1. Lipogenesis

Lipogenesis is the term used to describe the reactions for the formation of new endogenous lipids (Tocher, 2003) and is considered to be an identical process as in mammals (Sheridan, 1994). The key pathway in lipogenesis is catalyzed by the fatty acid synthase (FAS) complex that requires reduced nicotamide-adenine-dinucleotide phosphate (NADPH) during sequential condensation steps (Henderson and Sargent, 1985), obtaining as main products SFA (palmitic acid, 16:0 and stearic acid, 18:0) both in mammals and fish (Sargent *et al*., 1989), which can be desaturated by stearoyl-CoA desaturase (SCD) to form palmitoleic acid (16:1n-7) and oleic acid (18:1n-9) and further desaturated and elongated especially under EFA deficiency (Tocher, 2003). Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and malic enzyme (ME; EC 1.1.1.40) are the main suppliers of the reducing power in the form of NADPH, being the first one the major NADPH contributor (Dias *et al*., 1999), but the cellular content of both dehydrogenases varies depending on dietary and hormonal conditions (Tocher, 2003). Noteworthy, the use in aquaculture of diets rich in lipids induce fish not to biosynthesize *de novo* fatty acids to any significant extent. Actually, fish tend to accumulate large lipid depots in form of TAG, when energy supply exceeds expenditure, in specific storage sites (mainly in adipose tissue) that are mobilized when energy requirements of the organism exceed the available energy from the diet (Tocher, 2003).

1.6.2. Lipolysis

Fatty acid oxidation in an important source of energy in fish and it can occur in liver, red and white muscle and heart (Frøyland *et al*., 1998, 2000). The β-oxidation of fatty acids can occur by in two different organelles, mitochondria and peroxisomes, in both fish and mammals (Mannaerts *et al*., 1979; Reddy and Mannaerts, 1994). In fish, peroxisomal β-oxidation can account up to 50% of total β-oxidation (Crocket and Sidell, 1993a, b; Nanton *et al*., 2003), but it is half as efficient producing energy as mitochondrial β-oxidation since in peroxisomal β-oxidation half of the energy is lost as heat (Mannaerts and van Veldhoven, 1996; Stubhaug *et al*., 2007). Nevertheless, peroxisomes are more abundant than mitochondria, so fish appeared to utilize this system because of the global β-oxidation capacity (Stubhaug *et al*., 2007). The rate-limiting enzyme in mitochondrial β-oxidation is the carnitine palmitoyl transferase-1 (CPT-I; EC 2.3.1.21) (McGarry and Foster, 1979; Frøyland *et al*., 1998), whereas acyl-CoA oxidase (ACO; EC 1.3.3.6) catalyzes the rate-limiting step in the peroxisomal β-oxidation system (Inestrosa *et al*., 1979; Fraser *et al*., 1997). There are evidences that SFA and MUFA can readily be catabolized by mitochondrial β-oxidation in fish (Sargent *et al*., 1989), whereas PUFA β-oxidation depends on PUFA molecules being more complicated to be directly β-oxidized in mitochondria, so in fish may occurs as in mammals where peroxisomal β-oxidation seems to primarily function as a detoxification system, chain-shortening system and β-oxidation of very long-chain fatty acids (Jakobs and Wanders, 1991; Mannaerts and van Veldhoven, 1993).

1.7. Conjugated linoleic acid (CLA)

1.7.1. Introduction

Conjugated linoleic acid (CLA) refers to a generic group of geometric and positional isomers of linoleic acid with non-methylene interrupted double bonds. Although to date 58 isomers have been identified, occurring the double bonds in several positions (7, 9; 8, 10; 9, 11; 10, 12; 11, 13), and found in food (Kramer *et al*., 1998: Pariza *et al*., 2001; Park and Pariza, 2007), the main research has been focused in two main isomers, the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12, which are the isomers considered bioactive (Pariza *et al*., 2001) (Fig. 1.2). Naturally occurring CLA in dairy products, milk

and food such as beef primarily consist on the *cis*-9, *trans*-11 isomer (80-90 %), whereas other isomers are found in low proportion (Chin *et al*., 1994; Khnanal and Dhiman, 2004). The *cis*-9, *trans*-11 isomer is originated from biohydrogenation of linoleic acid to stearic acid by rumen bacteria (*Butyrivibrio fibrisolvens*) (Kepler *et al*., 1966), but also by Δ-9 desaturation of *trans*-11 vaccenic acid (18:1) in mammalian tissues (Corl *et al*., 2003; Kay *et al*., 2004; Mosley *et al*., 2006a, b), whereas the *trans*-10, *cis*-12 isomer used in research studies is considered to be "man-made" because it is found in negligible amounts (3-5 %) in natural sources (Park and Pariza, 1998; Khnanal and Dhiman, 2004; Dhiman *et al*., 2005). Consequently, most experimental studies are being carried out using *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers individually or together as CLA, where isomers are in 50:50 proportion, being produced from linoleic acid rich oils or linoleic acid itself via alkaline isomerization or partial hydrogenation (Banni, 2002). Nowadays, the main sources of CLA include dietary supplements such as capsules, which contain amounts up to 1014 mg CLA per capsule, and few derived dairy products, even though the levels in these products are usually lower than 1% of total fatty acids (Manning *et al*., 2006; Schmid *et al*., 2006). In humans, dietary CLA intake is estimated about 95-440 mg day^{-1} differing country to country (Schmid *et al*., 2006), but higher levels (2.4-3.5 g day^{-1}) seems to be needed to promote human health benefits (Terpstra, 2004). Generally, the 60% of human CLA ingestion comes from lactic products, whereas other 37% from meat products, being the relation among the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers from 30:1 to 70:1, respectively (Wahle *et al*., 2004; Haro *et al*., 2006).

Increase the animal-derived food quality products in the market is one of the goals of the agricultural sector as in aquaculture. So, additional focus has been given to promote foods enriched which components that have beneficial effects on human health, as could be CLA. The way to do it is by including CLA in animal diets to raise the CLA levels found naturally and to obtain the beneficial effects of CLA in the same animals, having CLA inclusion in aquafeeds the same objective as in terrestrial animals.

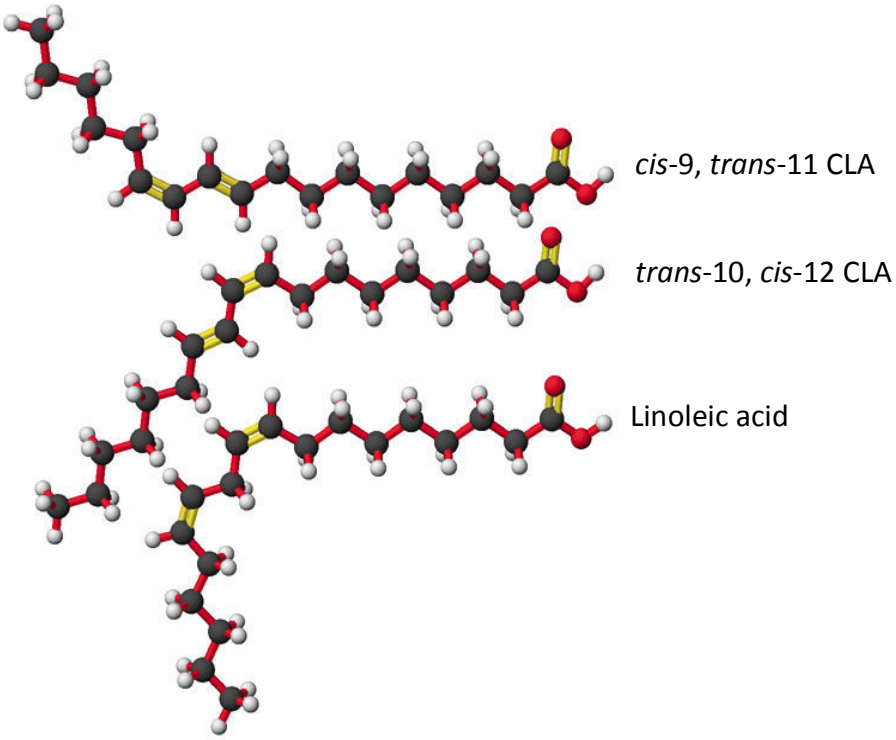


Figure 1.2. Isomer CLA structure of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 and linoleic acid (Modified from Bauman *et al*., 1999).

1.7.2. CLA and body composition in humans

During the last decade, the effects of CLA on humans have been tested. These studies reported the effects of CLA in humans, mainly regarding body deposition, immune system and anticarcinogenic properties, being the results obtained sometimes contradictory (Table 1.1). Regarding body composition, different studies reported a decrease in body fat and a reduction in sagittal abdominal diameter when CLA was included in the diet (Blankson *et al*., 2000; Riserus *et al*., 2001; Smedman and Vessby, 2001; Thom *et al*., 2001; Kreider *et al*., 2002: Gaullier *et al*., 2004, 2005) as well as a reduction in adipocyte size (Evans *et al*., 2002) and an increase in lean body mass coupled with an increase in the resting metabolic rate (Kamphuis *et al*., 2003; Gaullier *et al*., 2004). In the other hand, no effect was obtained in other studies (Kreider *et al*., 2002; Riserus *et al*., 2004; Bhattacharya *et al*., 2006). Studies in the immune system

obtained in most cases that CLA improved the immune response by enhancing the antibody formation (Sugano *et al*., 1998), by activating the nuclear factor kB (Chung *et al*., 2005; De Roos *et al*., 2005; Poirier *et al*., 2005) or by increasing immunoglobulin-A (IgA) and IgM levels (Song *et al*., 2005). On the contrary, no effect was found in the production of cytokines and eicosanoids in other studies (Kelley *et al*., 2001; Albers *et al.*, 2003; Nugent *et al*., 2005). Studies focused in breast cancer cells and the anticarcinogenic properties of CLA showed also controversial results. Durgam and Fernandes (1997) obtained a reduction of cancer cells, whereas no effect was obtained by Rissanen *et al*. (2003) and McCann *et al*. (2004). The same pattern was followed regarding its effects on diabetes and insulin resistance where Medina *et al*. (2000) reported increased levels of insulin in serum and Chung *et al*. (2005) obtained that CLA produced insulin resistance, and also in bone health where some studies reported an increase in bone mass (Brownbill *et al*., 2005; Gaullier *et al*., 2005) and others did not obtain any effect (Gaullier *et al*., 2004; Doyle *et al*., 2005).

1.7.3. CLA and body composition in experimental animals

Several studies have reported different properties of CLA in animal models. In most studies the effects on growth and body composition, anticarcinogenic properties, immunemodulative and diabetes modulator have been denoted, whereas in others, no effect was detected (Table 1.2). For example, growth was promoted in rodents and pigs (Chin *et al*., 1994; Bee, 2000; Dugan *et al*., 2004; Valeille *et al*., 2004; Storkson *et al*., 2005) as well as a better feed efficiency in the same models (Li and Watkins, 1998; Dugan *et al*., 2004). In most studies a decrease body fat is reported in different mammal species (Park *et al*., 1997, 1999; Cook *et al*., 1998; West *et al*., 1998, 2000; DeLany *et al*., 1999; Azain *et al.*, 2000; DeLany and West, 2000; Tsuboyama-Kasaoka *et al.*, 2000; Whigham *et al.*, 2000; Ryder *et al.*, 2001; Akahoshi *et al.*, 2002; Takahashi *et al.*, 2002; Azain, 2003; Yamasaki *et al.*, 2003; Wang and Jones, 2004) as well as an increase in lean body mass was obtained coupled with this fat reduction in other studies (Mirand *et al*., 2004; Park *et al*., 2005; Bhattacharya *et al*., 2006).

Table 1.1. Effects of CLA on human studies.

Function	Effects	References
Body composition	↓ Body fat	Blankson *et al*., 2000 Smedman and Vessby, 2001 Thom *et al*., 2001 Kreider *et al*., 2002 Gaullier *et al*., 2004, 2005
	↑ Resting metabolic rate and lean body mass	Kamphuis *et al*., 2003 Gaullier *et al*., 2004
	↓ Sagittal abdominal diameter	Riserus *et al*., 2001
	No change in body fat	Kreider *et al*., 2002 Riserus *et al*., 2004 Bhattacharya *et al*., 2006
	↓ Adipocyte size	Evans *et al*., 2002
Immune system	Enhances antibody formation	Sugano *et al*., 1998
	Activation of the nuclear factor kB	Chung *et al*., 2005 De Roos *et al*., 2005 Poirier *et al*., 2005
	↑ Immune response (IgA and IgM)	Song *et al*., 2005
	No effect on cytokines and eicosanoids	Kelley *et al*., 2001 Albers *et al*., 2003 Nugent *et al*., 2005
Anticarcinogenic	↓ Breast cancer cells	Durgam and Fernandes, 1997
	No effect on breast cancer	Rissanen *et al*., 2003 McCann *et al*., 2004
Diabetes and insulin resistance	↑ Serum insulin levels	Medina *et al*., 2000
	Produce insulin resistance	Chung *et al*., 2005
Bone health	↑ Bone mass	Brownbill *et al*., 2005 Gaullier *et al*., 2005
	No effect	Gaullier *et al*., 2004 Doyle *et al*., 2005

Different results on the effect of CLA in the immune system had been obtained. Akahoshi *et al*. in 2002 and 2004 found an inhibitory effect of dietary CLA on cytokines in mice, and Sugano *et al*. (1998) and Yamasaki *et al*. (2000) found that CLA enhanced the formation of IgA, IgM and IgG but not IgE. Also an enhancement of cellular immunity has been observed in pigs and mice (Bassaganya-Riera *et al*., 2001; Sugano *et al*., 2001) as well as a reduction of eicosanoids and histamine production in rodents in *in vitro* studies (Sugano *et al*., 1998; Whigham *et al*., 2001; Wahle *et al*., 2004). Besides, a reduction of the catabolic effects of immune stimulation after endotoxin injection was observed in rodents and chickens, as well as an increase of phagocytic activity (Cook *et al*., 1993; Miller *et al*., 1994; Sisk *et al*., 2001). The anticarcinogenic effect of CLA in rodents has also been demonstrated. An inhibition of chemically induced carcinogenesis and cancer cell proliferation were reported (Ha *et al*., 1987, 1999; Visonneau *et al*.,

1997; Hubbard *et al*., 2000; Ip *et al*., 2002), as well as the reduction or inhibition of angiogenesis (Masso-Welch *et al*., 2002; Toomey *et al*., 2003). Diabetes and insulin resistance are also affected by CLA, but whereas some studies reported a decrease in insulin levels in rats (Houseknecht *et al*., 1998; Ryder *et al*., 2001; Belury *et al*., 2002; Evans *et al*., 2002; Kritchevsky *et al*., 2004), in mouse there was an insulin resistance (Tsuboyama *et al*., 2000; Poirier *et al*., 2005) and an improve of glucose tolerance (Houseknecht *et al*., 1998; Henriksen *et al*., 2003). There has been few studies regarding the antiatherogenic properties of CLA, but it has been demonstrated the reduction in atherosclerosis in rabbits and hamsters (Lee *et al*., 1994; Nicolosi *et al*., 1997; Kritchevsky *et al*., 2004). In all these studies, the CLA ingestion reduced total and low density lipoprotein (LDL) and TAG in blood, as well as a reduction in the atherosclerosis effect on the aortas.

1.7.4. CLA in fish

Based on the effects of dietary CLA supplementation on experimental animals, it is hoped that similar effects could occur in fish when fed diets enriched with CLA. To the beginning of this thesis, some studies had been conducted in fish species. Some of them showed no significant effects of CLA supplementation on growth performance or feed efficiency such as in rainbow trout (*Oncorhynchus mykiss*) (Figueiredo-Silva *et al*., 2005; Bandarra *et al*., 2006), Atlantic salmon (*Salmo salar*) (Berge *et al*., 2004; Kennedy *et al*., 2005, 2006; Leaver *et al*., 2006), channel catfish (*Ictalurus punctatus*) (Twibell and Wilson, 2003), yellow perch (*Perca flavescens*) (Twibell *et al*., 2001), carp (*Cyprinus carpio*) (Schwarz *et al*., 2002), tilapia (*Orechromis niloticus*) (Yasmin *et al*., 2004). Whereas, other studies showed a reduction on growth in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) (Twibell *et al*., 2000) and channel catfish (Manning *et al*., 2006). An improvement in feed efficiency was reported in hybrid striped bass (Twibell *et al*., 2000). These results suggest that the effects of CLA on fish depend on fish species and experimental conditions. Furthermore, apparent digestibility coefficients (ADC) or nutrient retention were not affected by dietary CLA in rainbow trout (Figueiredo-Silva *et al*., 2005) (see Table 1.3 for detailed information).

Table 1.2. Effects of CLA on experimental animal studies.

Function	Effects	References
Growth	↑ Growth in rodents and pigs	Chin *et al*., 1994 Bee, 2000 Dugan *et al*., 2004 Valeille *et al*., 2004 Storkson *et al*., 2005
	↑ Feed efficiency in rodents and pigs	Li and Watkins, 1998 Dugan *et al*., 2004
Body composition	↓ Body fat deposition in rodents, chicks, pigs and dogs	Park *et al*., 1997, 1999 Cook *et al*., 1998 West *et al*., 1998, 2000 DeLany and West, 2000 DeLany *et al*., 1999 Azain *et al*., 2000 Tsuboyama-Kasaoka *et al*., 2000 Whigham *et al*., 2000 Ryder *et al*., 2001 Akahoshi *et al*., 2002 Takahashi *et al*., 2002 Azain, 2003 Yamasaki *et al*., 2003 Wang and Jones, 2004
	↓ Fat mass and ↑ lean mass	Mirand *et al*., 2004 Park *et al*., 2005 Bhattacharya *et al*., 2006
Immune system	Inhibits pro-inflammatory cytokine (TNF-α) in mice	Akahoshi *et al*., 2002, 2004
	↑ Antibody formation in mice and pigs	Sugano *et al*., 1998 Bassaganya-Riera *et al*., 2001
	↓ Catabolic effects of immune stimulation in rodents and chickens and increased phagocytosis	Cook *et al*., 1993 Miller *et al*., 1994 Sisk *et al*., 2001
	↓ Eicosanoid and histamine production in rodents and cell cultures	Sugano *et al*., 1998 Whigham *et al*., 2001 Wahle *et al*., 2004
	↑ IgA, IgB and IgM in rats	Sugano *et al*., 1998 Yamasaki *et al*., 2000
Anticarcinogenic	Inhibits chemically induced carcinogenesis in rodents	Ha *et al*., 1987, 1990 Ip *et al*., 2002
	Inhibits cancer cell proliferation and increases apoptosis in rodents	Visonneau *et al*., 1997 Hubbard *et al*., 2000
	↓ Angiogenesis in implanted rat breast tumors	Masso-Welch *et al*., 2002
	Inhibits angiogenesis in mice	Toomey *et al*., 2003
Diabetes and insulin resistance	↓ Diabetes in rats	Houseknecht *et al*., 1998 Kritchevsky *et al*., 2004
	Improves glucose tolerance in rats	Houseknecht *et al*., 1998 Henriksen *et al*., 2003
	Induces insulin resistance in mouse	Tsuboyama *et al*., 2000 Poirier *et al*., 2005
	↓ Insulin levels	Ryder *et al*., 2001 Belury *et al*., 2002 Evans *et al*., 2002
Antiatherogenic	↓ Atherosclerosis in rabbits, hamsters and mice	Lee *et al*., 1994 Nicolosi *et al*., 1997 Kritchevsky *et al*., 2004

Reductions in body fat and increased lean body mass have been obtained in terrestrial animal models when CLA was included in the diet. But in fish studies, contradictory effects have been observed. For example, no effect have been reported regarding proximate composition in rainbow trout (Figueiredo-Silva *et al*., 2005; Bandarra *et al*., 2006), Atlantic salmon (Berge *et al*., 2004), channel catfish (Twibell and Wilson, 2003) and tilapia (Yasmin *et al*., 2004), whereas an increase in liver lipid content was reported in Atlantic salmon (Kennedy *et al*., 2005), and a decrease of liver and whole fish lipid content was observed in the same species (Leaver *et al*., 2006). In yellow perch, liver lipid was reduced when CLA was included in the diet (Twibell *et al*., 2001).

The fatty acid composition of fish tissues depends on the tissue analyzed, but in general the SFA fraction was increased, mainly due to palmitic and stearic acids, and the MUFA fraction reduced, mainly due to palmitoleic and oleic acids in both muscle and liver (Twibell *et al*., 2000; Berge *et al*., 2004; Bandarra *et al*., 2006).

The effects on HUFA are contradictory and vary among species. There was a decrease of EPA and DHA in muscle of hybrid striped bass (Twibell *et al*., 2000), Atlantic salmon (Kennedy *et al*., 2005) and rainbow trout (Bandarra *et al*., 2006). In yellow perch, these n-3 HUFA were not affected by dietary CLA in fish muscle, whereas they were reduced in liver (Twibell *et al*., 2001). In Atlantic salmon no effect on these fatty acids was noticed (Berge *et al*., 2004; Kennedy *et al*., 2005; Leaver *et al*., 2006). Besides these effects of CLA on fish tissues fatty acid profiles, CLA was accumulated in all fish tissues analyzed (muscle, liver and perivisceral fat) and was positive correlated with dietary CLA content as reported in all fish published studies until the start of this thesis (Twibell *et al*., 2000, 2001; Twibell and Wilson, 2003; Berge *et al*., 2004; Kennedy *et al*., 2005; Bandarra *et al*., 2006; Leaver *et al*., 2006; Manning *et al*., 2006), being CLA incorporation in fish tissues greater than in other vertebrates (Dugan *et al*., 2001; Azain, 2003) (see Table 1.4 for detailed information).

Table 1.3. Effects of CLA supplementation in fish diets on growth parameters

Species	CLA dosage	Results	References
Atlantic salmon (*Salmo salar*)	0, 0.5, 1 and 2% in juveniles	No effect on growth parameters	Berge *et al*., 2004
	0, 1 and 2% in smolts	No effect on growth parameters	Kennedy *et al*., 2005, 2006
	0, 2 and 4% in post-smolts	No effect on feed conversion efficiency or growth	Leaver *et al*., 2006
Channel catfish (*Ictalurus punctatus*)	0, 0.5 and 1% in juveniles	No effect on weight gain, feed efficiency or feed intake	Twibell and Wilson, 2003
	0, 0.5 and 1% in juveniles	No effect on feed consumption and FCR ↓ Body weight gain with 0.5% CLA and 2.25% corn oil	Manning *et al*., 2006
Hybrid strip bass (*Morone saxatilis* x *M. chrysops*)	0, 1% CLA in juveniles	↓ Feed intake and weight gain ↑ Feed efficiency and HSI	Twibell *et al*., 2000
Rainbow trout (*Oncorhynchus mykiss*)	0, 0.5, 0.75, 1 and 2% in juveniles	No effect on growth parameters, FCR and nutrient or energy utilization	Figueiredo-Silva *et al*., 2005
	0, 0.5, 0.75, 1 and 2% in juveniles	No effect on growth parameters, FCR, HSI and VSI	Bandarra *et al*., 2006
Rock fish	0, 1 and 10%	↓ Growth with 10% CLA	Choi *et al*., 1999
Tilapia (*Oreochromis niloticus*)	0, 1 and 10%	↑ Growth with 1% ↓ Growth with 10%	Choi *et al*., 1999
	0 and 5% in juveniles	No effect on growth parameters	Yasmin *et al*., 2004
Yellow Perch (*Perca flavescens*)	0, 0.5 and 1% in juveniles	No effect on growth parameters	Twibell *et al*., 2001

Only three studies are published to date concerning the effect of dietary CLA on fish metabolism were performed (focused on lipogenic and lipolytic enzymes) and obtained inconclusive results. In rainbow trout, no effect was observed in liver G6PD and ME and FAS (Figueiredo-Silva *et al*., 2005) as well as the results found in Atlantic salmon for ME and FAS (Leaver *et al*., 2006). Liver and white muscle CPT-I were also not affected, whereas in red muscle, the activity was reduced by dietary CLA in Atlantic salmon (Leaver *et al*., 2006). On the contrary, CPT-I activity was increased in white and red muscle in the same species (Kennedy *et al*., 2006) (see Table 1.5 for detailed information).

Table 1.4. Effects of CLA supplementation in fish diets on biochemical properties

Species	CLA dosage	Results	References
Atlantic salmon (*Salmo salar*)	0, 0.5, 1 and 2% in juveniles	No effect on proximate composition ↑ Total n–3 fatty acid deposition ratio	Berge *et al*., 2004
	0, 1 and 2% in smolts	Clear trend of increased total lipid and TAG in both liver and fillet ↑ CLA levels in flesh and liver. ↓ Liver SFA and MUFA ↓ Muscle HUFA	Kennedy *et al*., 2005
	0, 1 and 2% in smolts	↑ Liver HUFA	Kennedy *et al*., 2006
	0, 2 and 4% in post-smolts	↓ Lipid content ↑ Protein content	Leaver *et al*., 2006
Channel catfish (*Ictalurus punctatus*)	0, 0.5 and 1% in juveniles	No effect on proximate composition	Twibell and Wilson, 2003
	0, 0.5 and 1% in juveniles	↓ Muscle n-3HUFA	Manning *et al*., 2006
Hybrid strip bass (*Morone saxatilis* x *M. chrysops*)	0, 1% CLA in juveniles	↓ Liver total lipid ↓ PFI ↓ Muscle EPA and DHA ↑ Liver EPA and DHA	Twibell *et al*., 2000
Rainbow trout (*Oncorhynchus mykiss*)	0, 0.5, 0.75, 1 and 2% in juveniles	No effect on proximate composition	Figueiredo-Silva *et al*., 2005
	0, 0.5, 0.75, 1 and 2% in juveniles	No effect on proximate composition ↑ Muscle and viscera SFA and PUFA ↓ Muscle and viscera MUFA	Bandarra *et al*., 2006
Tilapia (*Oreochromis niloticus*)	0 and 5% in juveniles	No effect on proximate composition	Yasmin *et al*., 2004
Yellow Perch (*Perca flavescens*)	0, 0.5 and 1% in juveniles	No effect on muscle proximate composition ↓ Liver lipid content	Twibell *et al*., 2001

Table 1.5. Effect of CLA supplementation in fish diets on lipogenic and lipolytic enzymes.

Species	CLA dosage	Results	References
Rainbow trout (*Oncorhynchus mykiss*)	0, 0.5, 0.75, 1 and 2% in juveniles	No effect in liver G6PD, ME and FAS activities	Figueiredo-Silva *et al*., 2005
Atlantic salmon (*Salmo salar*)	0, 1 and 2% in smolts	↑ Muscle (red and white) CPT-I	Kennedy *et al*., 2006
	0, 2 and 4% in post-smolts	No effect in liver ME and CPT-I No effect in muscle (red and white) CPT-I	Leaver *et al*., 2006

Only one study has been conducted in sensory analyses in fish fed CLA diets where no differences were denoted in any sensory attributes of rainbow trout when fed CLA up to 3% (Schabbel *et al*., 2004). So, more studies are needed to determine if CLA affects the sensory attributes of fish.

In summary, it has been shown that the effects of CLA are species related, dose dependent and closely related to the fish size and culture conditions. Besides, the chemical form of CLA supplementation (as free fatty acid, methyl ester or other) could influence the effects of CLA on the physiology of fish.

1.8. Objectives

Based on the previous literature review, considering that excess lipid deposition is one of the concerns of European sea bass producers and in view of the potential beneficial effects of CLA found in other vertebrates to reduce fat deposition and promote the nutritional value of food produced, the aim of this thesis was to study the effects of conjugated linoleic acid (CLA) supplementation on European sea bass culture.

For that purpose several specific objectives were proposed:

1. To determine the effects of graded levels of dietary CLA supplementation on European sea bass culture performance, particularly growth.
2. To study the effects of graded CLA supplementation on fat deposition and lipid and fatty acid composition of body tissues.
3. To find out the effects of dietary CLA supplementation on European sea bass muscle, liver and perivisceral fat morphology.
4. To better understand the effects of dietary CLA on and European sea bass metabolism, and the potential mechanisms involved, such as nutrient digestibility and liver lipogenic and lipolytic enzyme activities.
5. To investigate the effects of dietary CLA supplementation on European sea bass health in terms of immune system parameters.
6. To determine the effects of dietary CLA supplementation on European sea bass on CLA accumulation in fish tissues and its effects on fillet quality in terms of sensory and texture parameters.

Objectives 1 and 2 were addressed in Experiment I (Chapter 3), II (Chapter 4), III (Chapter 5) and IV (Chapter 6). Objective 3, 4 and 5 in Experiments I, II and III (Chapters 3-5) and objective 6 in Experiment IV (Chapter 6).

Chapter 2

Materials and Methods

2.1. Diets

In each experiment, several isonitrogenous and isolipidic diets based in a commercial formulation (Mistral 21 or Ecolife 64) were designed, to contain graded levels of CLA (CLA0=Control, CLA05=0.5%, CLA1=1%, CLA2=2% and CLA4=4%). CLA was supplied by BASF (Tarragona, Spain) as a supplement (50:50 mixture of each isomer; *cis*-9, *trans*-11 and *trans*-10, *cis*-12; LUTA-CLA60®) and added to the extruded diets in substitution of fish oil, to maintain a constant energy content among dietary treatments. Diets were formulated and manufactured by a commercial feed producer (Biomar Iberia, S.A., Spain). Diets formulation, proximate composition and fatty acid profile are shown in the chapters corresponding to each experiment.

2.2. Experimental conditions

All European sea bass used along the study were provided by a local fish farm (Alevines y Doradas, S.A., ADSA, San Bartolomé de Tirajana, Canary Islands, Spain). Fish were maintained in indoor stocking 1000 l fiberglass tanks until being well adapted to the environmental conditions (3 weeks). Afterwards, fish were distributed into 500 l, 1000 l experimental fiberglass or digestibility tanks (120 l), depending on the experiment (Figs. 2.1a, 2.1b and 2.1c, respectively), and fed the corresponding experimental diet.

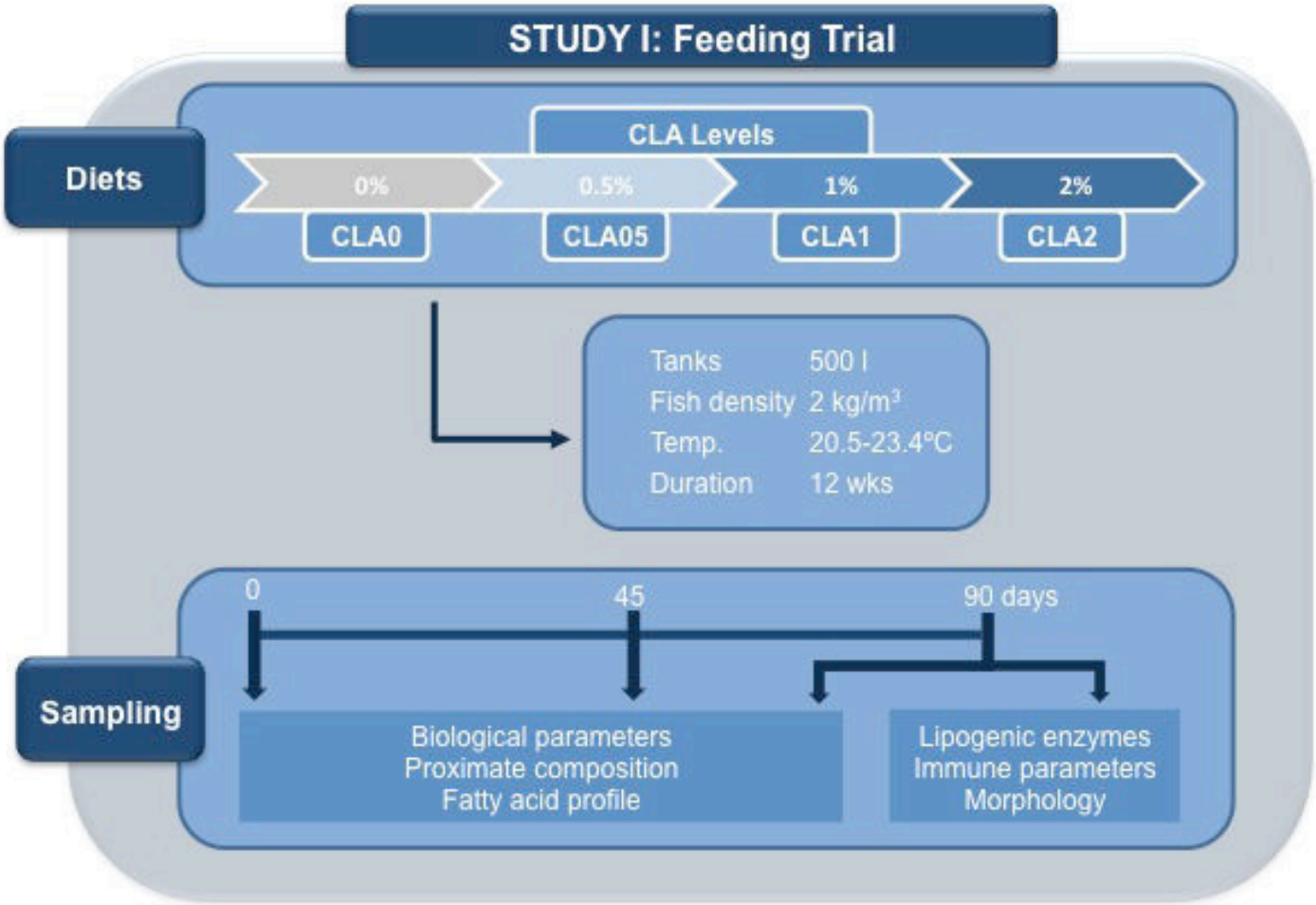
Standardized culture conditions were followed in all experiments. Tanks were supplied with filtered sea water (37 ppm salinity) in a open water system under natural photoperiod (approximately 12L:12D). Temperature and water dissolved

oxygen were daily measured using an Oxy Guard-Handy beta instrument (Zeigler Bros, Gardners, USA).

Figure 2.1. Tanks used along the study. (a) 1000 l; (b) 500 l; (c) 120 l (Digestibility tanks) provided with a faeces collection system described by Cho *et al.* (1985) and adapted to seawater fish by Robaina *et al.* (1995).

Detailed experimental designs and samplings protocols followed are described in the chapters corresponding to each study. A summarized schematic representation for each individual study has been included in this Materials and Methods section (Figs. 2.2, 2.3, 2.4 and 2.5 corresponding to studies I, II, III and IV, respectively).

Fig. 2.2 Schematic representation of experimental design and sampling followed in Study I.



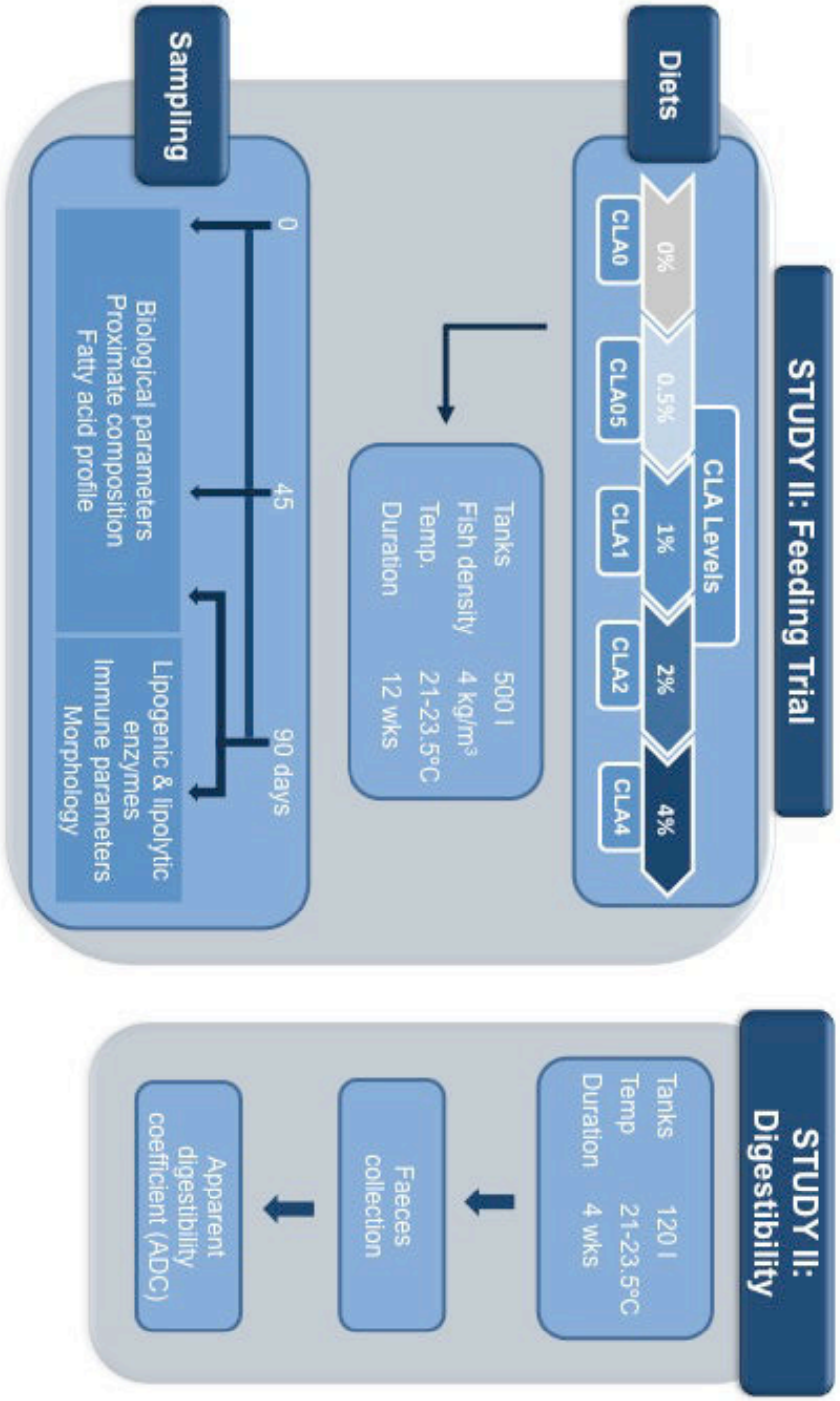
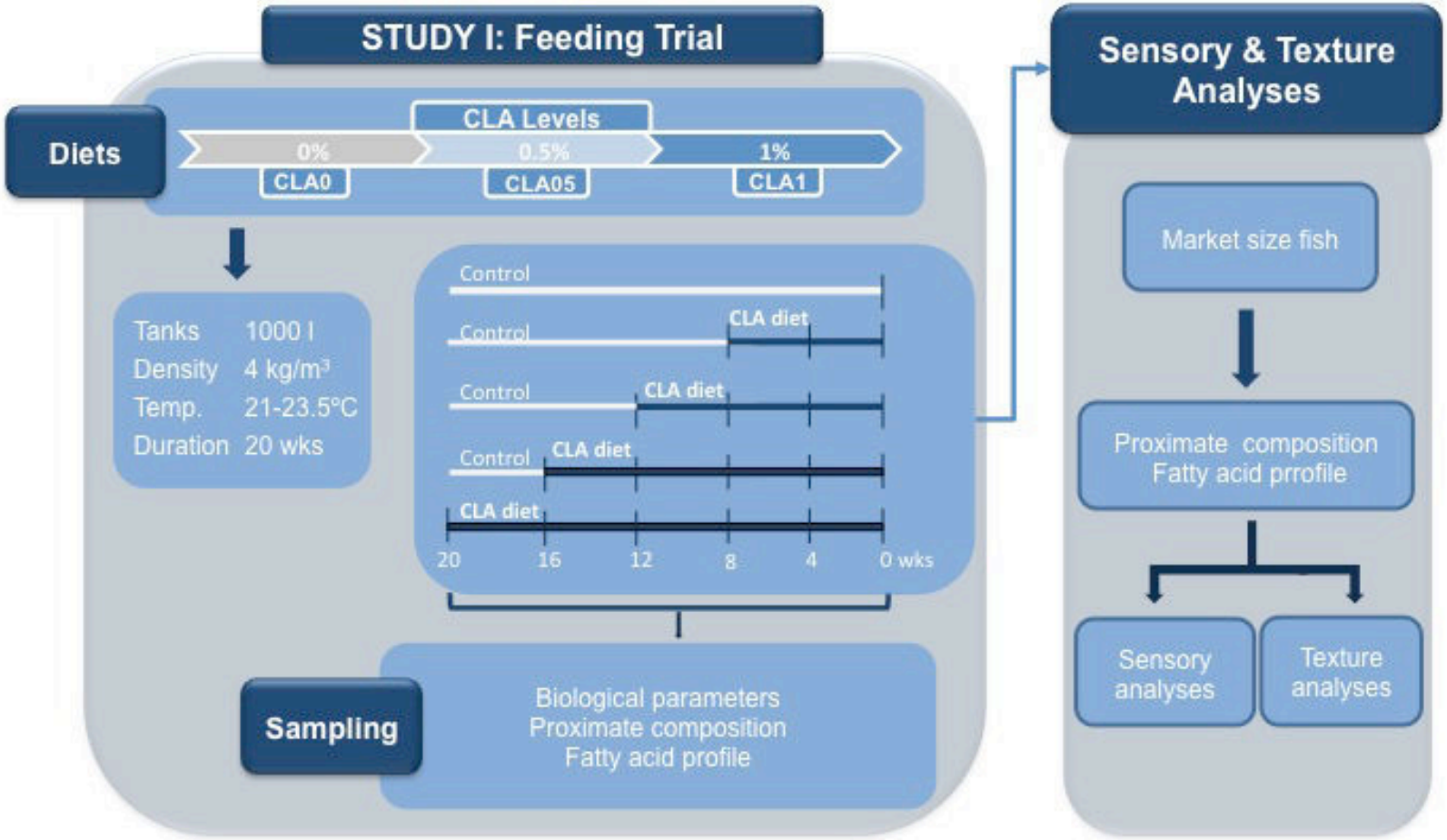


Fig. 2.3. Schematic representation of experimental design and sampling followed in Study II.

Fig. 2.4. Schematic representation of experimental design and sampling followed in Study III



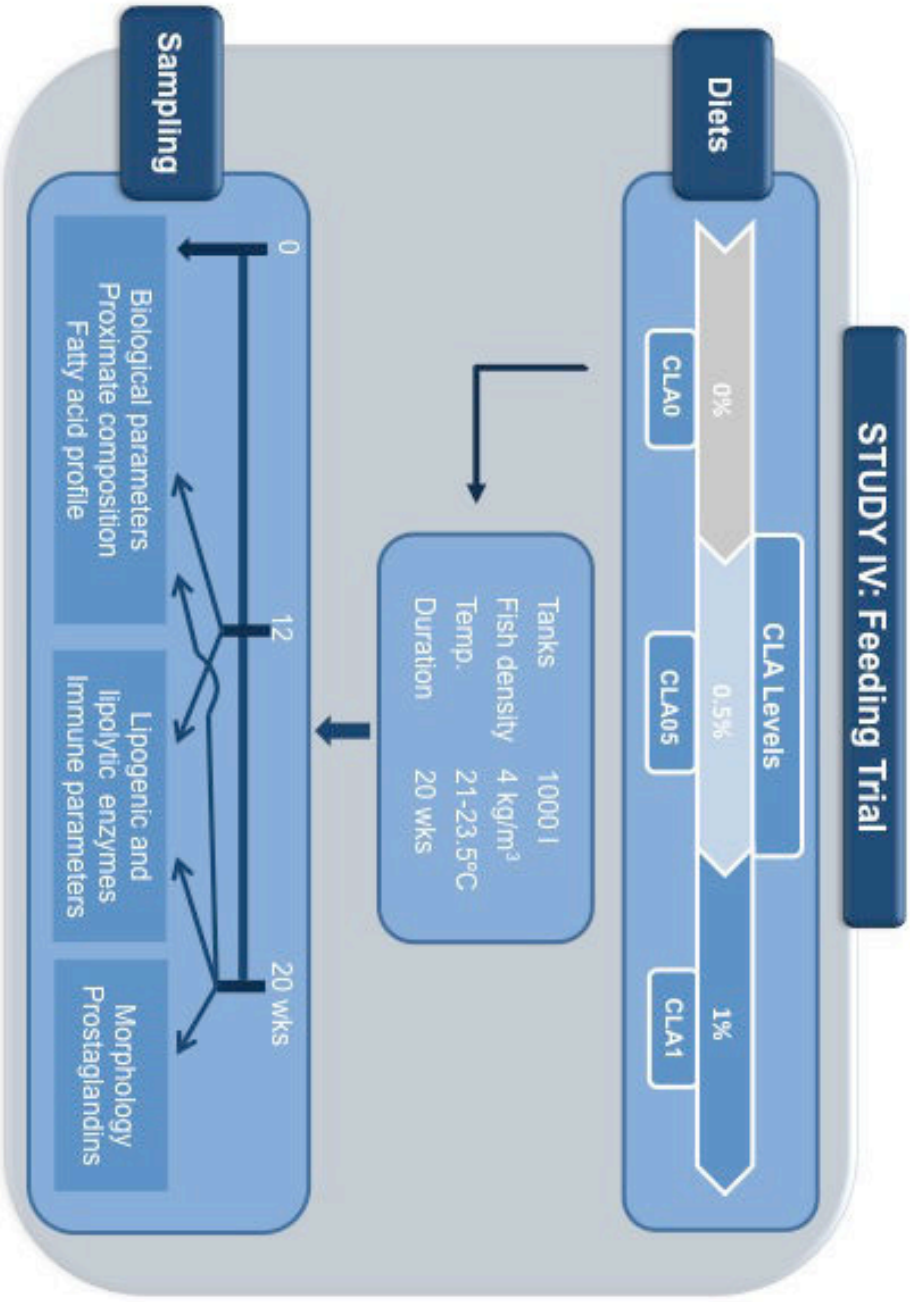


Fig. 2.5 Schematic representation of experimental design and sampling followed in Study IV.

2.3. Biological parameters

2.3.1. Relative growth

In order to observe the potential differences in efficacy among the different levels of CLA inclusion tested, relative growth was evaluated. Relative growth was defined as the relation between the biomass increase (g) and the initial weight (g).

$$\text{Relative growth} = [(\text{Final weight} - \text{Initial weight}) / \text{Initial weight}] \times 100$$

2.3.2. Specific growth rate (SGR)

This parameter shows the daily increase in weight gain.

$$\text{SGR} = [\ln \text{Final weight} - \ln \text{Initial weight}) / \text{days}] \times 100$$

2.3.3. Feed conversion ratio (FCR)

This parameter was evaluated to determine the efficiency of diets to promote growth in terms of ingested food, and was defined as the relation between the ingested food (g) and the generated biomass (g).

$$\text{FCR} = \text{Ingested food} / \text{Generated biomass}$$

2.4. Biochemical analyses

2.4.1. Total crude lipid content

Crude lipids from weighted samples were extracted by a mixture of chloroform:methanol (2:1) as described by Folch *et al*. (1957). Extracted lipids were diluted in chloroform and stored at -80°C under nitrogen atmosphere in order to avoid oxidation.

2.4.2. Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters were obtained by base-catalised methylation in dry toluene and 0.5M sodium methoxide in anhydrous methanol as described by Christie (1982). Fatty acid methyl esters were extracted with hexane and purified by adsorption chromatography on NH2 Sep-Pack cartridges (Waters S.A., Massachussets, USA). Fatty acid methyl esters were separated in a Gas-chromatograph (GC-14A, Shimadzu, Japan) equipped with a Supercolovax-10-fused silica capillary column (Length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA), according to conditions described by Izquierdo *et al.* (1992). Fatty acid methyl esters were quantified by flame ionization detector (FID) and identified in comparison to external standards of CLA isomers (Sigma-Aldrich and Matreya, LLC.) and well-characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan). All analyses were conducted in triplicate.

2.4.3. Total crude protein

Crude protein analyses were carried out according to the Kjeldahl method (AOAC, 2000). Samples were digested with 37% sulphuric acid in presence of a cupper catalyst. After digestion, samples were distilled in boric acid and total nitrogen was quantified by titration with 0.1N hydrochloric acid, converting to total protein value by multiplying by the empirical factor 6.25.

2.4.4. Moisture content

Moisture was determined according to Official Methods of Analysis (AOAC, 2000). Dry matter content was obtained by thermal drying to constant weight in an oven at 110°C, with a first 24 h period followed by 1h periods until reaching constant weight. Sample weight was recorded before drying and after each drying period, after a cooling period in a desiccator.

2.4.5. Ash content

Ash content was determined according to official methods of analysis (AOAC, 2000). Ash was obtained by combustion in a muffle furnace at 600 °C for 12h

(AOAC, 2000). Sample weight was recorded before and after the combustion period, after a cooling period in a desiccator.

2.5. Digestibility analysis

Faeces were collected from digestibility tanks following the faeces collection system described by Cho *et al*. (1985) modified by Robaina *et al.* (1995). Immediately after their daily collection, faecal matter was centrifuged, frozen and stored at -20 ºC. Pooled faeces from each group were freeze-dried prior to analysis. Apparent digestibility coefficients (ADC) were calculated using the formula reported by Maynard and Loosli (1969). Proximate composition of diets and faeces were used to calculate ADC for protein and lipid. Acid insoluble ash, which served as a marker of feed digestibility, was determined by the method of Atkinson *et al.* (1984). Each diet was assayed by triplicate.

$$ADC\ (\%) = 100 - [(100 \times (W / W_1) \times (W_2 / W_3))]$$

where,

W is the % of indicator in feed

W_1 is the % of indicator in faeces

W_2 is the % of nutrient in faeces

W_3 is the % of nutrient in feed

2.6. Enzymatic analyses

2.6.1. Lipogenic enzymes (G6PD and ME)

Muscle and liver samples were homogenized in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4) and homogenates were centrifuged at 20000 g for 40 min at 4°C. Enzyme activities of glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49) and malic enzyme (ME; EC 1.1.1.40) were assayed using spectrophotometric procedures on the supernatant following the method described by Dias *et al.* (1998). Soluble protein content of muscle and liver homogenates was determined by the method of Bradford (1976) using Bovine Serum

Albumin (BSA) as a standard. Care was taken to ensure that initial rates were measured in all assays and that enzymes were stable in the buffer solution used during the time and temperature required to perform the assays (Álvarez *et al.*, 1998). Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay temperature per minute, were expressed per mg of soluble protein (specific activity). All assays were performed in triplicate.

2.6.2. Lipolytic enzymes (ACO and L3HOAD)

Acyl-CoA oxidase (ACO; EC 1.3.3.6) was assayed in liver peroxisome enriched fractions prepared by homogenizing in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M Sucrose, 2 mM EDTA, pH 7.4). The homogenate was centrifuged at 7200 g for 10 min at 4°C and the supernatant fraction collected. The resulting pellet was washed once with 500 µl of the same buffer, centrifuged as above and the supernatant collected and combined with the first one. Combined supernatants were centrifuged at 18000 g for 30 min at 4°C and the resulting pellet re-suspended with 600 µl of the buffer prior to a sonication bath for 30 min. After sonication, supernatants were centrifuged at 18000 g for 45 min at 4°C and the supernatants collected for analyses. ACO was assayed by the spectrophotometric determination of H_2O_2 production, coupled to the oxidation of 2',7'-dichlorofluorescein diacetate (LDCF) at 502 nm. The reaction mixture contained 2.6 mM LDCF, 1 M aminotriazole, 5 mg/ml horseradish peroxidase type II, 5% triton X100, 1 M Tris-HCl pH 8.5, 15 mM Flavin adenine dinucleotide, 50 mg/ml bovine serum albumin (BSA). The reaction was started by the addition of 1mM Palmitoyl-CoA.

L-3-hydroxyacyl-CoA dehydrogenase (L3HOAD; EC 1.1.135) activity was assayed in mitochondrial preparations of liver samples performed as described by Menoyo *et al*. (2004) and centrifuged following the method of Harper and Saggerson (1975). L3HOAD activity was measured according to Bradshaw and Noyes (1975) on mitochondrial isolates disrupted by sonication in a 1% Triton X-100 solution. Soluble protein content of liver homogenates was determined on the supernatant by the method of Bradford (1976) using BSA as standard. Care was taken to ensure that initial rates were measured in all assays and that enzymes were stable in the buffer

solution used during the time and temperature required to perform the assays (Álvarez *et al*., 1998). Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay temperature per minute, were expressed per mg of hepatic and muscular soluble protein (specific activity). All enzyme assays were performed by triplicate.

2.7. Histological studies

Muscle, liver and perivisceral fat samples were fixed in 10% neutral-buffered formalin, embedded in paraffin and 5 µm-thick sections were stained with hematoxilin and eosin (H&E) for optical examination (Martoja and Martoja-Pierson, 1970). Micrographs were taken from the paraffin sections at a 400X final magnification using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. Hepatocyte and adipocyte areas as well as maximum and minimum longitude of cells were measured with an analySIS® (Image Pro Plus®) software package.

2.8. Immunological analyses

2.8.1. Blood collection and sample preparation

Blood was obtained by caudal sinus puncture with a 1 ml plastic syringe. No anaesthetic was used in order to avoid any possible effect in blood parameters and handle time was less than 1 min in order to minimize stress effects. The first aliquot of blood was transferred to a lithium heparin coated microcentrifuge tube and used for respiratory burst of blood circulating neutrophils (NBT) index. The second aliquot was transferred to a microcentrifuge tube and allowed to clot for 2 h for lysozyme and alternative complement pathway analyses. Serum was separated by centrifugation and stored at −80 °C until analysis.

2.8.2. Respiratory burst of circulating neutrophils (NBT)

Respiratory burst of blood circulating neutrophils was assayed as described by Siwicki *et al.* (1993), measuring the production of superoxide anions generated by

blood circulating neutrophils through the reduction of nitroblue tretrazolium (NBT) to formazan in the presence of oxygen radicals.

2.8.3. Lysozyme

Lysozyme level in blood serum was determined by turbidimetric assay according to Anderson and Siwiki (1994) using hens egg white lysozyme in PBS as a standard.

2.8.4. Alternative complement pathway

Alternative complement pathway was carried out as described by Sunyer and Tort (1995) for gilthead sea bream using rabbit red blood cells (RBC). The reciprocal of the serum dilution causing 50% lysis of RBC is designed as ACH50 and the results are presented as ACH50 units/ml.

2.8.5. Bactericidal activity

The bactericidal activity was determined against *Vibrio anguillarum* as described by Torrecillas *et al*. (2011a). Where a *V. anguillarum* culture in PBS was adjusted to 10^8 cfu/ml and the decrease in absorbance measured.

2.8.6. Phagocytic activity of head kidney leukocytes

Head kidneys of fish were removed and leukocytes were isolated by density gradient centrifugation technique. After collection, head kidneys were homogenized in supplemented Minimal Essential Medium (MEM) and filtered through nylon membrane. Resulting cell solution was layered onto preformed Percoll gradient of 51%-34% in 10% Hank´s Balanced salt solution (HBSS) and centrifuged at 800 g for 30 min. The interface layer was harvested and diluted in 1 ml of supplemented MEM and centrifuged again at 800 g for 5 min in order to remove residual Percoll. The resulting pellet was diluted in 1 ml of supplemented MEM and viability and concentration of leukocytes suspension was determined. The solution was re-suspended in supplemented MEM adjusting to the desired final concentration. Leukocyte solution was incubated against polystyrene microparticles (1 µm, Fluka-Sigma Chemicals, CA,

USA) as described by Esteban and Meseguer (1997) for *V. anguilllarum*. Ten series of one hundred leucocytes per fish were counted and the phagocytic index was determined as the percentage of leucocytes with phagocytic ability. Positive phagocytic activity was determined only for the leucocytes with presence of the microparticle of polyestyrene inside the cytoplasm.

2.8.7. Prostaglandins analysis

2.8.7.1. Tissue stimulation

Head kidneys were dissected out, weighed and digested in 4 ml of HBSS without Ca^{2+} plus 2% Collagenase during 30 min in continuous orbital agitation (100 U/min). After digestion, samples were filtered through nylon gauze and washed with 4 ml of HBBS without Ca^{2+} and isolated cells recollected in a glass tube. Cells were concentrated in a pellet by centrifugation at 2500 g for 5 min and the resultant pellet re-suspended in 1 ml of HBBS without Ca^{2+} in an Eppendorf tube. Cells were stimulated during 30 min with 50 µM Ca^{+} PMA (Phorbol 12-myristate 13-acetate, Sigma Chemicals, CA, USA) and 10 µM A321 (Sigma Chemicals, CA, USA). Stimulation reaction was stopped by adding 50 µl of 2 M formic acid. Samples were stored at -80°C until purification.

2.8.7.2. Purification of eicosanoids

The frozen stimulated fraction (pooled 3 fish tissues/tank) was centrifuged at 1000 g for 5 min in order to precipitate any remaining debris. The supernatants were extracted using an octadecylsilyl (C18) “Sep-Pak” mini-columns (Millipore, Watford, UK) by the method of Powell (1982) and as described in detail by Bell *et al.* (1994). One milliliter of supernatants was applied to the column, which had been prewashed with 5 ml of methanol and 10 ml of MiliQ water. The column was successively washed with 10 ml of MiliQ water, 5 ml of 15% ethanol (v/v) and 5 ml of hexane/chloroform (65:35, v/v) before elution of prostanoids with 10 ml of ethyl acetate. The extracts were dried under nitrogen and re-suspended in 100 µl of methanol and stored at -80°C in small glass vials until analysis.

2.8.7.3. Prostaglandins immunoassay

Measurement of prostaglandins was performed using an enzyme immunoassay (EIA) kit for PGE_2 (Cayman Chemical Co., MI, USA), that is based in the competition between PGE_2 and PGE_2-actylcholinesterase (AChE) conjugated PGE_2 (tracer) for a limited amount of PGE_2 monoclonal antibody.

2.9. Sensory analyses

Fish were starved for 24 h and slaughtered in a tank with ice and seawater, then gutted and kept at 4°C for 24 h until tests were performed. Organoleptic tests were conducted on fish fillets cooked in aluminum boxes for 10 min in a steam oven (120°C). Immediately after cooking, fillets were offered to a panel of eleven selected and trained judges (ISO 1985, ISO 1993). Tests were conducted in isolated and air conditioned rooms with standardized light (ISO 1988). Judges were randomly offered closed food boxes labeled with codes containing fillets (3x4 cm). Odour (intensity, marine and oily), appearance (colour, shininess and integrity), texture (firmness, juiciness, chewiness, adhesiveness and fatness) and flavour (intensity, marine, oily and aftertaste) were tested for samples of fish fed the experimental diets and classified by the judge in a continuous scale from 0 to 100 for each parameter.

2.10. Texture analyses

Raw fillets from the left side of the same fish used in sensory analyses were tested for texture parameters. Skin was removed and four square pieces (2.5x2.5cm) were collected from each fillet above the lateral line. The texture analyzer used was the TA-XT2 (Stable MicroSystems, England). A texture profile analysis (TPA) test was carried out using an aluminium compression plate with a 100 mm diameter. The compression rate was set to 0.8 mm/s and the strain to 80% penetration. Samples were compressed twice with a 60s interval between the two compressions (Tryggvadottir and Olafsdottir, 2000). Texture variables measured were fracture

ability, hardness, springiness, cohesiveness, gumminess, chewiness, adhesiveness and resilience and were calculated as described by Ginés *et al.* (2004).

2.11. Statistical analysis

Means and standard deviations (SD) were calculated for each measured parameter. Statistical analysis followed methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of CLA inclusion. When data did not pass a normality test, a log transformation was used to normalize data. When *F* values showed significance, individual means were compared using Tukey's or Duncan tests for multiple means comparison. Significant differences were considered for $P<0.05$. Analyses were performed using Statgraphics software (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA).

Chapter 3

STUDY I: Effect of conjugated linoleic acid (CLA) on growth performance and feed utilization in European sea bass juveniles (*Dicentrarchus labrax*)

This Chapter has been published as: Effect of conjugated linoleic acid on dietary lipids utilization, liver morphology and selected immune parameters in European sea bass juveniles (*Dicentrarchus labrax*) in Comp. Biochem. Physiol. B Mol. Biol., 154(2):179-87.

Abstract

Increased energy content in fish feeds has led to an enhanced fat deposition, particularly in European sea bass, concerning fish farmers. Inclusion of conjugated linoleic acid (CLA) could reduce fat deposition as in other vertebrates. To determine if dietary CLA affects fat deposition, lipid metabolism, lipid composition and morphology of different tissues, growth and selected immune parameters, European sea bass juveniles were fed 4 graded levels of CLA (0, 0.5, 1 and 2%). Growth and feed conversion were not affected by CLA, whereas feed intake was reduced ($P<0.05$) by feeding 2% CLA. In these fish perivisceral fat was also reduced ($P<0.05$), particularly reducing ($P<0.05$) monounsaturated fatty acids. CLA did not affected tissue proximal composition, but reduced ($P<0.05$) saturated and monounsaturated fatty acids and increased ($P<0.05$) the n-3 and n-3 highly unsaturated fatty acids in muscle and increase ($P<0.05$) CLA content in muscle, liver and perivisceral fat. A progressive reduction in lipid vacuolization of hepatocytes cytoplasm and regular-shaped morphology was found in fish fed increased CLA levels, together with a progressive increase in malic enzyme

Keywords:
Alternative complement pathway
Conjugated linoleic acid
Dicentrarchus labrax
Lipids
Lipogenic enzymes
Lysozyme
Morphology

activity (only significant in fish fed 1% CLA). Finally, inclusion of CLA up to 1% increased ($P<0.05$) plasma lysozyme activity and was positively correlated with alternative complement pathway.

3.1. Introduction

Increased energy contents in fish feeds and the inclusion of vegetable protein or lipid sources in substitution of fish meal and oil in fish diets (Caballero *et al*., 1999 and 2004) has led to an enhanced perivisceral, hepatic and subcutaneous fat deposition which may affect product quality and restrains optimization of fish production concerning fish farmers (Izquierdo *et al*., 2005). One of the marine fish species more susceptible to excess fat deposition is European sea bass, where this lipid accumulation may be a physiological response to the diet rather than a pathological situation (Izquierdo *et al*., 2003). Conjugated linoleic acid (CLA) includes a group of positional and geometric isomers of linoleic acid (18:2n-6) containing double bonds.

The two main isomers, *cis*-9, *trans*-11 (*c*9, *t*11) and *trans*-10, *cis*-12 (*t*10, *c*12), are considered the biologically-active CLA isomers. Meanwhile *trans*-10, *cis*-12 isomer is known to be responsible for body fat reduction, the *cis*-9, *trans*-11 isomer improves growth performance (Park and Pariza, 2007). But many physiological effects of CLA such as the immunemodulation seem to be the result of the interaction of both isomers (Pariza, 2004). In mammals, besides decreasing body fat, CLA has antioxidative, anticarcinogenic, immunomodulative and diabetes modulator properties (Belury, 2002; Risérus *et al*., 2003). A modification of tissue fatty acids profile has been also found in mammals, showing an increase in saturated and a decrease in monounsaturated fatty acids (Ramsay *et al*., 2001; Yang *et al*., 2003). There are scarce studies conducted with CLA in fish and they show that despite CLA reduces total lipid content in liver in hybrid striped bass (*Morone saxatilis* x *Morone chrysops*) and in yellow perch (*Perca flavescens*) (Twibell *et al*., 2000; Twibell *et al*., 2001), it does not in channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*) or Nile tilapia (*Oreochromis niloticus*) (Twibell and Wilson, 2003; Yasmin *et al*., 2004; Figueiredo-Silva *et al*., 2005; Bandarra *et al*., 2006). However, dietary CLA did not affect final weight, specific growth rate, feed conversion ratio and condition factor in most of the studied fish species (Kennedy *et al*., 2007b; Valente *et al*., 2007a; Ramos *et al*., 2008). On the contrary, in channel catfish

Peterson and Manning (2003) found a positive effect in growth and feed utilization after 3 weeks of CLA feeding, despite it disappear after 6 weeks. In some studies lipogenic enzymatic activity is not affected by dietary CLA (Figueiredo-Silva *et al*., 2005, Valente *et al*., 2007a) and in others lipid oxidation was markedly promoted (Du and Ahn, 2002; Takahashi *et al*., 2003). In fish, tissue fatty acid composition readily reflects that of the diet (Watanabe, 1982; Izquierdo, 1996; Izquierdo *et al*., 2003; Regost *et al*., 2003). Hence, among all production animal, fish show the highest accumulation of CLA in their tissues when fed with diets supplemented with this particular fatty acid (Twibell *et al*., 2000, 2001; Evans *et al*., 2002). The increase of CLA content in fish is particularly interesting for producing functional fish fillets enriched with CLA to enhance their quality for human consumption.

The aim of this study was to determine the effects of supplemented CLA diets on growth performance, dietary lipid utilization and selected immune parameters in European sea bass juveniles.

3.2. Materials and Methods

3.2.1. Experimental diets

Four isonitrogenous and isoenergetic diets, based in a commercial formulation, were designed with graded levels of CLA (CLA0=Control, CLA05=0.5%, CLA1=1% and CLA2=2%). CLA was supplied by BASF (Tarragona, Spain) as a supplement (50:50 mixture of each isomer; LUTA-CLA60®) and was added to the extruded diets in substitution of fish oil to maintain a constant energy content among dietary treatments. Diets were formulated and manufactured by a commercial feed producer (Biomar Iberia, S.A., Spain). Ingredients and proximate composition and fatty acid profiles are shown in Tables 3.1 and 3.2.

3.2.2. Growth trial

The trial was conducted with European sea bass juveniles produced by Alevines y Doradas S.A. (ADSA) in San Bartolomé de Tirajana (Las Palmas de Gran Canaria, Spain). Fish were maintained in stocking tanks and fed a commercial extruded diet (CLA0 diet) for 3 weeks (19-20.5°C) until being well adapted to the environmental conditions (2

kg/m^3 stocking density). Afterwards, 384 European sea bass juveniles were randomly distributed in 12 indoor 500 l, fibreglass tanks at initial stocking density of 2 kg/m^3 (32 fish/tank), in an open water system (5 l/min waterflow) under natural photoperiod (12L:12D). Fish average initial mass (g ± SD) and total length (cm ± SD) were 34.71 ± 7.61 and 13.52 ± 0.90 respectively. Water temperature ranged between 20.5-23.4°C and dissolved oxygen was kept at 8.0 ± 0.2 ppm, during the feeding period. Fish were manually fed three times a day (8, 12 and 16 h), 6 days a week for 12 weeks until apparent satiation. Feed conversion ratio was defined as the amount of feed ingested divided by the generated biomass; specific growth rate was defined as: [(ln final weight – ln initial weight)/number of days] x 100; relative growth (%) defined as: [(final weight – initial weight)/initial weight)] x 100 and perivisceral fat index defined as: (visceral fat weight/body weight) x 100.

Table 3.1. Ingredients and proximate composition of diets with different levels of CLA (0%, 0.5%, 1% and 2%)

	Dietary CLA treatments			
	CLA0	**CLA05**	**CLA1**	**CLA2**
Ingredients (%)				
Fish meal	41.00	41.00	41.00	41.00
Soyabean meal	25.00	25.00	25.00	25.00
Wheat	7.00	7.00	7.00	7.00
Corn gluten meal	4.50	4.50	4.50	4.50
Rapeseed meal	5.90	5.90	5.90	5.90
Vitamin & mineral mix	0.75	0.75	0.75	0.75
Fish oil[1]	15.85	15.12	14.38	12.93
CLA	0	0.73	1.47	2.92
Proximate composition (%)				
Crude protein	46.49	48.11	47.45	47.97
Crude fat	20.59	21.24	22.83	21.87
Ash	10.05	10.20	10.22	10.11
Moisture	6.49	6.46	6.47	7.06
Carbohydrates	22.77	20.45	19.50	20.05

Values are expressed as dry weight. Vitamin and mineral mix and CLA oil (LUTA-CLA 60®; containing 60% CLA methyl esters as a 50:50 mixture of *c*9, *t*11 and *t*10, *c*12 isomers), BASF (Spain), fish meal and fish oil (Sopropeche, France), soyabean meal (Cargill Inc., Spain), wheat (Hermanos Dueñas, Spain), corn gluten meal (SYRAL Iberia S.A.U., Spain), rapeseed meal (Esasa, Spain) [1] CLA was included at the expense of fish oil.

Table 3.2. Fatty acid composition (% of total identified fatty acids) of experimental diets

	Dietary CLA Treatments			
Fatty Acids	**CLA0**	**CLA05**	**CLA1**	**CLA2**
14:0	5.25	4.99	5.00	4.70
14:1n-5	0.04	0.04	0.04	0.04
14:1n-7	0.18	0.20	0.17	0.16
15:0	0.41	0.47	0.40	0.38
15:1n-5	0.08	0.03	0.03	0.03
16:0iso	0.03	0.09	0.03	0.07
16:0	15.60	15.08	15.46	14.77
16:1n-9	0.04	0.04	0.04	0.04
16:1n-7	6.16	5.72	5.82	5.80
16:1n-5	0.16	0.17	0.15	0.15
16:2n-6	0.22	0.25	0.21	0.19
16:2n-4	0.02	0.11	0.05	0.05
17:0	0.22	0.20	0.66	0.56
16:3n-4	0.41	0.51	0.42	0.30
16:3n-3	0.59	0.50	0.56	0.54
16:3n-1	0.06	0	0.06	0.06
16:4n-3	0.15	0.17	0.15	0.14
16:4n-1	0.40	0.27	0.32	0.35
18:0	3.03	3.67	3.20	3.11
18:1n-9	19.17	20.07	19.73	19.50
18:1n-7	3.15	3.49	3.13	3.15
18:1n-5	0.23	0.25	0.22	0.22
18:2n-9	0.00	0.04	0.03	0.03
18:2n-6	6.75	7.03	6.75	6.76
18:2n-4	0.19	0.20	0.18	0.18
18:3n-6	0.12	0.11	0.11	0.13
18:3n-4	0.20	0	0	0
18:3n-3	1.64	1.49	1.55	1.59
18:3n-1	0.04	0.07	0	0
18:2 *c*9,*t*11	0	0.70	1.34	2.19
18:4n-3	1.38	1.37	1.25	1.29
18:2 *t*10,*c*12	0	0.70	1.34	2.20
18:4n-1	0.01	0.17	0.20	0.20
20:0	0.20	0.25	0.21	0.20
20:1n-9	6.31	6.78	6.36	6.53
20:1n-7	0.27	0.30	0.26	0.37
20:2n-9	0	0.04	0.04	0.06
20:2n-6	0.06	0.07	0.06	0.05
20:3n-6	0.37	0.37	0.35	0.34
20:3n-9	0.11	0.10	0.10	0.10
20:4n-6	0.60	0.55	0.57	0.57
20:3n-3	0.16	0.15	0.15	0.15
20:4n-3	1.04	0.84	0.93	0.97
20:5n-3	6.28	5.79	5.73	5.46
22:1n-11	5.90	5.78	5.65	5.44
22:1n-9	0.71	0.78	0.68	0.76
22:4n-6	0.23	0.20	0.22	0.22
22:5n-6	0.08	0	0.08	0.07
22:5n-3	2.06	1.48	1.82	1.91
22:6n-3	9.71	8.32	8.19	7.96
Saturated	24.74	24.75	24.96	23.78
Monoenes	42.40	43.65	42.29	42.19
Σn-3	23.00	20.12	20.33	19.99
Σn-6	8.43	8.58	8.35	8.33
Σn-9	26.34	27.86	26.98	27.02
Σn-3 HUFA	19.24	16.59	16.82	16.43
Total CLA	0	1.40	2.68	4.39

CLA0, control diet; CLA05, CLA1 and CLA2, diets with 0.5%, 1% and 2% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

3.2.3. Sampling protocols

Growth parameters, hepatosomatic (HSI), viscerasomatic (VSI) and perivisceral fat index (PFI) were determined at days 0, 45 and 90. All fish were fasted for 24 hours before sampling. Eleven fish from the initial stock at day 0, ten fish per tank at day 45 and eleven fish per tank at day 90 were sampled for whole body, liver, muscle and perivisceral fat proximate and fatty acid composition (samples were stored under nitrogen atmosphere at -80ºC), as well as for hepatic and muscle tissue morphology studies. At day 90, lipogenic enzyme activities were determined (samples were frozen in liquid nitrogen and stored at -80°C). Blood samples were extracted from five fish per tank for plasma and serum collection at day 90 in order to determine the reduction potential of circulating neutrophils (NBT) index, lysozyme and alternative complement pathway (ACP) activities.

3.2.4. Biochemical analyses

Fish and diets biochemical composition were conducted following standard procedures (AOAC, 2000). Ash content was determined by combustion in a muffle furnace at 600°C for 12 h , moisture content was determined after drying at 105°C to constant weight, crude protein by acid digestion using Kjeldahl method (Nx6.25) and crude lipid was extracted following the method of Folch *et al*. (1957). Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo *et al*. (1992), being quantified by flame ionizator detector (FID) and identified in comparison to external standards of CLA isomers (Sigma-Aldrich and Matreya, LLC.). All analyses were conducted by triplicate.

3.2.5. Enzyme assays

Liver and muscle samples were homogenized in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4) and centrifuged at 20.000 g for 40 min at 4°C. Soluble protein content of muscle and liver homogenates was determined on the supernatant by the method of Bradford (1976) using bovine serum albumin (BSA)

as standard. Enzyme activities of glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) and malic enzyme (ME, EC 1.1.1.40), both lipogenic enzymes, were assayed using spectrophotometric procedures on the supernatant following the method described by Dias *et al*. (1998). Care was took to ensure that initial rates were measured in all assays and that enzymes were stable in the buffer solution used during the time and temperature required to perform the assays (Alvarez *et al*., 1998). Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay temperature per minute, were expressed per mg of hepatic and muscular soluble protein (specific activity). All enzyme assays were performed by triplicate.

3.2.6. Histological studies

Muscle and liver samples were fixed with 10% neutral-buffered formalin, embedded in paraffin prior to be stained with hematoxilin and eosin (H&E) for optical examination (Martoja and Martoja-Pierson, 1970). Micrographs of muscle and liver were taken at a final magnification of 400X using a Nikon Microphot-FXA microscope and an Olympus DP50 camera.

3.2.7. Blood collection and sample preparation

Blood was obtained by caudal sinus puncture. No anaesthetic was used to avoid any possible effect in blood parameters and handle time was less than 1 minute to minimize stress effects. The first aliquot of blood was transferred to a lithium heparin coated Eppendorf tube and used for Nitroblue tetrazolium (NBT) index, in order to measure the activity of circulating neutrophils spectrophotometrically as described by Siwicki *et al*. (1993), determining the reduction of NBT to formazan in presence of oxygen radicals. The second aliquot was divided in two parts, transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at –80**°**C for ACP and lysozyme activities determination.

3.2.8. Serum analysis

The Alternative complement pathway (ACP) was carried out as described by Sunyer and Tort (1995) for gilthead sea bream (*Sparus aurata*) using rabbit red blood cells (RBC). The reciprocal of the serum dilution causing 50% lysis of RBC is designed as

ACH50 and the results are expressed as ACH50 units/ml. Lysozyme level in blood serum was determined by turbidimetric assay according to Anderson and Siwicki (1994) using hens' egg white lysozyme (Sigma) in PBS as a standard. Results were expressed as lysozyme units ml^{-1}.

3.2.9. Statistical analyses

Means and Standard Deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of CLA inclusion. Where data did not pass a normality test, a log transformation was used to normalize data. When *F* values showed significance, individual means were compared using Tukey's test for multiple means comparison. Significant differences were considered for $P<0.05$.

3.3. Results

3.3.1. Growth performance

There were no mortalities during the experiment. Weight gain, SGR, FCR, K, relative growth and VSI were not affected by CLA inclusion during all the experiment (Table 3.3). However, a negative correlation was found ($R^2=0.93$; $y=-1.8234x+8.658$; $P<0.01$) between levels of CLA inclusion and PFI (Fig. 3.1), being PFI and feed intake (Table 3.3) reduced ($P<0.05$) in fish fed diet CLA2 at the end of the experiment.

3.3.2. Whole body, muscle and liver proximal composition

No significant differences were found in proximal composition of whole body, muscle and liver among treatments at the end of the experiment.

3.3.3. Muscle, liver and perivisceral fat fatty acid profile

The fatty acid profile of muscle is summarized in Table 3.4. In all tissues samples, fatty acids profiles were altered by the inclusion of CLA in diets. In muscle samples, saturated and monounsaturated fatty acids decreased ($P<0.05$) by the reduction in 16:0, 16:1n-7 and 18:1n-9. Besides, n-3 and n-3 Highly unsaturated fatty acids (HUFA) increased ($P<0.05$) in fish fed diets containing CLA, with the DHA/EPA ratio increased

(P<0.05) and the ARA/EPA ratio reduced (P<0.05) in fish fed diets CLA1 and CLA2. CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12, were detected in higher levels (P<0.05) in fish fed with supplemented CLA diets than fish fed control diet.

Liver fatty acid profile (Table 3.5) denoted a decrease (P<0.05) in saturated fatty acids in fish fed diet CLA05 compared with control fish, whereas monounsaturated group decreased (P<0.05) in fish fed with CLA2 diet. The deposition of the CLA isomers increased (P<0.05) with the inclusion of CLA in the diets.

The perivisceral fat fatty acid profile is presented in Table 3.6. The monounsaturated fatty acids decreased (P<0.05) in fish fed CLA diets compared to control fish. The n-3 fatty acids were reduced (P<0.05) in fish fed CLA1 and CLA2. The n-3 HUFA group decreased (P<0.05) in fish fed CLA1 diet. The deposition of the CLA isomers increased (P<0.05) in fish fed diets with CLA

Table 3.3. Biological and growth parameters (mean ± SD) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1 or 2%) for 12 weeks

	Dietary CLA treatments			
	CLA0	**CLA05**	**CLA1**	**CLA2**
Growth				
Food intake (g)	3191.50±93.13[a]	3268.64±460.85[ab]	3077.07±255.12[ab]	2902.92±49.78[b]
Initial mass (g)	35.37±1.45	34.31±1.92	33.73±1.08	35.77±2.38
Final mass (g)	108.38±4.55	110.71±10.69	102.67±7.23	104.26±8.82
Eviscerated mass (g)	89.69±12.2	98.68±23.42	87.52±15.72	86.99±21.25
Final Length (cm)	19.38±0.12	19.58±0.74	18.97±0.37	19.14±0.54
Relative growth (%)	206.61±15.07	222.86±27.79	204.23±13.83	192.94±44.13
SGR (%)	1.24±0.14	1.32±0.17	1.24±0.08	1.24±0.15
FCR	1.68±0.13	1.63±0.07	1.74±0.11	1.60±0.14
K	1.49±0.04	1.47±0.08	1.50±0.04	1.49±0
VSI (%)	16.23±0.69	15.19±1.58	16.52±1.52	14.61±1.48
HSI (%)	1.61±0.13	1.72±0.19	1.75±0.02	1.58±0.34

Data are presented as means±SD. Initial weight (n=96), final weight and length, relative growth, SGR, FCR and K (n=66), food intake (n=96). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA, conjugated linoleic acid; FCR, feed conversion ratio; HSI, Hepatosomatic index; K, condition factor; SGR, specific growth rate; VSI, Viscerosomatic index; CLA0, control diet; CLA05, CLA1 and CLA2, diets with 0.5%, 1% and 2% of CLA inclusion, respectively.

3.3.4. Muscle and liver morphology

Morphological analyses of liver for fish fed diets containing CLA resulted qualitatively in a regular-shaped morphology of the hepatocytes around sinusoidal

spaces and a reduction on the lipid vacuolization of the cytoplasm that decreased the number of hepatocytes with the nuclei displaced to the cellular periphery. This effect was more pronounced in fish fed with the higher concentration of CLA (Fig. 3.2). No differences were observed on muscle histological evaluation of fish fed different diets.

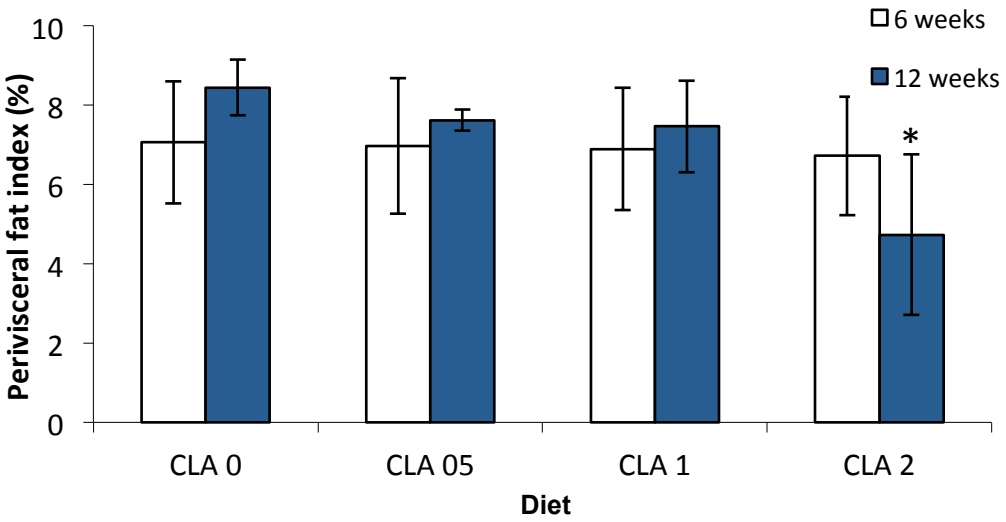


Figure 3.1. Effect of CLA on perivisceral fat index in European sea bass (n=24). Perivisceral fat index= (Visceral fat weight/body weight) x100. Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Significant differences (P<0.05) among treatments are indicated by (*).

3.3.5. Activities of lipogenic enzymes

Data on the activities of the two lipogenic enzymes assayed in liver are reported in Table 3.7. G6PD evidenced a 10 fold higher activity than ME. Dietary CLA did not affect G6PD values, but fish fed 1% CLA showed a higher (P<0.05) ME values than control fish. Furthermore, the activities of G6PD and ME showed a positive correlation when dietary CLA up to 1% inclusion level was included (R^2=0.91, y =0.0457x+0.0805, P<0.04; R^2=0.99, y=0.0085x+0.0052, P<0.05 respectively).

Table 3.4. Muscle fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1 or 2%) for 12 weeks

	Dietary CLA treatments			
	CLA0	**CLA05**	**CLA1**	**CLA2**
Fatty acids				
14:0	4.93±0.19	4.03±0.08	3.89±0.15	3.86±0.05
14:1n-5	0.07±0.01	0.05±0.00	0.05±0.00	0.05±0.00
14:1n-7	0.15±0.00	0.12±0.01	0.13±0.00	0.12±0.00
15:0	0.42±0.01	0.37±0.00	0.33±0.01	0.34±0.01
15:1n-5	0.08±0.00	0.07±0.00	0.07±0.00	0.07±0.00
16:0iso	0.07±0.00	0.11±0.01	0.07±0.02	0.08±0.01
16:0	20.19±0.28	17.94±0.36	16.56±0.15	16.49±0.12
16:1n-9	0.09±0.00	0.08±0.00	0.07±0.00	0.07±0.00
16:1n-7	6.81±0.15	6.26±0.51	5.76±0.12	5.60±0.03
16:1n-5	0.16±0.00	0.14±0.00	0.13±0.00	0.13±0.00
16:2n-6	0.22±0.00	0.20±0.00	0.18±0.01	0.18±0.01
16:2n-4	0.11±0.00	0.07±0.03	0.06±0.03	0.06±0.02
17:0	0.25±0.02	0.13±0.13	0.24±0.01	0.23±0.02
16:3n-4	0.41±0.01	0.34±0.04	0.35±0.01	0.36±0.01
16:3n-3	0.46±0.27	0.41±0.26	0.43±0.20	0.36±0.22
16:3n-1	0.05±0.00	0.04±0.00	0.03±'0.03	0.02±0.03
16:4n-3	0.15±0.00	0.13±0.00	0.12±0.00	0.12±0.01
16:4n-1	0.24±0.07	0.28±0.05	0.24±0.05	0.30±0.03
18:0	3.58±0.18	3.79±0.06	3.67±0.03	3.69±0.11
18:1n-9	22.22±0.13	20.89±1.04	20.97±0.51	20.22±0.01
18:1n-7	3.40±0.07	3.08±0.02	3.00±0.06	3.03±0.07
18:1n-5	0.22±0.00	0.20±0.01	0.20±0.00	0.19±0.01
18:2n-9	0.13±0.02	0.12±0.00	0.08±0.07	0.09±0.00
18:2n-6	5.92±0.08	5.69±0.18	5.89±0.04	5.81±0.16
18:2n-4	0.22±0.00	0.20±0.01	0.19±0.00	0.19±0.00
18:3n-6	0.11±0.04	0.08±0.00	0.12±0.03	0.10±0.04
18:3n-4	0.15±0.00	0.17±0.01	0.16±0.00	0.17±0.03
18:3n-3	1.18±0.03	1.20±0.08	1.25±0.02	1.22±0.06
18:3n-1	0.02±0.02	0.42±0.04	0.03±0.31	0.04±0.09
18:2 *c*9, t11	0.01±0.01[a]	0.42±0.05[b]	1.14±0.30[c]	1.28±0.09[c]
18:4n-3	0.95±0.02	1.06±0.02	1.07±0.05	1.07±0.04
18:2 *t*10, c12	0.01±0.00[a]	0.36±0.05[b]	1.02±0.28[c]	1.16±0.06[c]
18:4n-1	0.15±0.00	0.36±0.04	0.18±0.28	0,16±0.06
20:0	0.18±0.00	0.15±0.01	0.10±0.09	0.15±0.00
20:1n-9	4.96±0.01	3.77±0.31	4.77±0.39	4.23±0.13
20:1n-7	0.24±0.00	0.20±0.01	0.20±0.00	0.20±0.01
20:2n-9	0.01±0.02	0.01±0.01	0.02±0.04	0.03±0.04
20:2n-6	0.07±0.00	0.00±0.00	0.04±0.03	0.06±0.00
20:3n-6	0.45±0.02	0.41±0.03	0.41±0.01	0.42±0.00
20:3n-9	0.09±0.00	0.05±0.06	0.09±0.00	0.09±0.00
20:4n-6	0.59±0.01	0.73±0.00	0.63±0.04	0.65±0.00
20:3n-3	0.13±0.00	0.12±0.01	0.08±0.07	0.12±0.01
20:4n-3	0.20±0.28	0.82±0.07	0.85±0.03	0.84±0.02
20:5n-3	5.44±0.14	6.73±0.23	6.43±0.29	6.53±0.09
22:1n-11	3.87±0.09	3.22±0.44	3.42±0.00	3.19±0.11
22:1n-9	0.60±0.01	0.50±0.04	0.52±0.00	0.49±0.01
22:4n-6	0.22±0.00	0.27±0.00	0.25±0.01	0.26±0.01
22:5n-6	0.03±0.05	0.07±0.00	0.03±0.04	0.04±0.05
22:5n-3	1.63±0.03	2.04±0.04	1.63±0.56	2.01±0.02
22:6n-3	8.16±0.36	12.10±0.47	10.94±0.58	11.63±0.03
Saturated	29.55±0.31[a]	26.42±0.22[b]	24.78±0.31[c]	24.75±0.15[c]
Monoenes	42.87±0.10[a]	38.59±0.69[b]	39.28±0.81[b]	37.58±0.29[b]
Σn-3	18.31±0.29[a]	24.60±0.79[b]	22.80±0.54[b]	23.90±0.02[b]
Σn-6	7.60±0.19	7.46±0.20	7.54±0.17	7.51±0.16
Σn-9	28.11±0.15[a]	25.41±0.70[b]	26.52±0.71[ab]	25.23±0.09[b]
Σn-3 HUFA	13.61±0.22[a]	18.82±0.70[b]	19.93±0.37[b]	21.13±0.10[b]
DHA/EPA	1.50±0.11[a]	1.80±0.01[b]	1.70±0.03[b]	1.78±0.03[b]
ARA/EPA	0.11±0.00[a]	0.11±0.00[a]	0.10±0.00[b]	0.10±0.00[b]
ARA/DHA	0.07±0.00	0.06±0.00	0.06±0.00	0.06±0.00
Total CLA	0.02±0.01[a]	0.78±0.09[b]	1.83±0.04[c]	2.44±0.15[d]

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1 and CLA2, diets with 0.5%, 1% and 2% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 3.5. Liver fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1 or 2%) for 12 weeks

	Dietary CLA treatments			
	CLA0	**CLA05**	**CLA1**	**CLA2**
Fatty acids				
14:0	2.47±0.17	2.66±0.10	2.46±0.22	2.64±0.06
14:1n-5	0.06±0.01	0.05±0.01	0.04±0.00	0.04±0.00
14:1n-7	0.07±0.01	0.08±0.01	0.07±0.01	0.07±0.00
15:0	0.22±0.01	0.23±0.02	0.20±0.01	0.20±0.01
15:1n-5	0.02±0.00	0.02±0.00	0.02±0.00	0.02±0.00
16:0iso	0.06±0.00	0.06±0.00	0.06±0.00	0.06±0.00
16:0	23.96±1.48	20.47±0.66	20.92±0.51	21.09±0.70
16:1n-9	0.20±0.01	0.19±0.01	0.19±0.01	0.17±0.01
16:1n-7	4.77±0.17	4.68±0.04	4.08±0.19	4.15±0.06
16:1n-5	0.08±0.00	0.08±0.01	0.07±0.00	0.07±0.00
16:2n-6	0.21±0.01	0.20±0.01	0.20±0.00	0.19±0.00
16:2n-4	0.12±0.01	0.11±0.03	0.09±0.02	0.10±0.03
17:0	0.23±0.21	0.22±0.11	0.28±0.14	0.30±0.02
16:3n-4	0.39±0.00	0.40±0.02	0.42±0.01	0.40±0.00
16:3n-3	0.42±0.02	0.45±0.03	0.39±0.02	0.38±0.01
16:3n-1	0.00±0.00	0.04±0.00	0.02±0.02	0.03±0.00
16:4n-3	0.12±0.01	0.13±0.00	0.11±0.00	0.12±0.01
16:4n-1	0.05±0.00	0.06±0.02	0.05±0.01	0.06±0.01
18:0	4.70±0.41	5.25±0.42	6.46±0.39	6.68±0.16
18:1n-9	30.96±0.51	30.35±1.70	30.46±0.89	29.46±0.40
18:1n-7	3.12±0.14	3.12±0.13	2.94±0.04	2.81±0.05
18:1n-5	0.07±0.12	0.22±0.00	0.21±0.00	0.19±0.01
18:2n-9	0.38±0.01	0.28±0.06	0.27±0.05	0.19±0.01
18:2n-6	3.56±0.26	4.14±0.30	3.76±0.19	3.71±0.07
18:2n-4	0.14±0.01	0.16±0.01	0.14±0.01	0.14±0.01
18:3n-6	0.00±0.00	0.08±0.08	0.11±0.03	0.10±0.03
18:3n-4	0.14±0.01	0.15±0.00	0.14±0.01	0.12±0.01
18:3n-3	0.70±0.05	0.82±0.07	0.73±0.04	0.72±0.01
18:3n-1	0.01±0.02	0.42±0.01	0.79±0.02	1.27±0.02
18:2 *c*9,*t*11	0.05±0.00[a]	0.43±0.00[b]	0.80±0.03[c]	1.26±0.03[d]
18:4n-3	0.55±0.04	0.65±0.07	0.57±0.05	0.58±0.02
18:2 *t*10,*c*12	0.00±0.00[a]	0.28±0.01[b]	0.56±0.03[c]	0.96±0.05[d]
18:4n-1	0.08±0.01	0.10±0.01	0.08±0.01	0.09±0.01
20:0	0.87±0.09	0.86±0.02	0.87±0.02	0.88±0.04
20:1n-9	3.58±0.15	3.48±0.17	3.40±0.12	3.24±0.27
20:1n-7	0.16±0.01	0.16±0.01	0.15±0.01	0.15±0.01
20:2n-9	0.01±0.01	0.02±0.02	0.01±0.02	0.00±0.00
20:2n-6	0.04±0.03	0.03±0.03	0.04±0.00	0.04±0.00
20:3n-6	0.34±0.02	0.36±0.02	0.34±0.02	0.32±0.03
20:3n-9	0.06±0.00	0.06±0.00	0.06±0.00	0.05±0.00
20:4n-6	0.43±0.01	0.46±0.02	0.44±0.01	0.41±0.03
20:3n-3	0.09±0.01	0.11±0.01	0.10±0.00	0.05±0.07
20:4n-3	0.54±0.04	0.62±0.04	0.53±0.06	0.54±0.01
20:5n-3	3.31±0.08	3.79±0.35	3.44±0.16	3.43±0.02
22:1n-11	2.52±0.02	2.28±0.09	2.37±0.14	2.28±0.08
22:1n-9	0.45±0.01	0.41±0.02	0.41±0.02	0.39±0.02
22:4n-6	0.16±0.01	0.19±0.01	0.17±0.01	0.16±0.01
22:5n-6	0.06±0.01	0.04±0.04	0.04±0.04	0.06±0.00
22:5n-3	1.58±0.13	1.53±0.51	1.60±0.18	1.54±0.09
22:6n-3	7.89±0.70	9.04±0.69	8.34±0.91	8.09±0.12
Saturated	32.45±1.59[a]	29.68±0.86[b]	31.19±0.79[ab]	31.80±0.41[ab]
Monoenes	46.06±0.42[a]	45.12±1.29[a]	44.41±0.55[a]	43.05±0.08[b]
Σn-3	15.21±1.04	17.12±1.74	15.81±1.24	15.45±0.15
Σn-6	4.80±0.33	5.51±0.34	5.09±0.18	4.99±0.10
Σn-9	35.63±0.45	34.79±1.58	34.79±0.80	33.50±0.14
Σn-3 HUFA	13.42±0.95	15.07±1.57	14.01±1.22	13.66±0.11
DHA/EPA	2.38±0.15	2.39±0.07	2.42±0.24	2.36±0.05
ARA/EPA	0.13±0.00	0.12±0.01	0.13±0.01	0.12±0.01
ARA/DHA	0.05±0.00	0.05±0.00	0.05±0.01	0.05±0.00
Total CLA	0.05±0.00[a]	0.70±0.02[b]	1.36±0.06[c]	2.22±0.08[d]

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1 and CLA2, diets with 05%, 1% and 2% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 3.6. Perivisceral fat fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1 or 2%) for 12 weeks

	Dietary CLA treatments			
	CLA0	**CLA05**	**CLA1**	**CLA2**
Fatty acids				
14:0	4.81±0.24	4.72±0.09	4.64±0.06	4.63±0.14
14:1n-5	0.06±0.01	0.05±0.00	0.05±0.00	0.05±0.00
14:1n-7	0.14±0.01	0.14±0.00	0.14±0.00	0.13±0.00
15:0	0.38±0.02	0.38±0.01	0.38±0.01	0.36±0.01
15:1n-5	0.07±0.00	0.07±0.00	0.07±0.00	0.07±0.00
16:0iso	0.00±0.00	0.01±0.00	0.00±0.00	0.00±0.00
16:0	16.94±0.62	16.77±0.24	16.18±0.30	16.36±0.80
16:1n-9	0.06±0.00	0.06±0.00	0.06±0.00	0.05±0.00
16:1n-7	6.98±0.41	6.69±0.07	6.36±0.12	6.43±0.20
Me16:0	0.14±0.01	0.14±0.00	0.14±0.00	0.13±0.00
16:1n-5	0.17±0.01	0.18±0.00	0.17±0.00	0.17±0.00
16:2n-6	0.04±0.000	0.04±0.00	0.04±0.00	0.04±0.00
16:2n-4	0.72±0.06	0.74±0.06	0.75±0.03	0.72±0.04
17:0	0.27±0.04	0.32±0.03	0.33±0.00	0.30±0.07
16:3n-4	0.65±0.06	0.66±0.03	0.63±0.03	0.65±0.02
16:3n-3	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00
16:3n-1	0.12±0.01	0.12±0.01	0.12±0.01	0.12±0.00
16:4n-3	0.03±0.00	0.04±0.01	0.04±0.01	0.04±0.00
16:4n-1	0.34±0.07	0.35±0.04	0.35±0.05	0.38±0.03
18:0	2.95±0.20	3.22±0.10	3.40±0.08	3.45±0.13
18:1n-9	21.46±0.51	21.27±0.49	21.40±0.05	20.57±0.16
18:1n-7	3.14±0.02	3.13±0.02	3.13±0.04	3.08±0.07
18:1n-5	0.22±0.00	0.21±0.00	0.21±0.01	0.20±0.00
18:2n-9	0.15±0.01	0.12±0.02	0.12±0.01	0.10±0.00
18:2n-6	6.34±0.15	6.29±0.23	6.53±0.14	6.18±0.08
18:2n-4	0.21±0.01	0.21±0.00	0.20±0.01	0.21±0.00
18:3n-6	0.12±0.02	0.14±0.02	0.13±0.02	0.15±0.01
18:3n-4	0.22±0.03	0.22±0.02	0.22±0.02	0.22±0.00
18:3n-3	1.46±0.02	1.40±0.10	1.44±0.03	1.31±0.02
18:3n-1	0.00±0.00	0.00±0.00	0.01±0.04	0.02±0.02
18:2 *c*9, *t*11	0.04±0.00[a]	0.48±0.05[b]	1.03±0.05[c]	1.36±0.02[d]
18:4n-3	1.32±0.09	1.32±0.00	1.27±0.06	1.25±0.05
18:2 *t*10, *c*12	0.01±0.01[a]	0.43±0.04[b]	0.92±0.05[c]	1.24±0.02[d]
18:4n-1	0.21±0.02	0.20±0.01	0.20±0.01	0.19±0.01
20:0	0.16±0.02	0.17±0.01	0.16±0.00	0.17±0.00
20:1n-9	5.73±0.19	5.44±0.16	5.48±0.07	5.18±0.09
20:1n-7	0.21±0.01	0.22±0.01	0.22±0.01	0.21±0.00
20:2n-9	0.04±0.03	0.07±0.01	0.07±0.01	0.07±0.01
20:3n-6	0.46±0.03	0.42±0.01	0.44±0.01	0.41±0.03
20:2n-6	0.03±0.02	0.04±0.00	0.01±0.01	0.02±0.03
20:3n-9	0.12±0.01	0.12±0.00	0.07±0.06	0.10±0.01
20:4n-6	0.50±0.02	0.51±0.01	0.49±0.02	0.50±0.02
20:3n-3	0.14±0.00	0.14±0.01	0.14±0.00	0.12±0.00
20:4n-3	1.01±0.04	0.94±0.04	0.96±0.01	0.89±0.03
20:5n-3	6.53±0.57	6.62±0.13	6.37±0.25	6.70±0.27
22:1n-11	3.83±0.21	3.65±0.18	3.63±0.03	3.46±0.10
22:1n-9	0.57±0.02	0.55±0.02	0.55±0.00	0.53±0.01
22:4n-6	0.09±0.03	0.08±0.02	0.08±0.02	0.03±0.04
22:5n-6	0.18±0.07	0.22±0.00	0.20±0.00	0.21±0.00
22:5n-3	1.71±0.68	1.98±0.03	1.56±0.62	1.83±0.04
22:6n-3	8.93±0.10	8.705±0.13	7.93±0.27	8.06±0.15
Saturated	25.65±0.68	25.72±0.27	25.22±0.31	25.40±1.18
Monoenes	42.64±0.38[a]	41.66±0.17[b]	41.45±0.27[b]	40.12±0.40[c]
Σn-3	21.13±0.13[a]	21.15±0.07[a]	19.71±0.22[b]	20.22±0.56[b]
Σn-6	7.75±0.23	7.75±.25	7.93±0.15	7.53±0.14
Σn-9	28.11±0.58[a]	27.61±0.34[ab]	27.73±0.07[ab]	26.61±0.22[b]
Σn-3 HUFA	18.32±0.02[a]	18.38±0.17[ab]	16.95±0.31[c]	17.61±0.49[ac]
DHA/EPA	1.37±0.10	1.32±0.02	1.248±0.08	1.20±0.03
ARA/EPA	0.08±0.00	0.08±0.00	0.077±0.00	0.07±0.00
ARA/DHA	0.06±0.00	0.06±0.00	0.062±0.00	0.06±0.00
Total CLA	0.05±0.01[a]	0.91±0.09[b]	1.949±0.09[c]	2.60±0.04[d]

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1 and CLA2, diets with 05%, 1% and 2% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

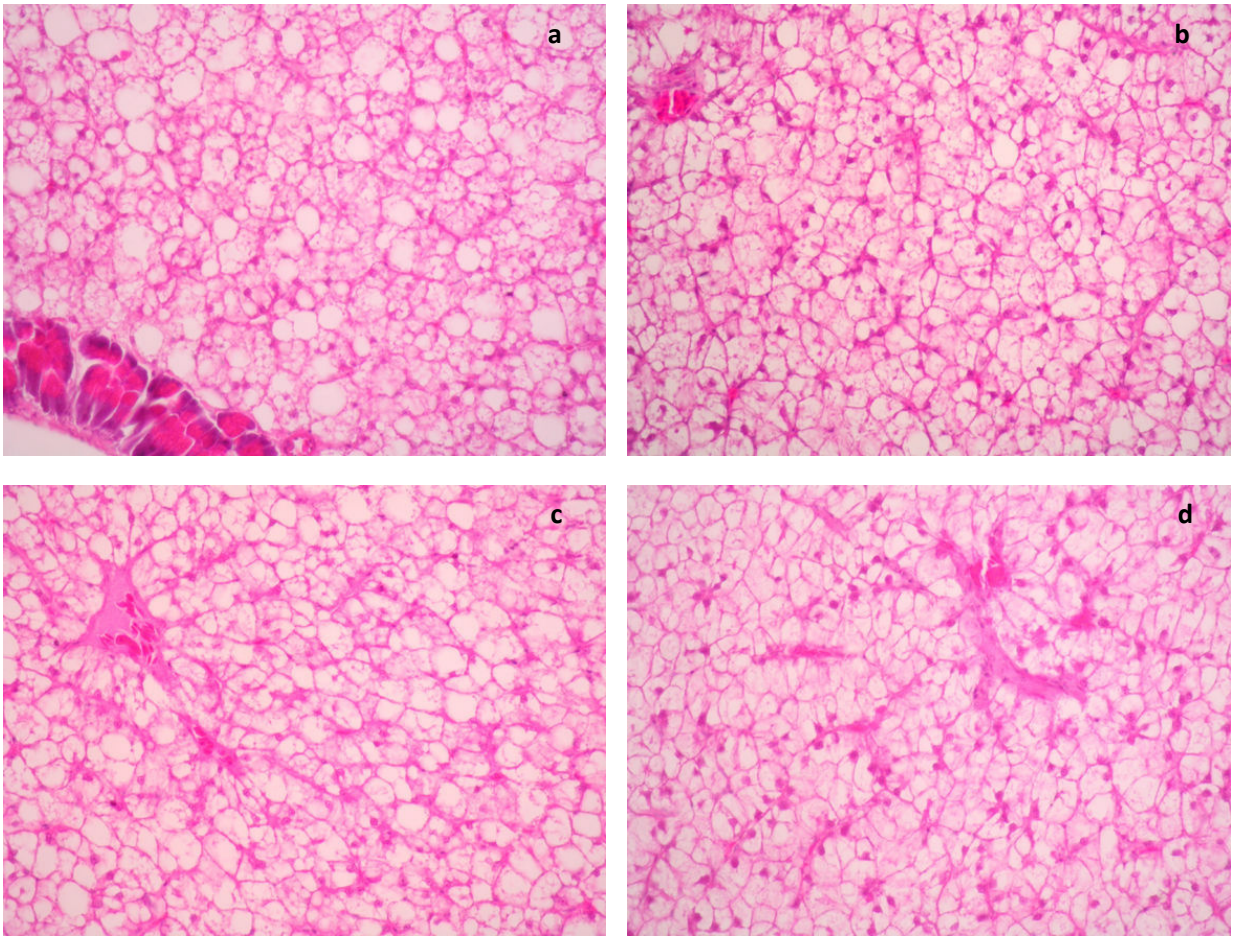


Figure 3.2. Liver micrographs (H&E; 400X) from fish fed (a) Control diet showing foci of swollen hepatocytes characterized by cytoplasm vacuolization and nuclei displaced to periphery cellular. (b) CLA05 diet with lower number of swollen hepatocytes. (c) CLA1 diet with lower number of swollen hepatocytes and (d) CLA2 diet with regular morphology of the hepatocytes located around sinusoidal spaces (n=12).

Table 3.7. Activities of glucose 6-phosphate dehydrogenase and malic enzyme in liver of European sea bass fed graded dietary levels of CLA (0, 0.5, 1 or 2%) for 12 weeks

	Dietary CLA treatments			
	CLA0	**CLA05**	**CLA1**	**CLA2**
G6PD IU / mg protein	0.1179±0.042	0.1884±0.065	0.2093±0.037	0.1957±0.028
ME IU / mg protein	0.0132±0.004^{a}	0.0231±0.008^{ab}	0.0302±0.005^{b}	0.0107±0.005^{a}

Data are presented as means ± SD (n=12). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1 and CLA2, diets with 0.5%, 1% and 2% of CLA inclusion, respectively; G6PD, glucose-6-phophate dehydrogenase (EC 1.1.1.49); ME, malic enzyme (EC 1.1.1.40); IU, enzyme activity units defined as micromoles of substrate converted to product at assay temperature per minute expressed per mg of soluble protein

3.3.6. Immune parameters

An improvement on lysozyme levels ($P<0.05$) was determined in fish fed diet 1% CLA. Furthermore, a positive correlation ($R^2=0.99$, $y =22.883x+78.028$; $P<0.01$) between the CLA level of inclusion and lysozyme levels was detected up to 1% inclusion level. The ACP values and the NBT index did not showed statistical difference among treatments, but a positive correlation between CLA inclusion levels and ACP was observed ($R^2=0.93$, $y=6.7552x+64.86$; $P<0.03$) (Fig. 3.3).

3.4. Discussion

Growth performance and feed utilization were not affected by the inclusion of dietary CLA, as found in previous studies with different fish species (Leaver *et al*., 2006; Valente *et al*., 2007b; Ramos *et al*., 2008).

In the present study, inclusion levels of 2% of dietary CLA significantly reduced feed intake as observed by Twibell *et al*. (2000) in hybrid striped bass fed 1% CLA. This effect was not found in other studies with different fish species (Leaver *et al*., 2006; Kennedy *et al*., 2007b). This reduction of feed intake could be related to an alteration in the metabolic homeostasis as proposed by House *et al*. (2005) or through the reduction in the leptin secretion found by Inoue *et al*. (2004).

Most interesting was the reduction of perivisceral fat deposition in fish fed CLA supplemented diets, in agreement with the reduced fat deposition found in hybrid striped bass (Twibell *et al*., 2000) and mammals (Yamasaki *et al*., 2003). Despite reduction in perivisceral fat could be partly related to the observed feed intake, this effect was only significant in fish fed 2% CLA and has been regarded to be insufficient *per se* for producing a significant reduction in fat mass in mammals (House *et al*., 2005). Therefore, these results suggest the activation of lipid catabolism as seen in mammals, where dietary CLA increased the activity of lipolitic enzymes in liver such as the carnitine-palmitoyltransferase I (CPT-I), a key enzyme in the mitochondrial β-oxidation of fatty acids in adipose tissues (Park and Pariza, 2007).

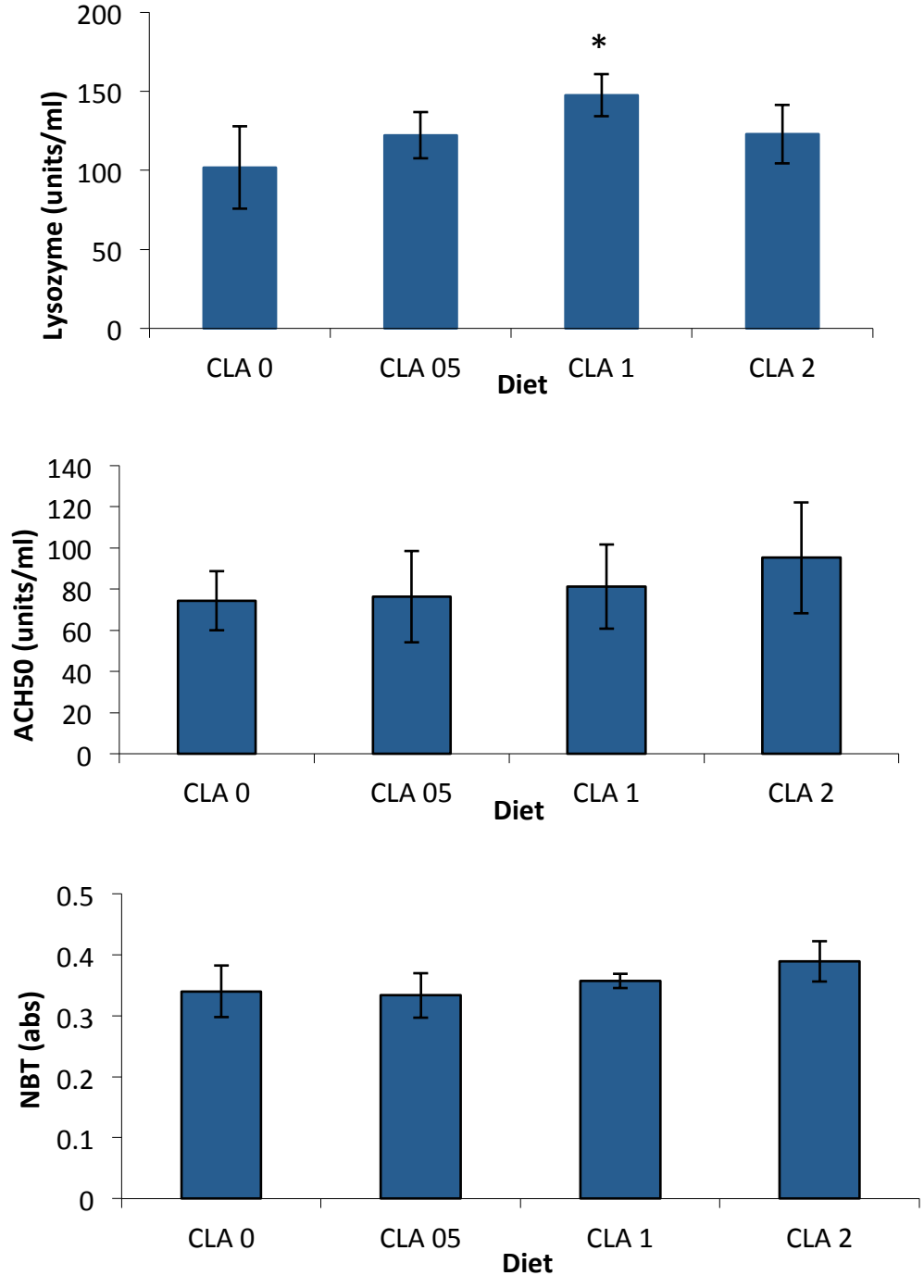


Figure 3.3. Influence of CLA on total (a) lysozyme activity (n= 15). (b) alternative complement pathway activity (n = 15) in European sea bass. (c) reduction potential of circulating neutrophils (n = 15). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Significant differences ($P<0.05$) among treatments are indicated by (*).

The reduction in European seabass perivisceral fat deposition and the potential increase in β-oxidation agree well with the reduction in monounsaturated fatty acids, particularly palmitoleic acid (16:1n-7), which are known to be primary sources for fat catabolism. Moreover, palmitoleic and oleic acid (18:1n-9) are synthesized through stearoyl-CoA desaturase (SCD) which is a rate-limiting enzyme for fat accumulation in adipose tissue and whose activity is inhibited by dietary CLA in mammals (Lin *et al*., 2004) and could be contributing to the reduction of perivisceral fat in sea bass. Nevertheless, as suggested by other authors, reduced perivisceral fat could be also related to an inhibition of the adipocyte lipoprotein lipase activity, the initiation of apoptosis in adipose tissue or the inhibition of SCD, which is the rate-limiting enzyme converting saturated fatty acids to monounsaturated fatty acids (Park and Pariza, 2007). Reduction of fat deposition in the visceral cavity without compromising fish growth performance suggests a better use of dietary energy and the potential value of CLA to improve European sea bass production quality.

The reduction in saturated and monounsaturated fatty acids and the increase in the DHA/EPA ratio found in muscle of fish fed CLA suggest the increase β-oxidation and SCD inhibition as discussed above. EPA has been found to be a better substrate for mitochondrial CPT-I than DHA (Madsen *et al*., 1998) and its preferential β-oxidation in muscle over DHA would increase the DHA/EPA ratio. This results are in agreement with the reduction in monounsaturated fatty acids found in Atlantic salmon (Kennedy *et al*., 2005; Leaver *et al*., 2006), European sea bass (Valente *et al*., 2007a) and sea bream (Diez *et al*., 2007) or in saturated fatty acids found in Atlantic salmon (Leaver *et al*., 2006) and sea bream (Diez *et al*., 2007). As a result, n-3 and n-3 HUFA increased in European sea bass muscle in agreement with other studies (Twibell *et al*., 2000; Manning *et al*., 2006; Valente *et al*., 2007a) and enhancing the beneficial effect of fish fillet for human consumption. Moreover, CLA was accumulated in muscle adding a potential nutritional value for human consumption (Gaullier *et al*., 2007). CLA accumulation in fish fillet has been also found in other species (Bandarra *et al*., 2006; Valente *et al*., 2007b), but the degree of deposition seems to depend on the type of isomer used. Thus, in European sea bass, the isomer *cis*-9, *trans*-11 was preferentially accumulated in fish tissues maybe due to a more efficient incorporation than *trans*-10, *cis*-12 isomer as reported in other studies (Lauridsen *et al*., 2005; Valente *et al*., 2007a) or because the *trans*-10, *cis*-12

isomer is more efficiently oxidized (Churruca *et al*., 2009). In liver, feeding CLA at 1 % increased the activity of ME, a lipogenic enzyme involved in NADPH generation for *de novo* lipogenesis. Nevertheless, this enhanced lipogenic activity did not lead to increase lipid deposition in the liver of fish fed CLA, which indeed showed a tendency to reduce its lipid and monounsaturated fatty acids content and a decreased lipid vacuolization of hepatic cells cytoplasm, suggesting lipid mobilization and hepatocytes turnover when fish are fed increased dietary CLA levels and in agreement with the lack of effect of CLA found in G6PD activity in this and other studies (Valente *et al*., 2007a). Further increase in CLA (2%) did not affect the activity of malic enzyme what maybe could be related with the lower feed intake or fat deposition found in these fish, or maybe with the previous reported effects of CLA on anaerobic glucose metabolism and insulin resistance as seen in other vertebrates (Pérez-Matute *et al*., 2007).

Finally, inclusion of CLA up to 1% increased plasma lysozyme activity, meanwhile 2% of CLA had not the same effect may be related to the reduced feed intake found in this treatment, although CLA levels were positively correlated with ACP activity. These are the first results about the effect of dietary CLA on fish immune system and they are in agreement with the improved immune resistance found also in birds and mammals. For instance, in broiler chicks phagocyte activity is promoted by CLA dietary inclusion (Zhang *et al*., 2005) and in rats CLA reinforces the specific immune system (Ramírez-Santana *et al*., 2009). Further studies are being conducted to determine the effect of CLA on fish immune system and resistance to potential pathogens.

These results indicate that European sea bass can successfully incorporate CLA in tissues up to 2%, and that CLA increases fillet quality due to the increase of n-3 and n-3 HUFA and the reduction of saturated fatty acids in muscle contributing to the production of a functional food. Even more, CLA inclusion at 2% in European sea bass diets reduced fat deposition in the perivisceral cavity.

Chapter 4

STUDY II: Effect of a range of dietary conjugated linoleic acid (CLA) levels on lipid metabolism and immune system in European sea bass (*Dicentrarchus labrax*)

Abstract

In the present study, the effects of dietary doses of conjugated linoleic acid (CLA) on growth performance, digestibility, lipid metabolism enzymes, liver morphology and immune parameters were assayed. Seventy hundred and fifty European sea bass (39.98±0.47 g) were randomly allocated in 15 indoor 500 l fibreglass tanks (4kg/m^3) and fed five diets containing graded amounts of CLA (0, 0.5, 1, 2 and 4%) for 12 weeks. CLA dietary inclusion did not affect growth performance and dietary nutrients digestibility. Muscle lipid and protein content were significantly reduced and increased, respectively, when fish were fed higher levels of CLA. CLA isomers were accumulated gradually depending on the CLA treatment in all analysed tissues. G6PD, ME and L3HOAD enzymes activities were not affected by dietary CLA, however ACO activity was increased in fish fed 2 and 4% of CLA level of inclusion. Dietary CLA up to 2% reduced hepatocyte area and minimum length. Finally, phagocytic activity of head kidney leukocytes was increased in fish fed diet with 4% of CLA inclusion. We can conclude that CLA tends to reduce perivisceral fat and muscle lipids, being 2% of CLA level of inclusion the optimal level to promote liver lipid mobilization by increasing lipolysis, and only 4% of CLA led to an improvement in immune system.

Keywords:
Cell morphology
Conjugated linoleic acid
Dicentrarchus labrax
Digestibility
Enzymes
Leukocytes
Lipids

4.1. Introduction

Increase in perivisceral and hepatic fat deposition in fish has been associated to the use of plant lipid sources or the use of so-called high energy diets (Tocher, 2003; Caballero *et al.*, 2004), particularly in certain species such as European sea bass (*Dicentrarchus labrax*) (Izquierdo *et al.*, 2005). Inclusion of conjugated linoleic acid (CLA) in European sea bass diets reduces perivisceral fat deposition (Makol *et al.*, 2009). CLA is a term used to describe a group of positional and geometric isomers of linoleic acid (18:2n−6) containing double bonds, which can be found for instance in dairy products or beef (Pariza *et al.*, 2001). The two main isomers, *cis*-9, *trans*-11 (*c*9, *t*11) and *trans*-10, *cis*-12 (*t*10, *c*12), are considered the biologically-active CLA isomers. The *trans*-10, *cis*-12 isomer is known to be responsible for body fat reduction in mammals and the *cis*-9, *trans*-11 isomer for growth performance improvement (Park and Pariza, 2007). In fish, dietary inclusion of CLA reduces total liver lipid content when included at 1% in hybrid striped bass (*Morone saxatilis* × *M. chrysops*), 1% in yellow perch (*Perca flavescens*) and 0.5-2% in yellow catfish (*Pelteobagrus fulvidraco*) (Twibell *et al.*, 2000, 2001; Tan *et al.*, 2010). However, CLA does not affect fat deposition when included at 0.5-1% in channel catfish (*Ictalurus punctatus*), 0.5-2% in rainbow trout (*Oncorhynchus mykiss*), 5% in Nile tilapia (*Oreochromis niloticus*) or 2-4% in sea bream (*Sparus aurata*) (Twibell and Wilson, 2003; Yasmin *et al.*, 2004; Figueiredo-Silva *et al.*, 2005; Bandarra *et al.*, 2006; Diez *et al.*, 2007). Accordingly, CLA may also affect lipid metabolism. Thus, a higher malic enzyme (ME, EC 1.1.1.40) activity is found in juvenile European sea bass fed 1% CLA (Makol *et al.*, 2009) and a reduced glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) activity is detected in sea bream fed 2-4% CLA (Diez *et al.*, 2007). L-3-hydroxyacyl-CoA dehydrogenase activity (L3HOAD, EC 1.1.135) and Acyl-CoA Oxidase (ACO, EC 1.3.3.6) activities were promoted when measured 6 hours post-feeding but not after 24 hours of feeding in sea bream (Diez *et al.*, 2007). On the contrary, G6PD and ME were not affected in rainbow trout and European sea bass fed 0.5-2% CLA (Figueiredo-Silva *et al.*, 2005; Valente *et al.*, 2007a).

Studies in mammals showed that, besides decreasing body fat, CLA has antioxidative, anticarcinogenic, immunomodulative and diabetes modulator

properties (Belury, 2002; Risérus *et al.*, 2003; Ramírez-Santana *et al.*, 2008). In yellow catfish, dietary administration of 0.5-2% CLA improves growth performance and reduces feed intake and FCR (Tan *et al.*, 2010), but not in other fish species when fed at the same level (Kennedy *et al.*, 2007a; Valente *et al.*, 2007b; Ramos *et al.*, 2008).

Studies regarding the effect of CLA in fish immune system are scarce, but suggest that fish fed diets with CLA show a higher disease resistance (Jiang *et al.*, 2010) and enhanced immune system (Makol *et al.*, 2009; Jiang *et al.*, 2010).

The different results found in the previous studies could be related to a dose-dependent effect of CLA, and therefore, higher levels of CLA inclusion would produce more pronounced effects on fish metabolism and immune system. Then, the objective of this study was to determine the effects of a range of CLA dietary levels (0, 0.5, 1, 2 and 4%) on European sea bass juveniles´ performance, lipid metabolism and immune system.

4.2. Materials and Methods

4.2.1. Experimental diets

Five isonitrogenous and isoenergetic diets, based in a commercial formulation (Mistral 21), were designed with graded levels of CLA (CLA0=Control, CLA05=0.5%, CLA1=1%, CLA2=2% and CLA4=4%). CLA was supplied by BASF (Tarragona, Spain) as a supplement (50:50 mixture of each isomer as LUTA-CLA60®) and was added to the extruded diets in substitution of fish oil to maintain a constant energy content among dietary treatments. Diets were formulated and manufactured by a commercial feed producer (Biomar Iberia, S.A., Spain). Ingredients and proximate composition and fatty acid profiles are shown in Tables 4.1 and 4.2.

4.2.2. Experimental conditions

4.2.2.1. Experiment I: feeding trial

European sea bass juveniles were maintained in stocking tanks and fed a commercial extruded diet (CLA0 diet) for 3 weeks (23-23.5°C) until being well adapted to the environmental conditions. Afterwards, 750 fish were randomly distributed in 15 indoor 500 l, fibreglass tanks at initial stocking density of 4 kg/m^3 (50 fish/tank),

supplied with filtered sea water (5l/min water flow) and natural photoperiod (12L:12D). Water temperature and dissolved oxygen (mean ± SD) ranged 21-23.5°C and 6.27 ± 0.13 mg/l, respectively. Fish average body weight and total length (mean ± SD) were 39.98 ± 0.47 g and 15.02 ± 0.69 cm, respectively. Fish were manually fed until apparent satiation, three times a day 6 days a week for 12 weeks. Feed utilization and somatic parameters calculated were: feed conversion ratio (FCR), as the amount of feed ingested divided by the generated biomass; specific growth rate (SGR) as: [(ln final weight – ln initial weight)/number of days] x 100; relative growth (%): [(final weight–initial weight)/initial weight)] x 100 and perivisceral fat index (PFI): (visceral fat weight/body weight)x100; hepatosomatic index (HSI): (liver weight/body weight)×100 and viscerosomatic index (VSI): (viscera weight/body weight)×100.

Table 4.1. Ingredients and proximate composition of diets with different levels of CLA (0, 0.5, 1, 2 and 4%)

	Dietary CLA treatments				
	CLA 0	**CLA05**	**CLA1**	**CLA2**	**CLA4**
Ingredients (%)					
Fish meal	41.00	41.00	41.00	41.00	41.00
Soyabean meal	25.00	25.00	25.00	25.00	25.00
Wheat	7.00	7.00	7.00	7.00	7.00
Corn gluten meal	4.50	4.50	4.50	4.50	4.50
Rapeseed meal	5.90	5.90	5.90	5.90	5.90
Vitamin and	0.75	0.75	0.75	0.75	0.75
Fish oil[1]	15.85	15.12	14.38	12.93	9.97
CLA	0	0.73	1.47	2.92	5.88
Proximate composition (%)					
Crude protein	46.75	46.64	46.14	46.51	47.23
Crude fat	23.60	23.61	24.60	25.70	23.30
Ash	7.90	7.74	7.60	7.60	7.90
Moisture	5.15	6.60	7.55	7.60	5.20
Carbohydrates	21.75	22.01	21.66	20.19	21.57

Values are expressed as dry weight. Vitamin and mineral mix and CLA oil (LUTA-CLA 60®; containing 60% CLA methyl esters as a 50:50 mixture of *c*9, *t*11 and *t*10, *c*12 isomers), BASF (Spain), fish meal and fish oil (Sopropeche, France), soyabean meal (Cargill Inc., Spain), wheat (Hermanos Dueñas, Spain), corn gluten meal (SYRAL Iberia S.A.U., Spain), rapeseed meal (Esasa, Spain) [1] CLA was included at the expense of fish oil.

Table 4.2. Fatty acid composition (% of total identified fatty acids) of experimental diets

	Dietary CLA treatments				
Fatty acids	**CLA0**	**CLA05**	**CLA1**	**CLA2**	**CLA4**
14:0	7.778	7.788	7.483	7.260	7.012
14:1n-5	0.047	0.047	0.045	0.040	0.034
15:0	0.280	0.277	0.269	0.256	0.191
15:1n-5	0.044	0.043	0.040	0.041	0.033
16:0ISO	0.579	0.579	0.561	0.572	0.500
16:0	19.956	20.033	18.971	18.496	18.247
16:1n-7	6.794	6.792	6.552	6.366	5.463
16:1n-5	0.393	0.387	0.379	0.360	0.283
16:2n-6	0.183	0.177	0.172	0.171	0.136
16:2n-4	1.078	1.086	1.055	0.989	0.849
17:0	1.031	1.039	0.967	0.912	0.876
16:3n-4	0.074	0.073	0.068	0.066	0.050
16:3n-3	0.174	0.170	0.167	0.161	0.154
16:3n-1	0.068	0.068	0.064	0.062	0.069
16:4n-3	0.889	0.902	0.781	0.683	0.502
18:0	3.143	3.172	3.265	3.104	2.940
18:1n-9	15.828	16.210	16.799	16.674	16.185
18:1n-7	2.494	2.419	2.465	2.376	2.168
18:1n-5	0.237	0.236	0.230	0.222	0.172
18:2n-9	0.044	0.050	0.054	0.047	0.044
18:2n-6	8.201	8.324	8.491	8.585	8.522
18:2n-4	0.245	0.242	0.236	0.235	0.200
18:3n-6	0.227	0.224	0.217	0.204	0.218
18:3n-4	0.101	0.087	0.084	0.090	0.078
18:3n-3	1.411	1.365	1.293	1.249	1.195
18:2 *c*9, *t*11	0.038	0.645	1.314	2.623	5.258
18:4n-3	1.553	1.510	1.367	1.172	1.289
18:2 *t*10, *c*12	0	0.627	1.308	2.598	5.201
18:4n-1	0.108	0.105	0.097	0.081	0.089
20:0	0.269	0.265	0.273	0.277	0.253
20:1n-9+n-7	3.453	3.334	3.372	3.236	2.413
20:1n-5	0.272	0.262	0.264	0.252	0.201
20:2n-9	0.055	0.053	0.054	0.054	0.048
20:2n-6	0.245	0.236	0.234	0.223	0.185
20:3n-6	0.081	0.077	0.076	0.070	0.069
20:4n-6	0.694	0.671	0.643	0.584	0.519
20:3n-3	0.067	0.065	0.063	0.056	0.055
20:4n-3	0.427	0.411	0.419	0.347	0.411
20:5n-3	8.077	7.567	7.509	7.188	6.782
22:1n-11	4.764	4.519	4.571	4.309	3.853
22:1n-9	0.342	0.341	0.337	0.319	0.305
22:4n-6	0.294	0.271	0.255	0.214	0.231
22:5n-6	0.191	0.182	0.173	0.151	0.192
22:5n-3	0.979	0.911	0.858	0.834	0.765
22:6n-3	6.792	6.158	6.107	6.189	5.754
Saturated	33.036	33.154	31.788	30.878	30.018
Monoenes	34.668	34.590	35.054	34.195	31.109
Σn-3	20.369	19.058	18.563	17.879	16.906
Σn-6	10.116	10.162	10.260	10.203	10.080
Σn-9	19.722	19.989	20.617	20.330	18.994
Σn-3 HUFA	16.342	15.112	14.956	14.614	13.767
Total CLA	0.038	1.272	2.622	5.221	10.459

CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; CLA, conjugated linoleic acid.

Growth parameters, HSI, PFI and VSI were determined at day 0, 45 and 90. All fish were fasted for 24 hours before sampling. Ten fish from the initial stock at day 0 and ten fish per tank at day 45 and 90 were sampled for liver, muscle and perivisceral fat proximate and fatty acid composition as well as for hepatic tissue morphology studies.

At day 90, liver samples were collected for lipogenic and lipolytic enzyme activities determination. Blood samples were also extracted from five fish per tank for serum collection in order to determine lysozyme and bactericidal activities. In addition head kidneys were removed from the same fish to determine phagocytic activity of head kidney leukocytes.

4.2.2. Digestibility trial

One hundred and twenty fish were randomly selected and distributed among 15 indoor 120 l fibreglass digestibility tanks. Water temperature ranged from 22.5 to 23.5°C during the trial with natural photoperiod (12L:12D). Fish were manually fed their respective diets until apparent satiation for 1 month (twice daily, 6 days a week). Faeces were collected following the faeces collection system described by Cho *et al.* (1985) modified by Robaina *et al.* (1995). Immediately after their daily collection, faecal matter was centrifuged, frozen and stored at -20°C. Pooled faeces from each group were freeze-dried prior to analysis. Apparent digestibility coefficients (ADC) were calculated using the formula reported by Maynard and Loosli (1969). Each diet was assayed by triplicate. Proximate composition of diets and faeces were used to calculate ADC for protein and lipid. Acid insoluble ash, which served as a marker of feed digestibility, was determined by the method of Atkinson *et al.* (1984).

4.2.3. Biochemical analyses

Fish tissues and diets biochemical composition were conducted following standard procedures (AOAC, 2000). Ash content was determined by combustion in a muffle furnace at 600ºC for 12h, moisture content was determined after drying at 105ºC to constant weight, crude protein by acid digestion using Kjeldahl method (Nx6.25) and crude lipid was extracted following the method of Folch *et al.* (1957).

Fatty acids from total lipids were prepared by base-catalysed transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo *et al.* (1992), being quantified by flame ionization detector (FID) and identified in comparison to external standards of CLA isomers (Sigma-Aldrich and Matreya, LLC.). All analyses were conducted by triplicate.

4.2.4. Lipogenic and β-oxidation enzyme activities.

Liver samples were homogenized in 3 volumes of ice-cold buffer (20 mM Tris–HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4) and centrifuged at 20000 g for 40 min at 4°C. Activities of G6PD and ME were assayed on the supernatant using spectrophotometric procedures following the method described by Dias *et al.* (1998). ACO was assayed in peroxisome enriched liver fractions prepared by homogenizing in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M Sucrose, 2 mM EDTA, pH 7.4). The homogenate was centrifuged at 7200 g for 10 min at 4°C and the supernatant fraction collected. The resulting pellet was washed once with 500 µl of the same buffer, centrifuged as above and the supernatant collected and combined with the first one. Combined supernatants were centrifuged at 18000 g for 30 min and the resulting pellet was suspended in 600 µl of the buffer prior to sonication bath for 30 min. After sonication, supernatants were centrifuged at 18000 g for 45 min and the supernatants collected for analyses. ACO was assayed by the spectrophotometric determination of H_2O_2 production, coupled to the oxidation of 2',7'-dichlorofluorescein diacetate (LDCF) at 502 nm. The reaction mixture contained 2.6 mM LDCF, 1 M Aminotriazole, 5 mg/ml Horseradish peroxidase type II, 5% Triton X-100, 1 M Tris-HCl pH 8.5, 15 mM Flavin adenine dinucleotide, 50 mg/ml Bovine serum albumin (BSA). The reaction was started by the addition of 1 mM Palmitoyl-CoA.

Mitochondrial preparations of liver samples were performed as described by Menoyo *et al.* (2004), centrifuged following the method of Harper and Saggerson (1975) and the activity of L3HOAD was measured according to Bradshaw and Noyes (1975) on mitochondrial isolates disrupted by sonication in a 1% Triton X-100 solution. All enzyme assays were performed in triplicate.

Soluble protein content of liver homogenates was determined on the supernatant by the method of Bradford (1976) using BSA as a standard. Care was

taken to ensure that initial rates were measured in all assays and that enzymes were stable in the buffer solution used during the time and temperature required to perform the assays (Álvarez *et al.*, 1998).

Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay temperature per minute, were expressed per mg of hepatic soluble protein (specific activity). All enzyme assays were performed by triplicate.

4.2.5. Histological studies

Liver samples were fixed with 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E) for optical examination (Martoja and Martoja-Pierson, 1970). Micrographs of liver were taken at a final magnification of 400X using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. Hepatocellular area as well as maximum and minimum length of cells taking hepatocyte nuclei as reference were measured with an analySIS® software package (Image Pro Plus®, Media Cybernetics, Silver Spring, MD, USA) using arbitrary units.

4.2.6. Blood collection and sample preparation

Blood was obtained by caudal vein puncture with a 1 ml plastic syringe. No anaesthetic was used to avoid any possible effect in blood parameters and handle time from first catch was less than 1 minute to minimize stress effects. The samples were transferred to Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at –80°C for lysozyme and bactericidal activities determination.

4.2.7. Serum analysis

Lysozyme activity in blood serum was determined by turbidimetric assay according to Anderson and Siwicki (1994) using hens' egg white lysozyme (Sigma) in Phosphate buffer solution (PBS) as a standard. Results were expressed as lysozyme units/ml. The bactericidal activity against *Vibrio anguillarum* was determined as described by Torrecillas *et al.* (2011a).

4.2.8. Head kidney leukocytes phagocytic activity

Head kidneys of 15 fish per diet were removed and leukocytes were isolated by density gradient centrifugation technique as described by Torrecillas *et al.* (2007). The final pellet with more than 92% viability was re-suspended in supplemented Minimum Essential Medium (MEM) adjusting the final concentration to 10^6 leukocytes/ml. Head kidney leukocytes solution was incubated in a ratio 1 : 1 with microparticles based on polystyrene (1 μm, Sigma, St. Louis, MO, USA) as described by Esteban and Meseguer (1997) for *Vibrio anguillarum*. Phagocytic activity was measured as described by Blazer (1991). Ten series of one hundred leukocytes per fish were counted, and the phagocytic index was determined as the percentage of leukocytes with phagocytic ability. Positive phagocytic activity was determined only for the leukocytes with presence of the microparticle of polystyrene inside the cytoplasm.

4.2.9. Statistical analyses

Means and Standard Deviations (SD) were calculated for each parameter measured. Statistical analyses followed the methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of CLA inclusion. Where data did not pass a normality test, a log transformation was used to normalize data. When *F* values showed significance, individual means were compared using Tukey's or Duncan tests for multiple means comparison. Significant differences were considered for $P<0.05$.

4.3. Results

4.3.1. Growth performance

There were no mortalities during the experiment. Feed intake, weight gain, SGR, FCR, VSI and HSI were not affected by CLA inclusion along the feeding trial (Table 4.3). Nevertheless, increase in dietary CLA was negatively correlated ($y=0.0283x^2-0.2203x+6.4325$, $R^2=0.9552$; $P=0.0019$ with the deposition of perivisceral fat (Table 4.3, Figure 4.1).

Table 4.3. Biological and growth parameters of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 and 4%) for 12 weeks

	Dietary CLA treatments				
	CLA0	**CLA05**	**CLA1**	**CLA2**	**CLA4**
Growth					
Feed intake (g)	3797.9±158.8	3823.9±262.3	3953.1±216.4	3831.6±185.1	3780.5±156.8
Initial weight (g)	40.0±0.8	39.9±0.5	40.0±0.2	40.3±0.2	39.8±0.6
Final weight (g)	104.8±3.9	106.2±1.2	104.8±6.0	104.8±3.9	102.6±2.3
Eviscerated weight (g)	90.9±8.2	91.6±16.2	91.5±8.7	90.2±15.5	86.3±14.3
Final total length (cm)	20.0±0.3	20.0±0.2	20.0±0.4	19.9±0.1	19.8±0.2
Relative growth (%)	161.9±4.8	166.1±3.9	162.1±14.0	160.2±10.9	157.9±3.8
SGR (%)	1.1±0.02	1.2±0.02	1.1±0.06	1.1±0.05	1.1±0.02
FCR	1.9±0.06	1.8±0.06	1.8±0.12	1.8±0.06	1.8±0.05
VSI (%)	14.7±2.0	14.6±2.0	14.2±1.9	14.5±1.3	14.6±3.2
HSI (%)	2.7±0.6	2.7±0.5	2.9±0.7	2.7±0.4	2.9±0.5
PFI (%)	6.5±2.0	6.3±1.4	6.2±1.2	6.1±1.2	6.0±1.5

Data are presented as means ± SD. Initial weight (n=150), final weight and length, relative growth, SGR and FCR (n=90), food intake (n=150). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA, conjugated linoleic acid; FCR, feed conversion ratio; HSI, Hepatosomatic index; SGR, specific growth rate; VSI, Viscerosomatic index; CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively.

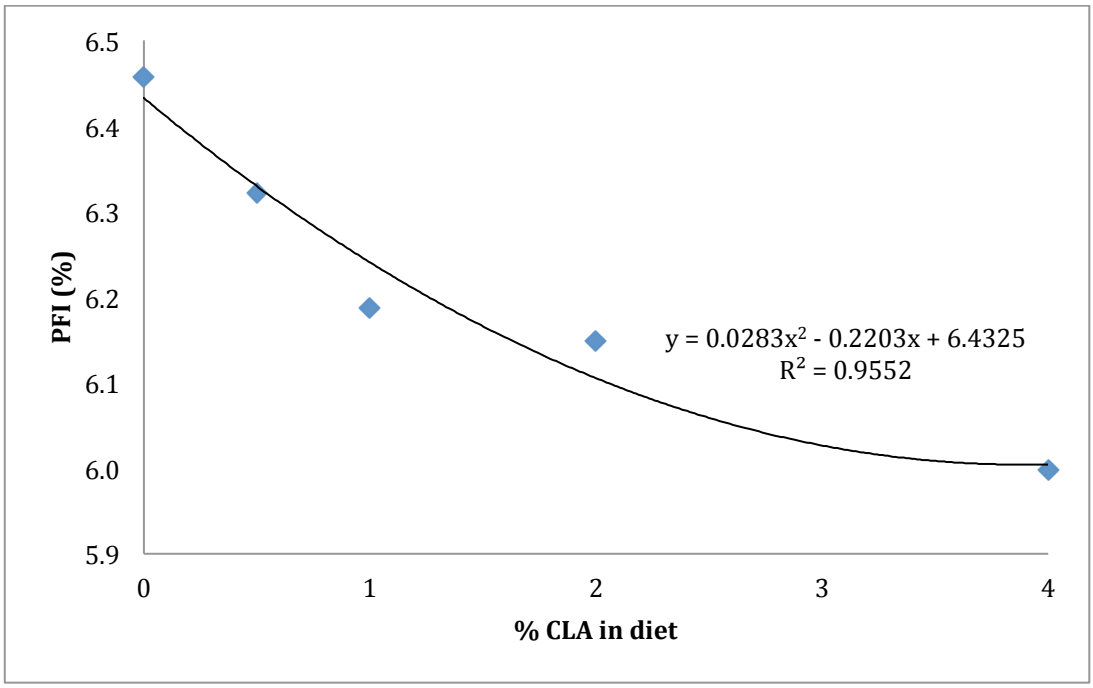


Figure 4.1. Negative correlation between level of CLA and PFI index.

4.3.2. Muscle and liver composition

Increase in dietary CLA markedly reduced lipids and increased protein contents in European sea bass muscle (Table 4.4). Thus, muscle of fish fed CLA4 diet showed the lowest lipid contents (P<0.05), followed by that of fish fed CLA2 diet, which in turn was significantly lower than in fish fed CLA0 and CLA05 diets. Besides, protein content of muscle in fish fed CLA2 and CLA4 diets was higher (P<0.05) than that of fish fed CLA0 and CLA05 diets. Liver lipid content was not affected by CLA inclusion, instead total liver protein content of fish fed CLA2 diet decreased (P<0.05) compared to fish fed CLA0 and CLA05 diets (Table 4.4).

4.3.3. Muscle, liver and perivisceral fatty acid profile

After 12 weeks of CLA supplementation, fish muscle fatty acids profiles showed that, despite fatty acids group contents (saturated, monounsaturated, n-3, n-6, n-9 and n-3 HUFA) were similar (Table 4.5), increase in dietary CLA reduced (P<0.05) the content of 14:0, 16:2n-6, 16:3n-1, 18:2n-9, 18:3n-6 and 22:1n-9 in fish. CLA isomers deposition was higher (P<0.05) among diets as dietary CLA inclusion level increased (Table 4.5) with no difference in deposition ratio among isomers. Besides, CLA deposition was positively correlated ($y=-0.071x^2+1.5997x-0.0608$, $R^2=0.9965$, P=0.0001) with the level of CLA included in the diet (Figure 4.2).

Table 4.4. Muscle and liver biochemical composition of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

		Dietary CLA treatments				
		CLA0	**CLA05**	**CLA1**	**CLA2**	**CLA4**
Muscle	**Lipids (%)**	24.86±1.94^{a}	27.23±4.35^{a}	23.78±1.30^{ab}	20.69±1.98^{b}	19.83±0.36^{c}
	Protein (%)	68.87±1.88^{a}	69.37±3.12^{a}	72.26±2.06^{ab}	74.62±0.71^{b}	74.96±3.32^{b}
Liver	**Lipids (%)**	66.24±4.32	66.37±1.06	62.94±4.08	67.15±2.11	63.55±3.57
	Protein (%)	13.12±0.63^{a}	13.35±1.57^{a}	11.52±1.54^{ab}	10.84±0.65^{b}	11.19±1.07^{ab}

Data are presented as means ± SD (n=12). Values are expressed as dry weight. Significance of differences between means was determined by one-way ANOVA followed by Duncan´s multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05).

In liver, feeding fish with CLA diets increased the contents of total saturated fatty acids, being higher (P<0.05) in fish fed CLA4 diet, mainly due to the increase in 18:0 (Table 4.6). Fish fed CLA diets also had lower (P<0.05) levels of 16:3n-3, 18:2n-9,

18:3n-6, 20:2n-9 and 20:4n-6 than fish fed CLA0 diet. Besides, the monounsaturated and the n-9 fatty acids were reduced (P<0.05) by feeding CLA1, CLA2 and CLA4 diets (mainly due to reductions in 16:1n-7, 16:1n-5, 18:1n-9 and 18:1n-5). The deposition of CLA isomers was higher (P<0.05) as dietary CLA inclusion level increased (Table 4.6). In addition, there was some selectivity on the individual isomer deposition, being the isomer *cis*-9, *trans*-11 more efficiently incorporated in liver than the *trans*-10, *cis*-12 isomer. Furthermore, CLA deposition was positively correlated ($y=-0.0751x^2+1.1884x-0.009$, $R^2=0.9991$, P=0.0001) with the level of CLA included in the diet (Figure 4.3).

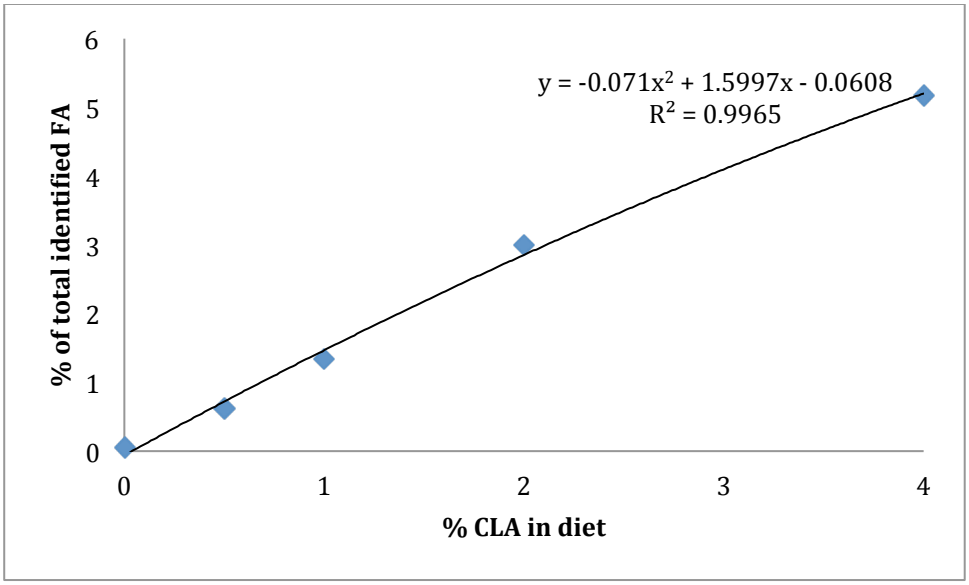


Figure 4.2. Positive correlation between level of CLA in the diet and the CLA deposition in muscle.

When compared to fish fed CLA0 diet, perivisceral fat fatty acids profiles of fish fed CLA4 diet presented reduced (P<0.05) values of monounsaturated fatty acids (particularly 16:1n-7, 18:1n-7, 20:1n-9+n7 and 20:1n-5) and 17:0, 18:3n-4, 18:4n-1 and 20:2n-9. Total n-6 fatty acids contents were reduced (P<0.05) in CLA fed fish independently of the dose supplemented (particularly 18:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6 in fish fed CLA4; 18:2n-6, 18:3n-6 and 20:3n-6 in fish fed CLA2; 18:2n-6, 18:3n-6 and 20:2n-6 in fish fed CLA1 and CLA05 diets. In fish fed diets CLA1, CLA2 and

CLA4, 18:0 content was increased (P<0.05) (Table 4.7). Even though total n-3 fatty acids values were similar between CLA0 diet and the different levels of CLA inclusion, fish fed CLA diets presented reduced (P<0.05) contents of 16:3n-3, 18:3n-3 and 20:3n-3. Deposition of CLA isomers was higher (P<0.05) as dietary CLA inclusion level increased (Table 4.7). CLA deposition was positively correlated ($y=-0.0244x^2+1.6515x-0.0254$, $R^2=0.9973$, P=0.0001) with the level of CLA included in the diet (Figure 4.4).

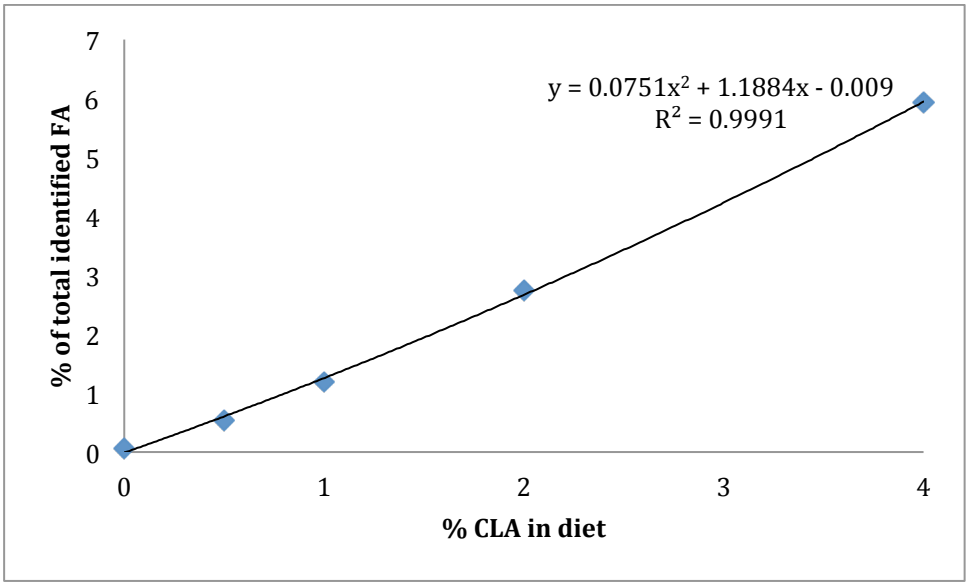


Figure 4.3. Positive correlation between level of CLA in the diet and the CLA deposition in liver.

4.3.4. Digestibility trial

Visual inspection of faeces daily collected confirmed that there were no signs of contamination from uneated feed. ADC (%) of lipids and protein ranged from 80.60 to 87.51 and from 79.49 to 85.55, respectively. No significant differences were found in protein and lipid digestibility coefficients among the experimental diets.

4.3.5. Lipogenic and β-oxidation enzyme activities

Despite liver G6PD and ME activities did not significantly differ among fish fed different CLA contents (Table 4.8), G6PD tend to be higher in fish fed CLA diets. Besides, liver ACO activity showed a positive correlation between enzyme activity and CLA inclusion levels ($y=-0.7185x^2+4.5182x+26.58$; $R^2=0.9036$, P=0.0181) being higher

(P<0.05) in fish fed diets CLA2 and CLA4 when compared to CLA0 and CLA05 diets (Table 4.8, Figure 4.5). Hepatic L3HOAD activity was not significantly affected by CLA inclusion.

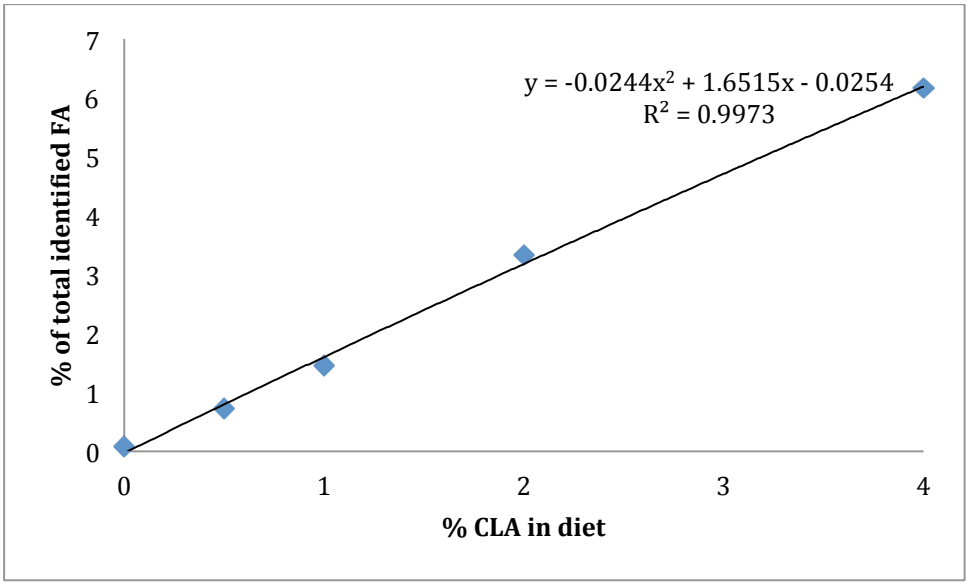


Figure 4.4. Positive correlation between level of CLA in the diet and the CLA deposition in perivisceral fat.

4.3.6. Liver morphology

Morphological analyses of fish liver resulted qualitatively in a regular-shaped morphology of the hepatocytes around the sinusoidal spaces and a reduction on the lipid vacuolization of the cytoplasm that reduced the number of hepatocytes with the nuclei displaced to the cellular periphery.

Quantitative morphometric analyses of liver showed that fish fed CLA05, CLA1 and CLA2 diets had smaller (P<0.05) hepatocellular area than fish fed CLA0 diet (Table 4.9, Figure 4.6). Besides, fish fed CLA05 and CLA2 diets also had smaller (P<0.05) area than fish fed CLA4 diet.

Maximum length was shorter (P<0.05) in fish fed diets CLA05, CLA1 than in fish fed diet CLA0 and between fish fed diet CLA4 and fish fed other CLA diets. Regarding minimum length, fish fed diets CLA05, CLA1 and CLA2 showed shorter (P<0.05) length than fish fed diets CLA0 and CLA4.

Table 4.5. Muscle fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

	Dietary CLA treatments				
Fatty Acids	**CLA0**	**CLA05**	**CLA1**	**CLA2**	**CLA4**
14:0	3.873±0.097^{a}	3.83±0.136^{ab}	3.667±0.123^{ab}	3.674±0.294^{ab}	3.406±0.064^{b}
14:1n-7	0.108±0.006	0.105±0.013	0.104±0.005	0.113±0.012	0.09±0.009
14:1n-5	0.029±0.001	0.027±0.005	0.027±0.003	0.029±0.004	0.023±0.001
15:0	0.352±0.019	0.351±0.033	0.333±0.023	0.327±0.017	0.3±0.019
15:1n-5	0.019±0.001	0.019±0.003	0.012±0.011	0.014±0.013	0.019±0
16:0ISO	0.057±0.004	0.054±0.006	0.054±0.002	0.058±0.006	0.05±0.006
16:0	23.527±4.036	24.793±5.99	23.318±0.615	19.791±1.113	21.459±4.356
16:1n-7	4.752±0.209	4.598±0.548	4.474±0.1	4.697±0.359	3.992±0.51
16:1n-5	0.149±0.007	0.148±0.02	0.141±0.002	0.15±0.015	0.146±0.045
16:2n-6	0.23±0.003^{a}	0.231±0.003^{a}	0.216±0.002^{b}	0.217±0.013^{abc}	0.187±0.004^{c}
16:2n-4	0.077±0.002^{a}	0.092±0.038^{ab}	0.084±0.029^{ab}	0.109±0.040^{b}	0.08±0.036^{ab}
17:0	0.734±0.046	0.717±0.103	0.694±0.012	0.751±0.058	0.609±0.039
16:3n-4	0.447±0.043	0.461±0.064	0.438±0.014	0.406±0.024	0.446±0.069
16:3n-3	0.655±0.043	0.628±0.072	0.611±0.006	0.628±0.034	0.382±0.251
16:3n-1	0.108±0.005^{a}	0.104±0.008^{ab}	0.094±0.015^{abc}	0.077±0.008^{b}	0.048±0.013^{c}
16:4n-3	0.424±0.031	0.399±0.036	0.365±0.003	0.359±0.035	0.359±0.044
18:0	5.715±1.101	6.167±1.606	6.788±0.277	6.088±0.341	7.829±2.219
18:1n-9	18.01±0.858	17.409±2.1	18.053±0.232	19.367±1.094	17.358±2.265
18:1n-7	2.178±0.185	2.11±0.284	2.11±0.031	2.276±0.255	1.985±0.184
18:1n-5	0.232±0.012	0.23±0.033	0.225±0.005	0.243±0.02	0.189±0.026
18:2n-9	0.275±0.012^{a}	0.232±0.017^{ab}	0.206±0.009^{bc}	0.212±0.005^{b}	0.161±0.030^{c}
18:2n-6	7.306±0.642	6.424±0.751	6.702±0.132	7.49±0.697	6.994±0.731
18:2n-4	0.239±0.016	0.231±0.024	0.233±0.005	0.249±0.013	0.208±0.014
18:3n-6	0.188±0.014^{a}	0.165±0.018^{ab}	0.163±0.005^{ab}	0.178±0.002^{ab}	0.151±0.014^{b}
18:3n-4	0.104±0.005	0.096±0.009	0.091±0.008	0.098±0.007	0.085±0.008
18:3n-3	1.513±0.102	1.327±0.142	1.309±0.027	1.376±0.08	1.287±0.105
18:2 *c*9, *t*11	0.039±0.008^{a}	0.336±0.057^{b}	0.722±0.036^{c}	1.567±0.045^{d}	2.642±0.282^{e}
18:4n-3	1.101±0.063	1.072±0.103	1.027±0.023	0.977±0.118	0.925±0.122
18:2 *t*10, *c*12	0.015±0.014^{a}	0.283±0.051^{b}	0.618±0.038^{c}	1.429±0.053^{d}	2.534±0.264^{e}
18:4n-1	0.111±0.006	0.109±0.011	0.103±0.009	0.09±0.012	0.091±0.001
20:0	0.212±0.06	0.221±0.078	0.203±0.011	0.151±0.011	0.208±0.072
20:1n-9+n-7	2.528±0.134	2.437±0.377	2.408±0.036	2.62±0.122	2.33±0.297
20:1n-5	0.189±0.01	0.183±0.026	0.18±0.004	0.208±0.028	0.169±0.02
20:2n-9	0.054±0.002	0.049±0.007	0.046±0.001	0.041±0.009	0.042±0.004
20:2n-6	0.498±0.039	0.45±0.048	0.448±0.028	0.425±0.084	0.452±0.049
20:3n-9	0.038±0.004	0.036±0.003	0.034±0.002	0.044±0.014	0.033±0.003
20:3n-6	0.084±0.006	0.078±0.01	0.079±0.004	0.085±0.011	0.07±0.008
20:4n-6	0.708±0.076	0.744±0.156	0.716±0.019	0.803±0.062	0.706±0.063
20:3n-3	0.074±0.006	0.068±0.008	0.069±0.001	0.069±0.004	0.063±0.008
20:4n-3	0.463±0.052	0.443±0.048	0.463±0.014	0.457±0.06	0.494±0.038
20:5n-3	8.557±0.844	8.369±0.899	8.181±0.329	8.529±0.514	7.929±0.62
22:1n-11	2.076±0.093	2.161±0.504	1.994±0.203	2.243±0.416	1.746±0.316
22:1n-9	0.069±0.000^{a}	0.068±0.003^{ab}	0.064±0.004^{ab}	0.066±0.007^{ab}	0.058±0.003^{b}
22:4n-6	0.364±0.027^{ab}	0.386±0.019^{a}	0.348±0.018ab	0.385±0.014^{a}	0.325±0.023^{b}
22:5n-6	0.226±0.029	0.228±0.036	0.226±0.009	0.242±0.011	0.223±0.022
22:5n-3	1.505±0.187	1.422±0.128	1.46±0.12	1.505±0.076	1.379±0.09
22:6n-3	9.788±1.642	9.915±1.441	9.7±0.796	10.732±0.524	9.669±0.974
Saturated	34.47±5.189	36.133±7.674	35.058±0.941	30.84±1.416	33.863±6.563
Monoenes	30.341±1.5	29.493±3.891	29.792±0.474	32.027±2.3	28.1±3.678
Σn-3	24.08±2.959	23.641±2.678	23.185±1.289	24.63±1.229	22.488±1.771
Σn-6	9.603±0.82	8.704±0.988	8.897±0.071	9.824±0.702	9.284±1.167
Σn-9	20.974±0.992	20.23±2.501	20.812±0.253	22.351±1.229	19.981±2.578
Σn-3 HUFA	20.388±2.727	20.217±2.442	19.872±1.256	21.291±1.077	19.535±1.68
Total CLA	0.055±0.022^{a}	0.618±0.109^{b}	1.34±0.072^{c}	2.996±0.098^{d}	5.177±0.543^{e}

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 4.6. Liver fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

	Dietary CLA treatments				
Fatty acids	**CLA0**	**CLA05**	**CLA1**	**CLA2**	**CLA4**
14:0	1.916±0.056	1.99±0.081	2.152±0.088	2.381±0.214	2.246±0.149
14:1n-7	0.058±0.004	0.053±0.002	0.061±0.001	0.066±0.001	0.062±0.001
14:1n-5	0.014±0.019	0.013±0.019	0.009±0.015	0.017±0.024	0.00±0.00
15:0	0.147±0.006	0.158±0.001	0.164±0.017	0.158±0.004	0.134±0.009
16:0ISO	0.054±0.001	0.049±0.003	0.051±0.002	0.051±0.003	0.048±0.001
16:0	19.984±0.444	24.34±3.144	23.833±0.502	22.498±0.861	20.085±1.79
16:1n-7	5.218±0.104	4.595±0.409^{ab}	3.952±0.520^{ab}	3.76±0.112^{ab}	2.785±0.212^{b}
16:1n-5	0.107±0.000^{a}	0.105±0.003^{a}	0.091±0.003^{bc}	0.088±0.001^{c}	0.068±0.004^{d}
16:2n-6	0.237±0.001	0.221±0.009	0.221±0.013	0.209±0.011	0.182±0.003
16:2n-4	0.182±0.004	0.209±0.02	0.196±0.009	0.2±0.012	0.171±0.014
17:0	0.536±0.013	0.484±0.02	0.481±0.009	0.501±0.015	0.462±0.017
16:3n-4	0.37±0.008	0.408±0.024	0.449±0.032	0.444±0.014	0.455±0.056
16:3n-3	0.552±0.017^{a}	0.493±0.026^{ab}	0.462±0.015^{b}	0.451±0.017^{b}	0.381±0.011^{b}
16:3n-1	0.106±0.004	0.08±0.022	0.094±0.004	0.089±0.003	0.085±0.002
16:4n-3	0.113±0.001	0.114±0.004	0.115±0.009	0.119±0.009	0.1±0.011
18:0	5.259±0.214^{a}	7.097±0.604^{b}	9.246±0.385^{c}	9.758±0.165^{c}	13.679±0.185^{d}
18:1n-9	36.272±1.178^{a}	32.162±1.972^{ab}	30.405±1.155^{b}	30.469±0.981^{ab}	28.282±0.823^{b}
18:1n-7	2.492±0.163	2.302±0.292	2.074±0.202	1.893±0.09	1.881±0.142
18:1n-5	0.328±0.004^{a}	0.291±0.024^{ab}	0.279±0.010^{ab}	0.268±0.009^{ab}	0.244±0.002^{b}
18:2n-9	0.709±0.032^{a}	0.438±0.033^{b}	0.335±0.027^{b}	0.318±0.006^{b}	0.282±0.010^{b}
18:2n-6	4.416±0.383	3.86±0.309	4.069±0.261	4.247±0.083	4.222±0.219
18:2n-4	0.226±0.012	0.21±0.025	0.209±0.021	0.198±0.005	0.18±0.008
18:3n-6	0.274±0.040^{a}	0.16±0.007^{b}	0.144±0.001^{b}	0.151±0.000^{b}	0.129±0.002^{b}
18:3n-4	0.094±0.002	0.087±0.011	0.084±0.007	0.081±0.006	0.071±0.004
18:3n-3	0.744±0.038	0.7±0.078	0.713±0.054	0.684±0.004	0.614±0.037
18:2 *c*9, *t*11	0.060±0.005^{a}	0.345±0.011^{b}	0.714±0.022^{c}	1.567±0.079^{d}	3.151±0.030^{e}
18:4n-3	0.666±0.010^{a}	0.668±0.031^{a}	0.648±0.014^{a}	0.622±0.030^{ab}	0.519±0.017^{b}
18:2 *t*10, *c*12	0±0.000^{a}	0.19±0.010^{b}	0.477±0.010^{c}	1.178±0.048^{d}	2.782±0.031^{e}
18:4n-1	0.065±0.001	0.071±0.008	0.066±0.007	0.054±0.005	0.054±0.003
20:0	0.14±0.022	0.152±0.043	0.178±0.006	0.16±0.014	0.164±0.026
20:1n-9+n-7	2.641±0.129	2.274±0.14	2.332±0.05	2.459±0.072	2.447±0.104
20:1n-5	0.164±0.008	0.141±0.007	0.146±0.003	0.151±0.008	0.157±0.005
20:2n-9	0.061±0.002a	0.041±0.003b	0.037±0.002b	0.035±0.001b	0.03±0.002b
20:2n-6	0.402±0.034	0.356±0.06	0.374±0.036	0.387±0.006	0.397±0.01
20:3n-9	0.033±0	0.031±0.004	0.031±0.003	0.028±0.002	0.027±0.001
20:3n-6	0.066±0.007	0.053±0.002	0.05±0.004	0.047±0	0.044±0.004
20:4n-6	0.451±0.001^{a}	0.399±0.003^{b}	0.367±0.002^{c}	0.409±0.005^{b}	0.399±0.004^{b}
20:3n-3	0.059±0.003	0.055±0.007	0.058±0.006	0.055±0.002	0.051±0.002
20:4n-3	0.309±0.021	0.322±0.064	0.264±0.02	0.346±0.001	0.39±0.01
20:5n-3	5.075±0.306	4.82±0.296	4.786±0.196	4.662±0.027	4.289±0.159
22:1n-11	1.455±0.091	1.354±0.104	1.394±0.149	1.392±0.015	1.305±0.027
22:1n-9	0.04±0.003	0.163±0.002	0.126±0.075	0.173±0.014	0.036±0.073
22:4n-6	0.265±0.031	0.291±0.072	0.286±0.046	0.246±0.017	0.219±0.011
22:5n-6	0.145±0.005	0.139±0.018	0.142±0.012	0.13±0.004	0.128±0.003
22:5n-3	1.058±0.079	1.056±0.18	1.103±0.171	0.968±0.017	0.886±0.014
22:6n-3	6.438±0.123	6.457±0.67	6.533±0.461	5.834±0.097	5.678±0.213
Saturated	28.036±0.605^{a}	34.27±3.851^{ab}	33.953±0.397^{ab}	33.125±1.026^{ab}	36.818±0.243^{b}
Monoenes	48.789±0.860^{a}	43.454±1.945^{ab}	40.869±1.480^{b}	40.735±1.025^{b}	37.11±2.137^{b}
Σn-3	15.013±0.596	14.571±1.351	14.683±0.888	13.740±0.051	12.907±0.064
Σn-6	6.040±0.804	5.480±0.480	5.652±0.371	5.826±0.125	5.720±0.386
Σn-9	39.756±1.011^{a}	35.11±2.149^{ab}	33.266±1.057^{b}	33.454±1.063^{ab}	31.103±2.540^{b}
Σn-3 HUFA	12.938±0.532	12.71±1.216	12.744±0.809	11.864±0.103	11.294±0.065
Total CLA	0.060±0.005^{a}	0.535±0.021^{b}	1.191±0.032^{c}	2.746±0.127^{d}	5.932±0.085^{e}

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 4.7. Perivisceral fat fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

	Dietary CLA treatments				
Fatty acids	**CLA0**	**CLA05**	**CLA1**	**CLA2**	**CLA4**
14:0	4.568±0.054^{ab}	4.808±0.165^{a}	4.544±0.219^{ab}	4.545±0.059^{ab}	4.24±0.070^{b}
14:1n-7	0.132±0.008	0.197±0.10	0.130±0.006	0.136±0.001	0.118±0.004
14:1n-5	0.034±0.003	0.072±0.062	0.034±0.002	0.036±0.001	0.03±0.001
15:0	0.342±0.009	0.405±0.101	0.334±0.016	0.338±0.006	0.306±0.008
15:1n-5	0.016±0.014	0.058±0.079	0.015±0.013	0.012±0.017	0.000±0.000
16:0ISO	0.066±0.002	0.123±0.095	0.066±0.002	0.067±0.001	0.06±0.001
16:0	16.779±0.285	17.432±0.497	17.72±0.598	17.537±1.222	16.72±0.142
16:1n-7	6.078±0.087^{ab}	6.156±0.070^{a}	5.85±0.081^{bc}	5.593±0.095^{c}	5.136±0.129^{d}
16:1n-5	0.217±0.011	0.254±0.052	0.214±0.003	0.222±0.006	0.195±0.003
16:2n-6	0.099±0.005	0.107±0.017	0.089±0.003	0.086±0.009	0.081±0.007
16:2n-4	0.143±0.011	0.165±0.043	0.137±0.002	0.141±0.006	0.124±0.002
17:0	0.913±0.015^{ab}	0.94±0.023^{a}	0.874±0.027^{ab}	0.853±0.029^{b}	0.797±0.022^{c}
16:3n-4	0.362±0.021	0.385±0.041	0.366±0.008	0.374±0.007	0.348±0.01
16:3n-3	0.863±0.022^{a}	0.89±0.007^{a}	0.821±0.029^{ab}	0.77±0.047^{b}	0.761±0.021^{b}
16:3n-1	0.044±0.003	0.047±0.005	0.041±0.003	0.035±0.02	0.069±0.002
16:4n-3	0.602±0.055	0.638±0.052	0.557±0.046	0.495±0.076	0.564±0.018
18:0	3.581±0.081^{a}	3.738±0.245^{ac}	4.284±0.230^{b}	4.596±0.350^{b}	5.163±0.195^{c}
18:1n-9	21.368±0.386	20.922±0.99	21.319±0.945	20.511±1.065	20.326±0.612
18:1n-7	2.557±0.096a	2.548±0.061a	2.532±0.087a	2.432±0.029ab	2.244±0.053b
18:1n-5	0.227±0.007	0.233±0.004	0.235±0.021	0.255±0.001	0.224±0.005
18:2n-9	0.246±0.007	0.220±0.032	0.214±0.04	0.227±0.017	0.199±0.015
18:2n-6	10.049±0.530^{a}	8.497±0.189^{b}	8.715±0.285^{b}	8.592±0.451^{b}	8.935±0.213^{b}
18:2n-4	0.274±0.012	0.271±0.007	0.274±0.017	0.284±0.013	0.264±0.015
18:3n-6	0.267±0.023^{a}	0.216±0.008^{b}	0.211±0.011^{b}	0.211±0.005^{b}	0.194±0.010^{b}
18:3n-4	0.046±0.002^{a}	0.043±0.001^{ab}	0.043±0.001^{ab}	0.042±0.001^{ab}	0.035±0.007^{b}
18:3n-3	2.3±0.170^{a}	2.009±0.103^{ab}	1.968±0.089^{b}	1.766±0.012^{b}	1.868±0.064^{b}
18:2 *c*9,*t*11	0.055±0.014^{a}	0.384±0.062^{b}	0.767±0.069^{c}	1.739±0.096^{d}	3.172±0.329^{e}
18:4n-3	1.328±0.08	1.423±0.143	1.302±0.099	1.236±0.137	1.159±0.006
18:2 *t*10,*c*12	0.027±0.024^{a}	0.335±0.065^{b}	0.679±0.066^{c}	1.589±0.123^{d}	2.991±0.372^{e}
18:4n-1	0.151±0.019^{a}	0.158±0.005^{ab}	0.151±0.005^{ab}	0.121±0.013^{ab}	0.038±0.066^{b}
20:0	0.159±0.015	0.152±0.023	0.147±0.009	0.169±0.041	0.144±0.007
20:1n-9+n-7	3.204±0.085^{a}	3.143±0.096^{a}	3.12±0.021a	3.192±0.001a	2.873±0.074b
20:1n-5	0.25±0.010^{a}	0.247±0.010^{ab}	0.245±0.009^{ab}	0.241±0.001^{ab}	0.221±0.008^{b}
20:2n-9	0.075±0.005^{a}	0.07±0.004^{ab}	0.068±0.002^{ab}	0.061±0.000^{b}	0.058±0.003^{c}
20:2n-6	0.611±0.025^{a}	0.542±0.019^{b}	0.54±0.033^{b}	0.569±0.022^{ab}	0.536±0.024^{b}
20:3n-9	0.042±0.001	0.041±0.004	0.042±0.003	0.038±0.007	0.039±0.001
20:3n--6	0.104±0.005^{a}	0.095±0.002^{ab}	0.094±0.000^{ab}	0.087±0.003^{b}	0.084±0.004^{c}
20:4n-6	0.613±0.011^{ab}	0.62±0.021^{a}	0.598±0.024^{ab}	0.568±0.036^{ab}	0.549±0.015^{b}
20:3n-3	0.09±0.001^{a}	0.083±0.002^{b}	0.084±0.000^{b}	0.082±0.002^{b}	0.074±0.001^{c}
20:4n-3	0.568±0.016	0.565±0.02	0.572±0.015	0.573±0.046	0.596±0.017
20:5n-3	8.833±0.486	9.062±0.66	8.603±0.51	8.111±1.05	8.109±0.122
22:1n-11	2.699±0.275	2.831±0.271	2.669±0.093	2.738±0.038	2.285±0.115
22:1n-9	0.072±0.006	0.074±0.006	0.071±0.003	0.073±0.003	0.062±0.004
22:4n-6	0.397±0.025	0.398±0.027	0.385±0.018	0.361±0.05	0.356±0.005
22:5n-6	0.191±0.013	0.188±0.02	0.185±0.005	0.185±0.025	0.17±0.002
22:5n-3	1.465±0.087	1.417±0.125	1.441±0.049	1.361±0.202	1.29±0.021
22:6n-3	6.892±0.762	6.800±0.997	6.650±0.253	6.751±1.277	6.193±0.124
Saturated	26.408±0.424	27.598±1.09	27.969±0.696	28.105±1.648	27.43±0.102
Monoenes	36.855±0.287^{a}	36.734±1.091^{a}	36.433±0.856^{a}	35.44±1.153^{ab}	33.714±0.459
Σn-3	22.942±1.278	22.248±1.959	21.999±0.902	21.144±2.848	20.612±0.1
Σn-6	12.331±0.492^{a}	10.662±0.211^{b}	10.816±0.348^{b}	10.659±0.301^{b}	10.905±0.200
Σn-9	25.006±0.333	24.47±0.958	24.833±0.987	24.103±1.079	23.499±0.546
Σn-3 HUFA	17.849±1.299	17.926±1.791	17.351±0.641	16.877±2.575	16.261±0.021
Total CLA	0.082±0.038^{a}	0.719±0.127^{b}	1.447±0.134^{c}	3.329±0.219^{d}	6.164±0.700^{e}

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 4.8. Lipogenic and lipolytic enzymatic activities in liver of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

	Dietary CLA treatments				
	CLA0	**CLA05**	**CLA1**	**CLA2**	**CLA4**
G6PD					
IU / mg protein	0.181±0.01	0.189±0.02	0.226±0.01	0.216±0.02	0.195±0.01
ME					
IU / mg protein	0.061±0.01	0.067±0.01	0.071±0.00	0.054±0.01	0.061±0.00
L3HOAD					
mIU / mg protein	49.39±14.00	50.71±7.02	60.73±5.98	57.69±11.23	50.28±10.91
ACO					
mIU / mg protein	27.35±0.59^{a}	27.15±0.90^{a}	30.96±1.79^{ab}	32.97±1.43^{b}	33.09±2.66^{b}

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively; G6PD, glucose-6-phophate dehydrogenase (EC 1.1.1.49); ME, malic enzyme (EC 1.1.1.40); L3HOAD, L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.135); ACO, acyl-CoA oxidase (EC 1.3.3.6); IU, enzyme activity units defined as micromoles of substrate converted to product at assay temperature per minute expressed per mg of soluble protein.

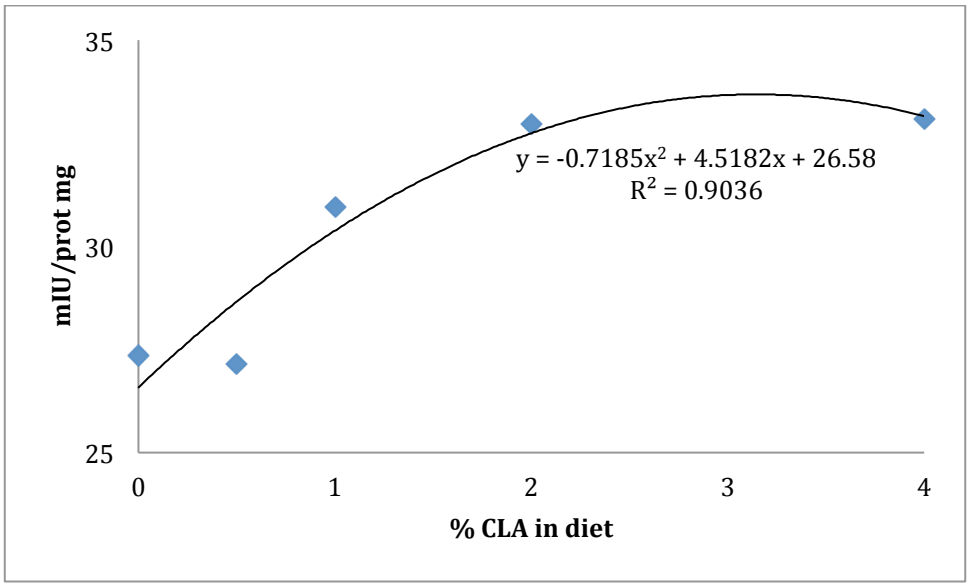


Figure 4.5. Positive correlation between level of CLA and ACO activity.

4.3.7. Immune parameters

Phagocytic activity of head kidney leukocytes increased (P<0.05) in fish fed diet CLA4 when compared to fish fed CLA0 and CLA05 diets (Table 4.10). Bactericidal and lysozyme activities were not affected by the inclusion of CLA in fish diets.

Table 4.9. Quantitative image analysis (arbitrary units) of hepatocytes from European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

	Dietary CLA treatments				
	CLA0	**CLA 05**	**CLA1**	**CLA2**	**CLA4**
Area	3.528±0.176^{a}	3.012±0.179^{b}	3.065±0.102^{bc}	2.778±0.181^{b}	3.311±0.176^{ac}
Maximum length	0.718±0.128^{ac}	0.675±0.166^{b}	0.674±0.171^{b}	0.700±0.204^{ab}	0.744±0.189^{c}
Minimum length	0.463±0.127^{a}	0.397±0.106^{b}	0.384±0.126^{b}	0.411±0.112^{b}	0.478±0.146^{a}

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05, CLA1, CLA2 and CLA4, diets with 0.5%, 1%, 2% and 4% of CLA inclusion, respectively.

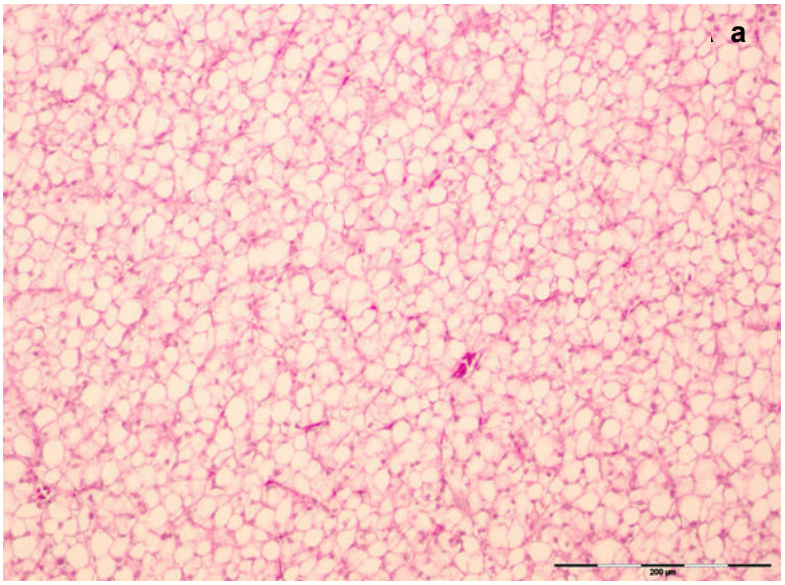
Table 4.10. Immune parameters of European sea bass fed graded dietary levels of CLA (0, 0.5, 1, 2 or 4%) for 12 weeks

	Dietary CLA treatments				
	CLA0	**CLA 05**	**CLA1**	**CLA2**	**CLA4**
Lysozyme (Units/ mg prot)	12.396±1.605	10.549±1.711	11.399±1.301	9.584±1.686	10.468±0.698
Phagocytic index (%)	24.7±4.6^{a}	22.7±4.1^{a}	26.0±3.8^{ab}	33.2±3.2^{ab}	37.3±6.4^{b}
Bactericidal activity (ΔAbs / min)	0.0155±0.005	0.0161±0.005	0.0135±0.001	0.0181±0.001	0.014±0.003

Data are presented as means ± SD (n=12). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05).

4.4. Discussion

Even the highest level of dietary CLA inclusion did not negatively affected growth performance in agreement with previous studies in the same (Valente *et al.*, 2007a; Makol *et al.*, 2009) and other fish species (Choi *et al.*, 1999; Twibell *et al.*, 2001; Twibell and Wilson, 2003; Berge *et al.*, 2004; Kennedy *et al.*, 2005, 2007a; Manning *et al.*, 2006; Valente *et al.*, 2007b; dos Santos *et al.*, 2011). Apparent digestibility coefficients (ADC) of dietary components were not affected by CLA inclusion in fish diets, which is in accordance with previous findings in rainbow trout (Figueiredo-Silva *et al.*, 2005) and European sea bass (Valente *et al.*, 2007a). Nevertheless, there was a progressive reduction in perivisceral fat deposition as the dietary CLA levels increased, in agreement with previous studies in this (Makol *et al.*, 2009) and other species (Twibell *et al.*, 2000), as well as in mammals (Yamasaki *et al.*, 2003; Park and Pariza, 2007).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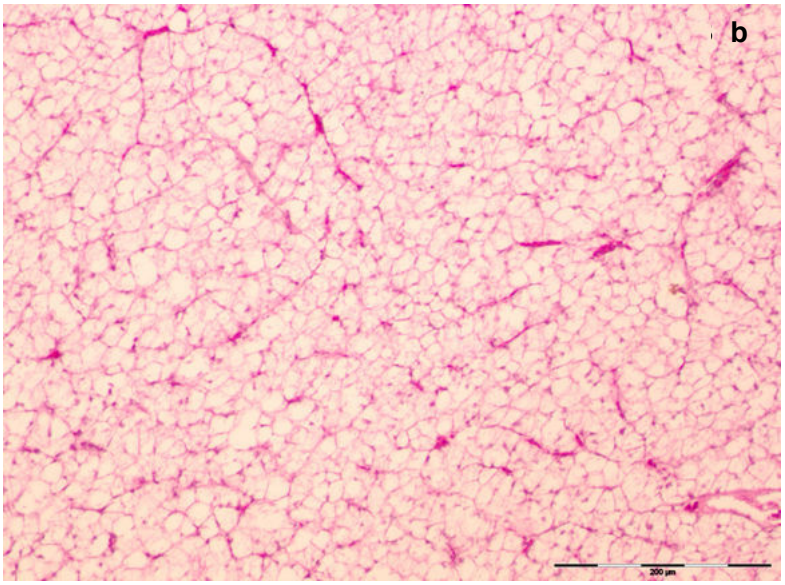
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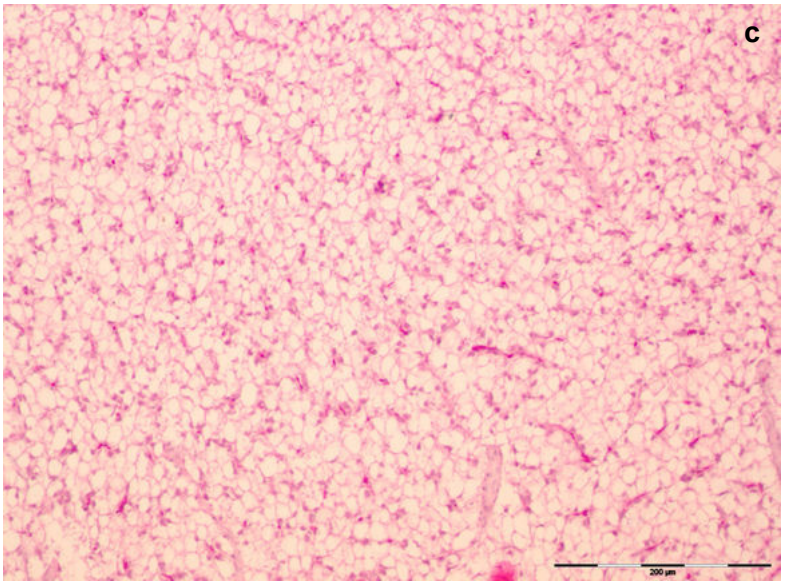

c
200 µm

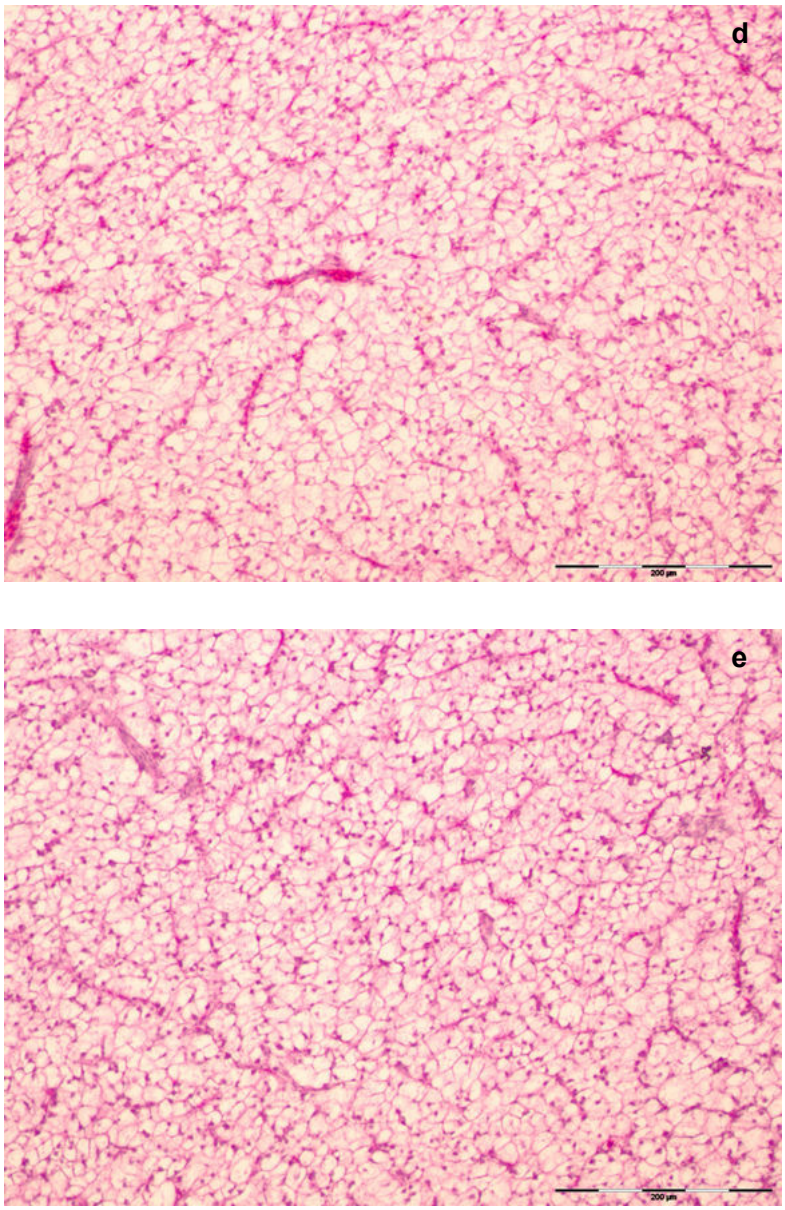


Figure 4.6. Liver micrographs (H&E; 400X) from fish fed (a) Control diet showing foci swollen hepatocytes characterized by cytoplasm vacuolization and nuclei displaced to cellular periphery, (b) CLA05 diet with lower number of swollen hepatocytes, (c) CLA1 diet with lower number of swollen hepatocytes, (d) CLA2 diet with lower number of swollen hepatocytes and (e) CLA4 diet with regular morphology of the hepatocytes located around sinusoidal spaces.

This marked reduction in perivisceral fat was associated with the deposition of CLA in comparison to other tissues, particularly of the isomer *trans*-10, *cis*-12. Some authors have attributed this lower perivisceral fat accumulation to a reduction in the size or in the number of adipose cells in the adipose tissue due to a reduced fatty acid uptake in adipocytes by the inhibition of lipoprotein lipase activity (Belury, 2002; Corino *et al.*, 2005; Park and Pariza, 2007, Rosa *et al.*, 2010). But it also could be related with

the modulation effect of dietary CLA on peroxisome proliferator-activated receptors (PPAR´s) expression, which are transcription factors known to modulate lipid metabolism in fish and mammals (Diez *et al.*, 2007; Park and Pariza, 2007). The higher reduction in PFI found in our previous study with the same species (Makol *et al.*, 2009) could be related to the lower culture densities in comparison to the present study, since stress affects lipid metabolism in fish (Vijayan and Leatherland, 1989; Tort, 2011). For example, certain metabolic routes as gluconeogenesis (Janssens and Waterman, 1998) are markedly affected by stress.

In several animal species, CLA has been found to reduce body fat and increase body lean mass (Cook *et al.*, 1998; Park, 1996; Park *et al.*, 1997; Park *et al.*, 1999a; Park *et al.*, 1999b; Park and Pariza, 2007; Park and Park, 2010). However, CLA did not affect fish muscle when hybrid striped bass was fed 1% CLA for 8 weeks (Twibell *et al.*, 2000), yellow perch fed 0.5-1% CLA for 9 weeks (Twibell *et al.*, 2001), channel catfish fed 0.5-1% CLA for 6 weeks (Manning *et al.*, 2006) or for 8 weeks (Twibell and Wilson, 2003), Atlantic salmon fed 2% CLA for 12 weeks (Berge *et al.*, 2004), Nile tilapia fed 5% CLA for 9 weeks (Yasmin *et al.*, 2004), rainbow trout fed 0.5-1% CLA for 8 weeks (Kennedy *et al.,* 2007a) or for 12 weeks (Bandarra *et al.*, 2006; Valente *et al.*, 2007b; Ramos *et al.*, 2008) and European sea bass fed 0.5-2% CLA for 12 weeks (Valente *et al.*, 2007a). These variety of results found in body composition could be related to different factors such as the different fish species being most of them freshwater species, different fish size which could influence metabolism status, different culture conditions (temperature, rearing density and photoperiod), shorter time of CLA administration, and also the chemical form of CLA used on the studies, that varies from free fatty acid, methyl ester or unknown that could affect the CLA isomers deposition in fish tissues and consequently affect the effects on fish metabolism and tissue composition.

Increase in CLA isomers in muscle added a potential nutritional value (Gaullier *et al.*, 2007) to European sea bass fillet. CLA accumulation in fish fillet has been also found in other species (Bandarra *et al.*, 2006; Valente *et al.*, 2007b, Ramos *et al.*, 2008; Tan *et al.*, 2010; dos Santos *et al.*, 2011), but the degree of deposition seems to depend on the type of isomer used. In the present study, the isomer *cis*-9,

trans-11 was preferentially accumulated in fish tissues than *trans*-10, *cis*-12 isomer as reported in other studies (Lauridsen *et al.*, 2005; Valente *et al.*, 2007b; Makol *et al.*, 2009) possibly due to a higher oxidation of the *trans*-10, *cis*-12 isomer (Churruca *et al.*, 2009).

Increase in dietary CLA up to 2% lead to smaller liver hepatocytes cytoplasm vacuolization and size, in agreement with our previous study (Makol *et al*., 2009) and suggesting a higher lipid mobilization and hepatocytes turnover. These results are in agreement with those obtained in rats where CLA proved to prevent liver steatosis (Andreoli *et al.*, 2010; Kostogrys and Pisulewski, 2010). However, further increase of CLA dietary levels up to 4% did not further reduced hepatocyte size. These results are in agreement with the significantly higher ACO activity found in fish fed dietary CLA up to 2%, and the similar trend followed by L3HOAD, denoting an increased β-oxidation in lipids, responsible in turn for the reduction of muscle total lipids.

Accordingly, monounsaturated fatty acids, the primary sources in lipid catabolism, were reduced in liver denoting the increase in β-oxidation capacity as seen in mammals (Park and Pariza, 2007). These results are in agreement with the reduction in monounsaturated fatty acids found in Atlantic salmon (Kennedy *et al.*, 2005; Leaver *et al.*, 2006), European sea bass (Valente *et al.*, 2007a), sea bream (Diez *et al.*, 2007) and yellow catfish (Tan *et al.*, 2010). However, further increase in dietary CLA up to 4% did not significantly increased ACO activity.

On the contrary, lipogenic enzymes G6PD and ME were not affected by CLA diet inclusion in agreement with the study of Valente *et al.* (2007a) for the same species. G6PD levels were higher (over 10 fold) than ME as reported previously (Dias *et al.*, 1998, Valente *et al.*, 2007b; Makol *et al.*, 2009; Torrecillas *et al.*, 2011b), confirming that in this species G6PD is the main NADPH generating enzyme. Thus, the higher ME activity found in the previous study, where European sea bass was fed CLA at lower fish densities, disappeared in the present study suggesting the interference of stress in gluconeogenesis as discussed above.

Although bactericidal and lysozyme activities were not affected by the inclusion of CLA in fish diets, phagocytic activity of head kidney leukocytes was significantly increased by dietary inclusion of CLA up to 4%, in agreement with the improved immune resistance found previously in birds and mammals. For instance,

phagocyte activity was promoted in broiler chicks and porcine by CLA dietary inclusion (Zhang *et al.*, 2005; Kang *et al.*, 2007), whereas in rats CLA reinforces the specific immune system (Ramírez-Santana *et al.*, 2009). This enhanced phagocytosis could be related to an inhibition COX-2 expression which contributed to a reduction of PGE_2 synthesis by CLA dietary inclusion as seen in chickens (He *et al.*, 2007) and humans (Stachowska *et al.*, 2007, 2009). Besides, dietary CLA modified fatty acid composition of head kidney leukocytes in European sea bass juveniles (unpublished data) pointing to an improvement in membrane flexibility and facilitating the phagocytic activity.

We can conclude that dietary CLA lead to a reduction of PFI and muscle lipids. Besides, 2% of CLA is the optimum level to improve liver lipid utilization by enhancing lipolysis and lipid use as energy source. Only the inclusion of CLA at 4% leads to an improvement of the immune system.

Chapter 5

STUDY III: Effect of long term feeding with conjugated linoleic acid (CLA) in growth performance and lipid metabolism of European sea bass (*Dicentrarchus labrax*)

Abstract

In the present study, the effects of long term feeding dietary conjugated linoleic acid (CLA) on growth, liver and perivisceral fat morphology, lipid metabolism enzymes and prostaglandin production potential were assessed. Two hundred and three European sea bass (152.4 ± 1.96 g) were randomly allocated in 9 indoor 1 m^3 fibreglass tanks (4.1 kg/m^3) and assigned three diets containing graded amounts of CLA (0, 0.5 and 1%) for 20 weeks. Dietary CLA did not affect growth performance or tissue proximate composition. Dietary CLA altered fatty acid profile in both muscle and liver, whereas only 1% of CLA increased the saturated fatty acid fraction in muscle. CLA content in fish tissues increased when CLA was included in fish diets. Liver lipogenic and lipolytic enzymes were not affected by dietary CLA. Hepatocyte area was reduced in fish fed 1% of CLA, as well as adipocyte area in fish fed 0.5% of CLA where adipocyte number also increased. Head kidney prostaglandins production potential was reduced in fish fed 1% of CLA, having also a negative correlation with dietary CLA. The present results suggest that CLA can be incorporated up to 1% in 150 g European sea bass for 20 weeks without affecting fish performance and enzyme activities and at the time improves liver and adipose tissue morphology and it is accumulated in fish tissues.

Keywords:
Conjugated linoleic acid
European sea bass
Dicentrarchus labrax
Fatty acids
Lipogenesis
Lipolysis
Prostaglandins
Cell morphometry

5.1. Introduction

There is a current practice in intensive aquaculture to substitute fish oil and meal by vegetables sources to reduce cost production (Tocher, 2003). Although fish diets produced with vegetable sources provide more cost-effective diets, their use could increase fat deposition in adipose and hepatic tissues particularly in European sea bass (*Dicentrarchus labrax*) (Izquierdo *et al*., 2005). Conjugated linoleic acid (CLA) has raised attention during the last decade as fat reducing fatty acid. Whereas the *cis*-9, *trans*-11 (*c*9, *t*11) isomer is the naturally occurring isomer present in food such as beef, dairy products and milk (Chin *et al*., 1994; Park and Pariza, 2007), the *trans*-10, *cis*-12 (*t*10, *c*12) isomer, responsible for body fat reduction and inhibition of stearoyl-CoA desaturase (SCD) (Cook *et al*., 1999; Storkson *et al*., 2005; Park and Pariza, 2007, 2009) is presented in negligible amounts in food, being considered as "man-made" isomer (Chin *et al*., 1994; Park and Pariza, 1998; Dhiman *et al*., 2005).

In fish, most studies have shown that there are no effects on growth performance and feed efficiency as seen in rainbow trout (*Oncorhynchus mykiss*) when fed 0.5-2% CLA for 12 weeks (Figueiredo-Silva *et al*., 2005; Valente *et al*., 2007b, 0.5-1% CLA for 8 weeks (Kennedy *et al*., 2007a) or 0.5-1% CLA for 12 weeks (Bandarra *et al*., 2006; Ramos *et al*., 2008), in Atlantic salmon (*Salmo salar*) when fed 0.5-2% for 12 weeks (Kennedy *et al*., 2005, 2006), 2-4% CLA for 12 weeks (Leaver *et al*., 2006) and European sea bass (*Dicentrarchus labrax*) fed 0.5-2% CLA for 12 weeks (Valente *et al*., 2007a; Makol *et al*., 2009). Whereas growth performance was improved in common carp (*Cyprinus carpio*) when fed 1% CLA for 12 weeks (Choi *et al*., 1999), in channel catfish (*Ictalurus punctatus*) fed 0.5-1% CLA for 6 weeks (Manning *et al*., 2006), yellow catfish (*Pelteobagrus fulvidraco*) fed 0.5-2% CLA for 8 weeks (Tan *et al*., 2010) and sea bream (*Sparus aurata*) fed 2-4% CLA for 12 weeks (Diez *et al*., 2007).

Body composition also showed controversial results, CLA reduced total liver lipid content in hybrid striped bass (*Morone saxatilis* x *M. chrysops*) fed 1% CLA for 8 weeks (Twibell *et al*., 2000), yellow perch (*Perca flavescens*) fed 0.5-1% for 8 weeks (Twibell *et al*., 2001) and yellow catfish fed 0.5-2% CLA for 8 weeks (Tan *et al*., 2010). Whereas no effect was detected in channel catfish fed 0.5-1% CLA for 8 weeks

(Twibell and Wilson, 2003), in rainbow trout fed 0.5-2% for 12 weeks (Figueiredo-Silva *et al*., 2005), 0.5-1% CLA for 12 weeks (Bandarra *et al*., 2006), in Nile tilapia (*Oreochromis niloticus*) fed 5% CLA for 9 weeks (Yasmin *et al*, 2004), sea bream fed 2-4% CLA for 12 weeks (Diez *et al*., 2007) and European sea bass fed 0.5-2% CLA for 12 weeks (Makol *et al*., 2009). The effect of CLA could be related with the time of administration.

Fish enzymatic activity showed different results depending on fish species. Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49) enzymatic activity was not affected by dietary CLA in rainbow trout when fish were fed 0.5-2% CLA for 12 weeks (Figueiredo-Silva *et al*., 2005) as G6PD and malic enzyme (ME, EC 1.1.1.40) in yellow croacker when fed 1-4% CLA for 10 weeks (Zhao *et al*., 2008), however G6PD was reduced in sea bream fed 2-4% CLA for 12 weeks (Diez *et al*., 2007) and ME increased in European sea bass juveniles fed 0.5-2% CLA for 12 weeks (Makol *et al*., 2009). Acyl-CoA oxidase (ACO, EC 1.3.3.6) and L-3-hydroxyacyl-CoA dehydrogenase activity (L3HOAD, EC 1.1.135) were not affected in sea bream fed 2-4% CLA for 12 weeks when measured 24 h post-feeding (Diez *et al*., 2007), whereas ACO was increased in rainbow trout fed 0.5-1% CLA for 8 weeks (Kennedy *et al*., 2007a) and Atlantic cod fed 0.5-1% CLA for 12 weeks (*Gadus morhua*) (Kennedy *et al*., 2007b). In European sea bass juveniles, when CLA was administered at 0.5-2% CLA for 12 weeks produced a decreased lipid vacuolization of hepatic cells cytoplasm (Makol *et al*., 2009) and a reduction of hepatocytes cells area and minimum length (Chapter 4).

The objective of this study was to determine the effects of supplemented CLA diets (0, 0.5 and 1%) for 20 weeks on growth performance, body composition, tissue fatty acid composition, hepatic metabolism and histological analysis in larger European sea bass juveniles.

5.2. Material and methods

5.2.1. Experimental diets

Three isonitrogenous and isoenergetic diets, based in a commercial formulation (Ecolife64), were designed with graded levels of CLA (CLA0=Control, CLA05=0.5% and CLA1=1%). CLA was supplied by BASF (Tarragona, Spain) as a

supplement (50:50 mixture of each isomer; LUTA-CLA60®) and was added to the extruded diets in substitution of fish oil to maintain a constant energy content among dietary treatments. Diets were formulated and manufactured by a commercial feed producer (Biomar Iberia, Spain). Ingredients and proximate composition and fatty acid profiles are shown in Tables 5.1 and 5.2.

Table 5.1 Ingredients and proximate composition of diets with different levels of CLA (0, 0.5 and 1%)

	Dietary CLA treatments		
	CLA0	**CLA05**	**CLA1**
Ingredients (%)			
Fish meal	41.00	41.00	41.00
Soyabean meal	25.00	25.00	25.00
Wheat	7.00	7.00	7.00
Corn gluten meal	4.50	4.50	4.50
Rapeseed meal	5.90	5.90	5.90
Vitamin and mineral mix	0.75	0.75	0.75
Fish oil[1]	15.85	15.12	14.38
CLA	0	0.73	1.47
Proximate composition (%)			
Crude protein	45.45	46.17	45.84
Crude fat	22.20	21.19	20.35
Ash	8.09	8.18	8.22
Moisture	3.66	3.44	3.82
Carbohydrates	24.25	24.46	25.59

Values are expressed as dry weight. Vitamin and mineral mix and CLA oil (LUTA-CLA 60; containing 60% CLA methyl esters as a 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers), BASF (Spain), fish meal and fish oil (Sopropeche, France), soyabean meal (Cargill Inc., Spain), wheat (Hermanos Dueñas, Spain), corn gluten meal (SYRAL Iberia S.A.U., Spain), rapeseed meal (Esasa, Spain) [1] CLA was included at the expense of fish oil.

5.2.2. Experimental conditions

European sea bass juveniles were maintained in stocking tanks and fed a commercial extruded diet (CLA0 diet) for 3 weeks until being well adapted to the environmental conditions. Afterwards, 243 fish were randomly distributed in 9 indoor 1 m^3, fibreglass tanks at initial stocking density of 4.1 kg/m^3 (27 fish/tank), supplied with filtered sea water (6 l/min water flow) and natural photoperiod (12L:12D). Water temperature and dissolved oxygen along the feeding trial ranged 22-24°C and 6.27 ± 0.19 mg/l, respectively. Fish initial weight of 152.4±1.96 g (mean±SD) were manually fed until apparent satiation with one of the three experimental diets for 20 weeks (twice daily, 6 days a week). Each treatment was tested in triplicate. Feed

intake was calculated daily and growth parameters were determined every 4 weeks. Feed conversion ratio (FCR) was defined as the amount of feed ingested divided by the generated biomass; specific growth rate (SGR) was defined as: [(ln final weight – ln initial weight)/number of days] x 100; relative growth (%) defined as: [(final weight –initial weight)/initial weight)] x 100 and perivisceral fat index (PFI) defined as: (visceral fat weight/body weight) x 100; hepatosomatic index (HSI) defined as: (liver weight/body weight)×100 and viscerosomatic index (VSI) defined as: (viscera weight/body weight)×100.

After a fasting period of 24 hours, 10 fish from the initial stock and 3 fish per tank every 4 weeks were sampled for liver and muscle proximate and fatty acid composition, HSI, VSI and PFI. At the end of the experiment 3 fish per tank were sampled for hepatic and perivisceral fat tissue morphology, lipogenic and lipolytic enzymes activities and head kidney samples were extracted to determine stimulated PG activity.

5.2.3. Biochemical analyses

Fish tissues and diets biochemical composition were conducted following standard procedures (AOAC, 2000). Ash content was determined by combustion in a muffle furnace at 600°C for 12 h, moisture content was determined after drying at 105°C to constant weight, crude protein by acid digestion using Kjeldahl method (Nx6.25) and crude lipid was extracted following the method of Folch *et al*. (1957). Fatty acids from total lipids were prepared by base-catalysed transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo *et al*. (1992), being quantified by flame ionizator detector (FID) and identified in comparison to external standards of CLA isomers (Sigma-Aldrich and Matreya, LLC.). All analyses were conducted by triplicate.

Table 5.2. Fatty acid composition (% of total identified fatty acids) of experimental diets

	Dietary CLA treatments		
Fatty acids	**CLA0**	**CLA05**	**CLA1**
14:0	7.76±0.05	7.70±0.03	7.68±0.06
14:1n-7	0.16±0.01	0.16±0.01	0.15±0.02
14:1n-5	0.12±0.01	0.09±0.02	0.09±0.03
15:0	0.51±0.02	0.59±0.06	0.52±0.04
16:0ISO	0.08±0.01	0.08±0.02	0.06±0.03
16:0	19.90±0.12	19.80±0.41	19.49±0.30
16:1n-7	9.57±0.25	9.28±0.31	8.96±0.39
16:1n-5	0.47±0.03	0.43±0.02	0.37±0.03
16:2n-6	1.55±0.01	1.60±0.03	1.48±0.04
16:2n-4	0.70±0.06	0.73±0.02	0.62±0.03
17:0	1.79±0.06	1.80±0.03	1.79±0.03
16:3n-4	0.11±0.02	0.10±0.02	0.11±0.01
16:3n-3	0.13±0.01	0.12±0.02	0.10±0.02
16:3n-1	0.05±0.01	0.05±0.00	0.05±0.01
16:4n-3	1.61±0.21	1.82±0.20	2.04±0.13
18:0	4.37±0.21	4.36±0.15	4.68±0.19
18:1n-9	13.03±0.09	13.05±0.02	13.04±0.04
18:1n-7	3.96±0.09	3.66±0.10	3.39±0.19
18:1n-5	0.20±0.02	0.18±0.03	0.17±0.02
18:2n-9	0.19±0.02	0.18±0.01	0.17±0.02
18:2n-6	4.02±0.10	4.14±0.09	4.24±0.06
18:2n-4	0.48±0.05	0.50±0.02	0.46±0.03
18:3n-6	0.24±0.04	0.26±0.03	0.27±0.02
18:3n-4	0.16±0.02	0.18±0.02	0.17±0.02
18:3n-3	0.83±0.06	0.84±0.03	0.90±0.05
18:3n-1	0.07±0.00	0.07±0.01	0.08±0.02
18:2 *c*9, *t*11	0.00±0.00	0.62±0.02	1.23±0.09
18:4n-3	1.65±0.13	1.76±0.19	1.99±0.11
18:2 *t*10, *c*12	0.00±0.00	0.74±0.03	1.40±0.10
18:4n-1	0.24±0.02	0.26±0.02	0.28±0.02
20:0	0.25±0.04	0.21±0.02	0.20±0.03
20:1n-9+n-7	1.05±0.05	0.95±0.04	0.88±0.04
20:1n-5	0.40±0.06	0.34±0.05	0.30±0.03
20:2n-9	0.20±0.05	0.17±0.03	0.15±0.04
20:2n-6	0.14±0.03	0.13±0.02	0.13±0.02
20:3n-6	0.20±0.02	0.15±0.03	0.15±0.02
20:4n-6	0.80±0.03	0.81±0.02	0.76±0.02
20:3n-3	0.06±0.01	0.06±0.00	0.06±0.00
20:4n-3	0.62±0.02	0.64±0.01	0.68±0.03
20:5n-3	12.71±0.26	12.12±0.15	11.73±0.29
22:1n-11	0.50±0.06	0.45±0.04	0.42±0.04
22:1n-9	0.22±0.02	0.18±0.06	0.18±0.05
22:4n-6	0.07±0.01	0.08±0.01	0.08±0.01
22:5n-6	0.25±0.02	0.26±0.01	0.29±0.02
22:5n-3	1.70±0.08	1.77±0.05	2.10±0.07
22:6n-3	6.87±0.26	6.49±0.34	5.91±0.21
Saturated	34.67±1.23	34.54±1.13	34.42±1.10
Monoenes	29.68±1.10	28.77±1.23	27.97±1.08
Σn-3	26.17±1.32	25.61±0.94	25.51±0.64
Σn-6	7.28±0.54	7.43±0.31	7.39±0.21
Σn-9	14.68±0.23	14.58±0.19	14.42±0.26
Σn-3 HUFA	21.95±0.91	21.07±0.46	20.47±0.39
Total CLA	0.00±0.00	1.36±0.09	2.63±0.10

CLA0, control diet; CLA05 and, diets with 0.5% and 1%,respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; CLA, conjugated linoleic acid.

5.2.4. Enzyme analyses

Liver samples were homogenized in 3 volumes of icecold buffer (20 mM Tris–HCl, 0.25 M sucrose, 2 mM EDTA, pH 7.4) and centrifuged at 20000 g for 40 min at 4 °C. Activities of G6PD and were assayed using spectrophotometric procedures on the supernatant following the method described by Dias *et al*. (1998). Acyl-CoA oxidase (ACO; EC 1.3.3.6) was assayed in peroxisome enriched liver fractions prepared by homogenizing in 3 volumes of ice-cold buffer (20 mM Tris-HCl, 0.25 M Sucrose, 2 mM EDTA, pH 7.4). The homogenate was centrifuged at 7200 g for 10 min at 4°C and the supernatant fraction collected. The resulting pellet was washed once with 500 µl of the same buffer, centrifuged as above and the supernatant collected and combined with the first one. Combined supernatants were centrifuged at 18000 g for 30 min and the resulting pellet re-suspended with 600 µl of the buffer prior to sonication bath for 30 min. After sonication, supernatants were centrifuged at 18000 g for 45 min and the supernatants collected for analyses. ACO was assayed by the spectrophotometric determination of H_2O_2 production, coupled to the oxidation of 2',7'-dichlorofluorescein diacetate (LDCF) at 502 nm. The reaction mixture contained 2.6 mM LDCF, 1M aminotriazole, 5 mg/ml horseradish peroxidase type II, 5% triton X100, 1 M Tris-HCl pH 8.5, 15 mM flavin adenine dinucleotide, 50 mg/ml BSA. The reaction was started by the addition of 1 mM Palmitoyl-CoA.

Mitochondrial preparations of liver samples were performed as described by Menoyo *et al*. (2004) and centrifuged following the method of Harper and Saggerson (1975) and the activity of L-3-hydroxyacyl-CoA dehydrogenase (L3HOAD; EC 1.1.135) was measured according to Bradshaw and Noyes (1975) on mitochondrial isolates disrupted by sonication in a 1% Triton X-100 solution. All enzyme assays were performed in triplicate.

Soluble protein content of liver homogenates was determined on the supernatant by the method of Bradford (1976) using bovine serum albumin (BSA) as standard. Care was taken to ensure that initial rates were measured in all assays and that enzymes were stable in the buffer solution used during the time and temperature required to perform the assays (Álvarez *et al*., 1998). Enzyme activity units (IU), defined as micromoles of substrate converted to product at assay

temperature per minute, were expressed per mg of hepatic soluble protein (specific activity). All enzyme assays were performed by triplicate.

5.2.5. Liver and perivisceral fat morphology

Liver and perivisceral fat samples were fixed with 10% neutral-buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H&E) for optical examination (Martoja and Martoja-Pierson, 1970). Micrographs of liver and fat were taken at a final magnification of 400X using a Nikon Microphot-FXA microscope and an Olympus DP50 camera. Adipocyte micrographs were converted to binary format with Photoshop (Photoshop CS5, Adobe Systems, San Jose, CA) in order to facilitate measurements. Hepatocellular and adipocyte areas as well as maximum and minimum longitude of cells, taking hepatocyte nuclei as reference, were measured with an analySIS® software package (Image Pro Plus®, Media Cybernetics, Silver Spring, MD, USA) using arbitrary units.

5.2.6. Head kidney prostaglandins analysis

Head kidneys were dissected out, weighed and digested in 4 ml of Hank´s Balanced Salt Solution (HBSBS) without Ca^{2+} plus 2% Collagenase during 30 minutes in continuous orbital agitation (100 U/min). After digestion, samples were filtered through nylon gauze and washed with 4 ml of HBBS without Ca^{2+} and isolated cells recollected in a glass tube. Cells were concentrated in a pellet by centrifugation (2500 g, 5 min) and the resultant pellet re-suspended in 1 ml of HBBS without Ca^{2+} in an Eppendorf tube. Cells were stimulated during 30 min with 50 µM Ca^{+} PMA (Phorbol 12-myristate 13-acetate, Sigma Chemicals, CA, USA) and 10 µM A321 (Sigma Chemicals, CA, USA). Stimulation reaction was stopped by adding 50 µl of formic acid 2M. Samples were stored at -80°C until purification. The frozen stimulated fraction (pooled 3 fish tissues/tank) samples were centrifuged (1000 g, 5 min) in order to precipitate any remaining debris. The supernatants were extracted using an octadecylsilyl (C18) “Sep-Pak” mini-columns (Millipore, Watford, UK) by the method of Powell (1982) and as described in detail by Bell *et al*. (1994). One milliliter of supernatants was applied to the column, which had been prewashed with 5 ml of methanol and 10 ml of MiliQ water. The column was washed successively with 10 ml

of MiliQ water, 5 ml of 15% ethanol (v/v) and 5 ml of hexane/chloroform (65:35, v/v) before elution of prostanoids with 10 ml of ethyl acetate. The extracts were dried under nitrogen and re-suspended in 100 µl of methanol and stored at -80°C in small glass vials until analysis. Measurement of prostaglandins was performed using an enzyme immunoassay (EIA) kit for PGE_2 according to manufacturer's protocol (Cayman Chemical Co., MI, USA), that is based in the competition between PGE_2 and PGE_2-actylcholinesterase (AChE) conjugated PGE_2 (tracer) for a limited amount of PGE_2 monoclonal antibody.

5.2.7. Statistical analysis

Means and Standard Deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of CLA inclusion. Where data did not pass a normality test, a log transformation was used to normalize data. When *F* values showed significance, individual means were compared using Tukey's test for multiple means comparison. Significant differences were considered for $P<0.05$.

5.3. Results

5.3.1. Growth performance

There were no mortalities during the experiment. European sea bass fed experimental diets during 20 weeks did not show differences in growth performance, FCR, SGR or K among diets (Table 5.3). Furthermore, HSI, VSI or PFI were not affected by dietary CLA (Table 5.3).

5.3.2. Muscle and liver composition

Muscle and liver proximate composition were not affected by dietary treatments along the experiment (Table 5.4).

Table 5.3. Biological and growth parameters of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments		
	CLA0	**CLA05**	**CLA1**
Feed intake (g)	8322.0±162.2	8267.8±162.6	8178.3±291.3
Initial weight (g)	153.0±2.5	153.1±2.3	151.3±1.0
Final weight (g)	355.0±55.3	359.3±48.4	363.2±50.6
Eviscerated weight (g)	311.6±28.9	333.7±43.8	299.4±45.0
Final length (cm)	29.0±1.5	29.3±1.2	29.5±1.2
K	1.7±0.1	1.8±0.1	1.8±0.01
SGR (%)	0.6±0.0	0.7±0.0	0.7±0.0
FCR	1.9±0.1	1.8±0.2	1.7±0.2
VSI (%)	12.2±1.5	10.9±1.2	16.3±11.0
HSI (%)	2.3±0.3	2.2±0.4	2.3±0.6
PFI (%)	6.0±1.8	5.0±1.3	6.4±1.6

Data are presented as means ± SD. Initial weight (n=486), final weight and length, relative growth, SGR, FCR and feed intake (n=54). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA, conjugated linoleic acid; FCR, feed conversion ratio; HSI, Hepatosomatic index; SGR, specific growth rate; VSI,Viscerosomatic index; PFI, perivisceral fat index; CLA0, control diet; CLA05 and CLA1 diets with 0.5% and 1% of CLA inclusion, respectively.

Table 5.4. Muscle and liver proximate composition of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments					
	Muscle			**Liver**		
	CLA0	**CLA05**	**CLA1**	**CLA0**	**CLA05**	**CLA1**
Lipids (%)	20.5±1.8	24.6±3.9	19.1±3.9	59.2±2.7	62.6±6.9	63.1±2.1
Protein (%)	74.5±0.8	72.3±0.5	75.4±2.5	12.4±0.2	10.6±0.4	11.0±0.9
Ash (%)	1.4±0.1	1.4±0.0	1.5±0.0	0.4±0.2	0.2±0.1	0.4±0.0
Moisture(%)	71.3±1.0	69.4±2.5	71.9±1.6	44.0±1.7	43.5±1.9	42.5±2.8

Data are presented as means ± SD (n=9). Values are expressed as dry weight. Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively.

After 20 weeks of supplementation fish muscle main fatty acids groups (monounsaturated, n-3, n-6, n-9 and n-3 HUFA) were similar among fish fed the different diets. However fish fed CLA1 diet had higher (P<0.05) levels of saturated fatty acids than fish fed the other diets, mostly due to the increase (P<0.05) in the 18:0 fatty acid. Specifically, fish fed CLA05 diet showed higher (P<0.05) content of 15:0 than fish fed CLA0 diet. Fish fed CLA1 diet had higher (P<0.05) levels of 18:0, 18:2n-9 and 18:2n-4 when compared to fish fed CLA0 diet, whereas 16:1n-7, 18:2n-9 were reduced (P<0.05) when compared to fish fed CLA05 and CLA0 diets, respectively. The 18:2n-4 fatty acid was reduced (P<0.05) in fish fed CLA1 diet compared to the other diets. Both CLA isomers, as well as total CLA was increased

(P<0.05) in fish fed CLA diets when compared to CLA0 diet as well as among CLA diets, were fish fed CLA1 diet showed two-fold higher levels of CLA isomers than fish fed CLA05 diet (Table 5.5). Besides, CLA deposition was positively correlated ($y=0.022x^2+1.433x+9E^{-16}$, $R^2=1$, P=0.0001) with the CLA level of inclusion in the diets (Figure 5.1)

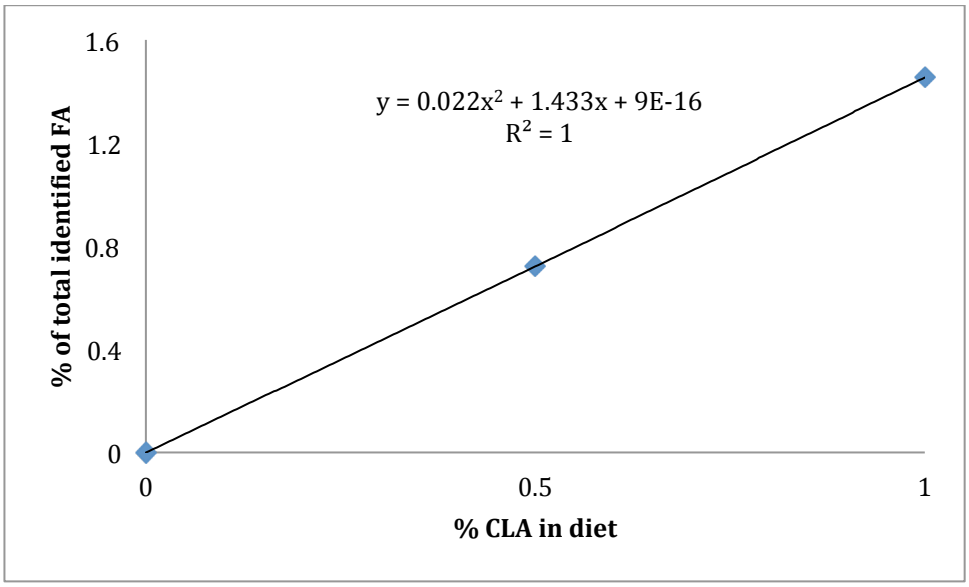


Figure 5.1. Positive correlation of dietary CLA level of inclusion and CLA deposition in muscle.

In liver, fish fed CLA1 diet had lower (P<0.05) levels of 16:1n-7, 17:0,18:1n-7, 18:2n-4, 18:3n-6 and 20:2n-9 when compared to fish fed CLA0 diet. Besides, fish fed diets supplemented with CLA had lower (P<0.05) levels of 16:0ISO and 18:3n-4, and higher (P<0.05) levels of 18:0 when compared to fish fed CLA0 diet. As in muscle, CLA isomers and total CLA was increased (P<0.05) in fish fed CLA diets (Table 5.6). Besides, CLA deposition was positively correlated ($y=-0.008x^2+1.524x+9E^{-16}$, $R^2=1$, P=0.0001) with the CLA level of inclusion in the diets (Figure 5.2).

5.3.3. Liver enzyme activities

After 12 and 20 weeks supplementation, both lipogenic and lipolytic enzyme activities were not affected by CLA inclusion (Table 5.7).

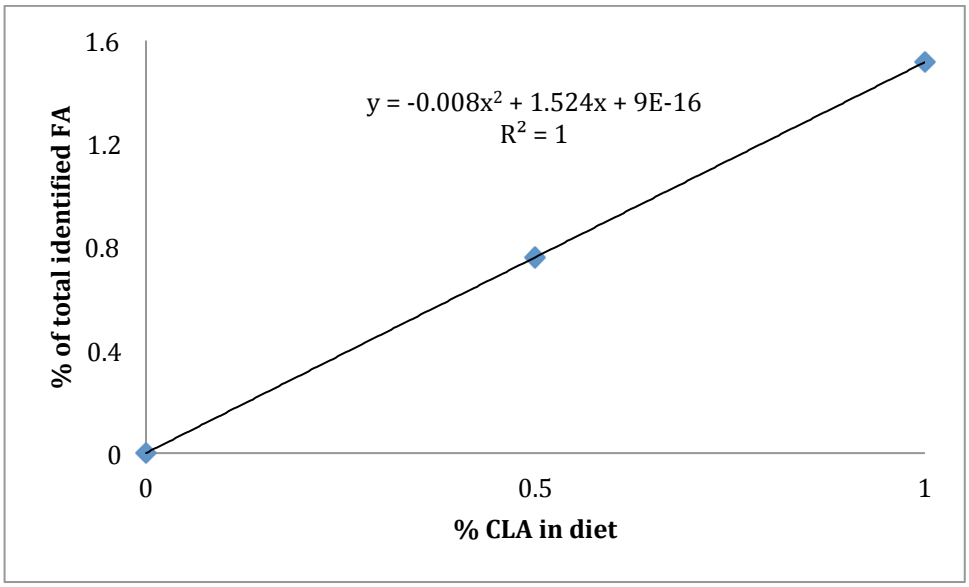


Figure 5.2. Positive correlation of dietary CLA level of inclusion and CLA deposition in liver.

5.3.4. Liver and perivisceral fat morphology

At the end of the experiment, liver morphometric analysis showed negatives correlations between hepatocytes area ($y=-89.764x+291.17$; $R^2=0.9996$; $P=0.0032$), maximum length ($y=-5.4396x^2+1.3313x+62.769$; $R^2=1$; $P=0.0901$) and minimum length ($y=-3.1932x^2-0.0229x+19.022$; $R^2=1$; $P=0.0207$) and dietary CLA level of inclusion (Figure 5.3). Besides, hepatocyte area was reduced ($P<0.05$) in fish fed CLA1 diet when compared to fish fed CLA0 diet (Table 5.8 and Figure 5.4). Perivisceral fat, showed smaller ($P<0.05$) adipocytes area and higher ($P<0.05$) number of adipocytes per area in fish fed CLA05 diet than fish fed CLA0 diet (Table 5.9 and Figure 5.5).

5.3.5. Prostaglandins analyses

Head kidney released PGs levels, when tissue was stimulated, were reduced ($P<0.05$) in fish fed CLA1 diet when compared to the other two diets, being also slight lower in fish fed CLA05 diet than CLA0 diet (Figure 5.6).

Table 5.5. Muscle fatty acids profile (% of total identified fatty acids) of European sea bass (*Dicentrarchus labrax*) fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments		
Fatty Acids	**CLA0**	**CLA05**	**CLA1**
14:0	4.103±0.119	4.467±0.193	4.199±0.15
14:1n-7	0.083±0.003	0.089±0.006	0.088±0.004
14:1n-5	0.073±0.012	0.063±0.002	0.081±0.024
15:0	0.32±0.006^{a}	0.342±0.011^{b}	0.338±0.003^{ab}
16:0ISO	0.053±0.002	0.054±0.002	0.052±0.002
16:0	18.06±0.262	18.61±1.076	18.763±0.168
16:1n-7	7.097±0.055^{ab}	7.214±0.211^{a}	6.665±0.259^{b}
16:1n-5	0.262±0.006	0.261±0.004	0.255±0.008
16:2n-6	1.017±0.027	1.053±0.049	0.996±0.023
16:2n-4	0.441±0.013^{a}	0.473±0.015^{b}	0.488±0.007^{b}
17:0	1.12±0.026	1.122±0.069	1.026±0.05
16:3n-4	0.061±0.001	0.059±0.008	0.055±0.004
16:3n-3	0.079±0.003	0.081±0.004	0.082±0.003
16:3n-1	0.033±0.001	0.032±0.004	0.031±0.001
16:4n-3	0.767±0.029	0.753±0.088	0.668±0.067
18:0	3.684±0.105^{a}	4.041±0.383^{a}	4.754±0.207^{b}
18:1n-9	17.722±0.281	17.784±0.909	17.199±1.38
18:1n-7	3.285±0.054	3.323±0.087	3.298±0.083
18:1n-5	0.274±0.002	0.261±0.004	0.268±0.023
18:2n-9	0.349±0.025^{a}	0.314±0.017^{ab}	0.294±0.023^{b}
18:2n-6	7.339±0.943	7.739±0.297	7.016±0.696
18:2n-4	0.482±0.007a	0.479±0.014a	0.456±0.011b
18:3n-6	0.255±0.005	0.246±0.01	0.238±0.009
18:3n-4	0.169±0.008	0.168±0.009	0.166±0.007
18:3n-3	1.158±0.064	1.082±0.05	1.079±0.045
18:3n- 1	0.05±0.001	0.049±0.005	0.046±0.001
18:2 *c*9, *t*11	0±0.000^{a}	0.375±0.016^{b}	0.766±0.047^{c}
18:4n-3	1.281±0.023	1.244±0.119	1.165±0.057
18:2 *t*10, *c*12	0±0.000^{a}	0.346±0.016^{b}	0.688±0.044^{c}
18:4n-1	0.206±0.011	0.209±0.025	0.203±0.012
20:0	0.127±0.013	0.135±0.003	0.128±0.003
20:1n-9+n-7	1.114±0.093	1.14±0.025	1.269±0.169
20:1n-5	0.226±0.02	0.237±0.008	0.23±0.007
20:2n-9	0.11±0.018	0.113±0.008	0.104±0.01
20:2n-6	0.419±0.029	0.423±0.014	0.41±0.029
20:3n-6	0.124±0.005	0.103±0.039	0.082±0.036
20:4n-6	0.891±0.056	0.821±0.027	0.896±0.114
20:3n-3	0.056±0.002	0.053±0.002	0.057±0.004
20:4n-3	0.57±0.019	0.57±0.051	0.581±0.02
20:5n-3	12.498±0.225	11.692±1.062	11.226±0.66
22:1n-11	0.241±0.056	0.247±0.012	0.366±0.15
22:1n-9	0.142±0.018	0.15±0.005	0.169±0.023
22:4n-6	0.082±0.004	0.08±0.004	0.08±0.004
22:5n-6	0.306±0.011	0.282±0.016	0.298±0.029
22:5n-3	2.197±0.074	2.039±0.182	2.117±0.221
22:6n-3	11.072±0.531	9.583±0.879	10.557±1.534
Saturated	27.467±0.235^{a}	27.938±0.361^{a}	29.262±0.171^{b}
Monoenes	30.52±0.074	30.769±1.012	29.89±1.899
Σn-3	29.678±0.712	27.096±2.272	27.533±2.237
Σn-6	10.433±0.904	10.746±0.324	10.016±0.622
Σn-9	19.438±0.184	19.502±0.944	19.036±1.537
Σn-3 HUFA	26.394±0.769	23.936±2.059	24.539±2.247
Total CLA	0±0.000^{a}	0.722±0.032^{b}	1.455±0.090^{c}

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively. FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 5.6. Liver fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments		
Fatty acids	**CLA0**	**CLA05**	**CLA1**
14:0	2.474±0.228	2.279±0.271	2.273±0.172
14:1n-7	0.058±0.002	0.053±0.008	0.054±0.001
14:1n-5	0.037±0.016	0.039±0.021	0.027±0.005
15:0	0.232±0.021	0.199±0.029	0.202±0.014
16:0ISO	0.061±0.001[a]	0.054±0.003[b]	0.055±0.002[b]
16:0	16.862±1.566	17.384±0.873	17.305±0.414
16:1n-7	7.345±0.551a	6.28±0.306ab	5.628±0.603b
16:1n-5	0.303±0.019	0.264±0.016	0.27±0.019
16:2n-6	0.573±0.307	0.495±0.266	0.563±0.223
16:2n-4	0.616±0.193	0.594±0.141	0.549±0.015
17:0	0.919±0.053[a]	0.843±0.032[ab]	0.78±0.043[b]
16:3n-4	0.051±0.007	0.016±0.028	0.016±0.027
16:3n-3	0.092±0.006	0.069±0.025	0.062±0.021
16:3n-1	0.041±0.004	0.042±0.036	0.032±0.056
16:4n-3	0.325±0.048	0.291±0.019	0.284±0.064
18:0	3.561±0.327[a]	5.352±0.737[b]	6.541±0.571[b]
18:1n-9	26.743±0.895	27.66±2.917	26.883±3.095
18:1n-7	4.129±0.115[a]	3.649±0.090[ab]	3.566±0.308[b]
18:1n-5	0.386±0.037	0.348±0.032	0.326±0.089
18:2n-9	0.526±0.03	0.488±0.047	0.417±0.133
18:2n-6	4.105±0.376	3.757±0.375	3.715±0.395
18:2n-4	0.585±0.024[a]	0.524±0.030[ab]	0.488±0.054[b]
18:3n-6	0.259±0.013[a]	0.239±0.016[ab]	0.219±0.018[b]
18:3n-4	0.206±0.012[a]	0.185±0.010[b]	0.182±0.006[b]
18:3n-3	0.681±0.047	0.61±0.073	0.612±0.073
18:3n-1	0.022±0.002	0.019±0.002	0.012±0.02
18:2 *c*9, *t*11	0±0.000[a]	0.464±0.030[b]	0.879±0.067[c]
18:4n-3	1.029±0.099	0.982±0.09	0.94±0.097
18:2 *t*10, *c*12	0±0.000[a]	0.297±0.028[b]	0.636±0.068[c]
18:4n-1	0.191±0.016	0.181±0.015	0.193±0.008
20:0	0.072±0.012	0.09±0.007	0.082±0.005
20:1n-9+n-7	0.946±0.078	0.984±0.021	1.034±0.073
20:1n-5	0.173±0.021	0.183±0.008	0.173±0.006
20:2n-9	0.109±0.001[a]	0.102±0.007[ab]	0.096±0.003[b]
20:2n-6	0.297±0.002	0.291±0.014	0.291±0.028
20:3n-9	0.019±0.034	0.019±0.034	0.000±0.000
20:3n-6	0.106±0.005	0.075±0.037	0.077±0.037
20:4n-6	0.67±0.039	0.592±0.095	0.617±0.069
20:3n-3	0.056±0.002	0.051±0.005	0.057±0.011
20:4n-3	0.398±0.229	0.364±0.191	0.558±0.071
20:5n-3	10.658±1.06	10.073±1.493	9.602±1.046
22:1n-11	0.11±0.029	0.117±0.01	0.141±0.038
22:1n-9	0.087±0.022	0.097±0.01	0.09±0.011
22:4n-6	0.112±0.077	0.112±0.087	0.065±0.01
22:5n-6	0.281±0.038	0.262±0.026	0.261±0.028
22:5n-3	2.535±0.38	2.403±0.315	2.517±0.543
22:6n-3	10.955±1.951	10.529±1.373	10.63±2.103
Saturated	24.18±1.915	26.201±1.307	27.238±0.97
Monoenes	40.323±1.174	39.675±2.564	38.193±3.146
Σn-3	26.73±3.186	25.37±3.194	25.261±3.648
Σn-6	6.00±0.321	5.823±0.392	5.809±0.609
Σn-9	28.429±0.943	29.351±2.937	28.519±3.212
Σn-3 HUFA	24.603±3.068	23.419±3.006	23.364±3.596
Total CLA	0±0.000[a]	0.76±0.057[b]	1.516±0.135[c]

Data are presented as means ± SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively. FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 5.7. Lipogenic and lipolytic enzymatic activities in liver of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 12 and 20 weeks

	Dietary CLA treatments					
	12 weeks			20 weeks		
	CLA0	CLA05	CLA1	CLA0	CLA05	CLA1
G6PD						
IU / mg	0.147±0.028	0.182±0.021	0.165±0.035	0.195±0.020	0.173±0.016	0.198±0.014
ME						
IU / mg	0.028±0.007	0.025±0.002	0.024±0.007	0.024±0.007	0.030±0.015	0.027±0.007
L3HOAD						
mIU / mg	44.808±5.919	35.14±8.721	35.103±5.867	19.363±1.344	19.317±2.594	23.304±7.648
ACO						
mIU / mg	126.992±35.693	121.227±22.157	129.133±16.814	168.812±70.9000	203.779±94.734	166.041±32.710

Data are presented as means±SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1 diets with 0.5% and 1% of CLA inclusion, respectively; G6PD, glucose-6-phophate dehydrogenase (EC 1.1.1.49); ME, malic enzyme (EC 1.1.1.40); L3HOAD, L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.135); ACO, acyl-CoA oxidase (EC 1.3.3.6); IU, enzyme activity units defined as micromoles of substrate converted to product at assay temperature per minute expressed per mg of soluble protein.

Table 5.8. Quantitative image analysis (μm) of hepatocytes from European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

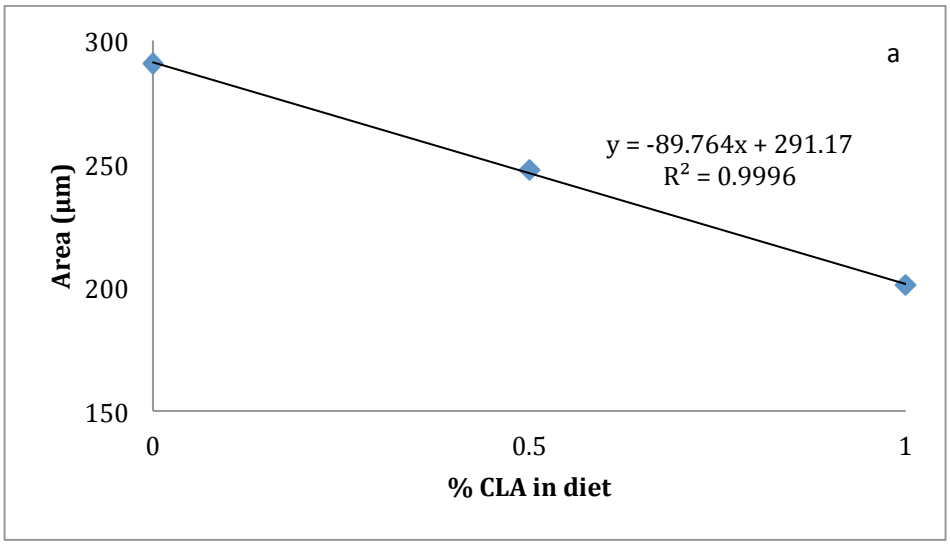
	Dietary CLA treatments		
	CLA0	CLA05	CLA1
Area (μm^2)	290.7 ± 50.8^{a}	247.3 ± 78.8^{ab}	200.9 ± 43.1^{b}
Maximum length (μm)	62.8±4.9	62.1±3.0	58.7±6.4
Minimum length (μm)	19.0±2.7	18.2±3.9	15.8±0.8

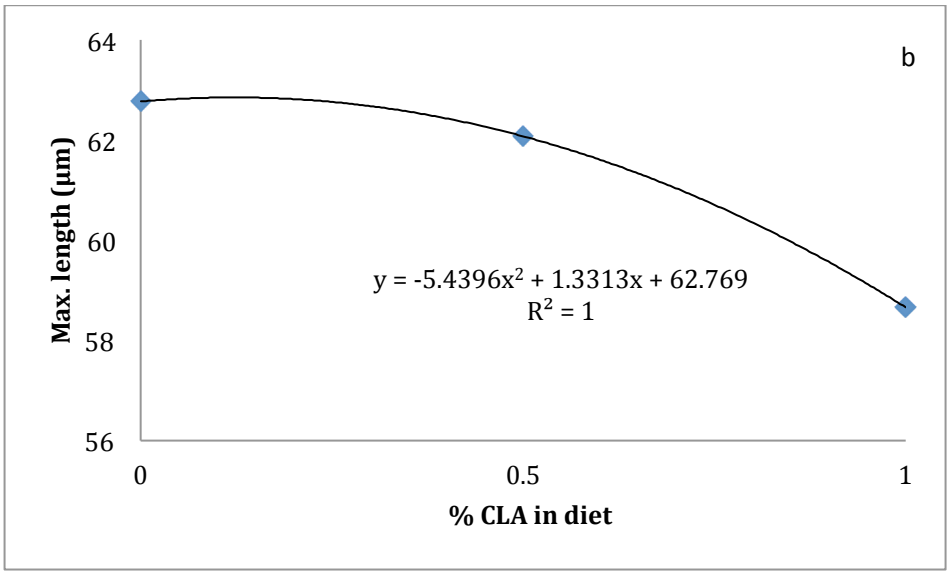
Data are presented as means±SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively.

Table 5.9. Quantitative image analysis (μm) of adipocytes from European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments		
	CLA0	CLA05	CLA1
Area (μm^2)	325.7 ± 55.8^{a}	291.7 ± 66.0^{b}	315.1 ± 57.9^{ab}
Adipocyte/Area	36.5 ± 4.4^{a}	43.7 ± 4.9^{b}	41.2 ± 4.5^{ab}

Data are presented as means±SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively.





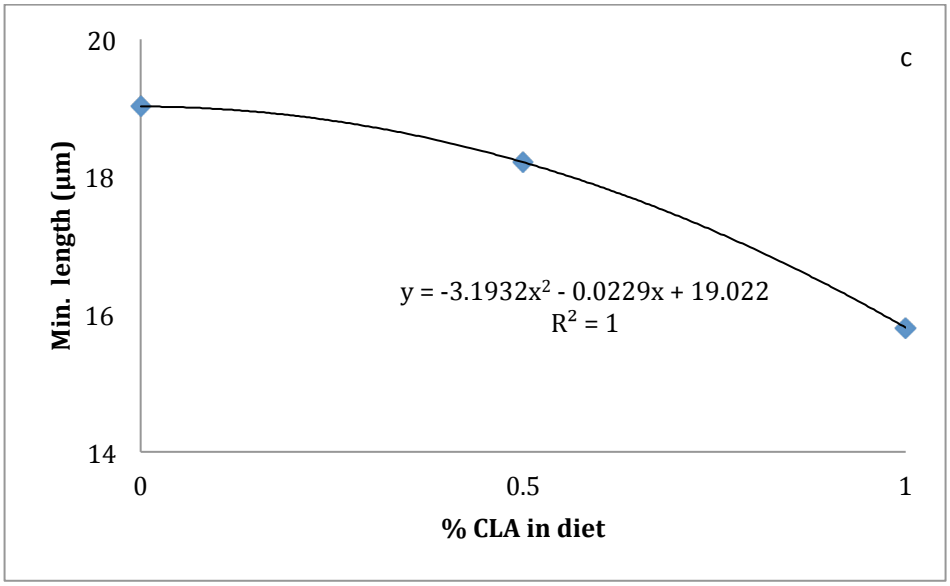


Figure 5.3. Negative correlations of (a) Hepatocyte area, (b) maximum length, (c) minimum length and dietary CLA inclusion.

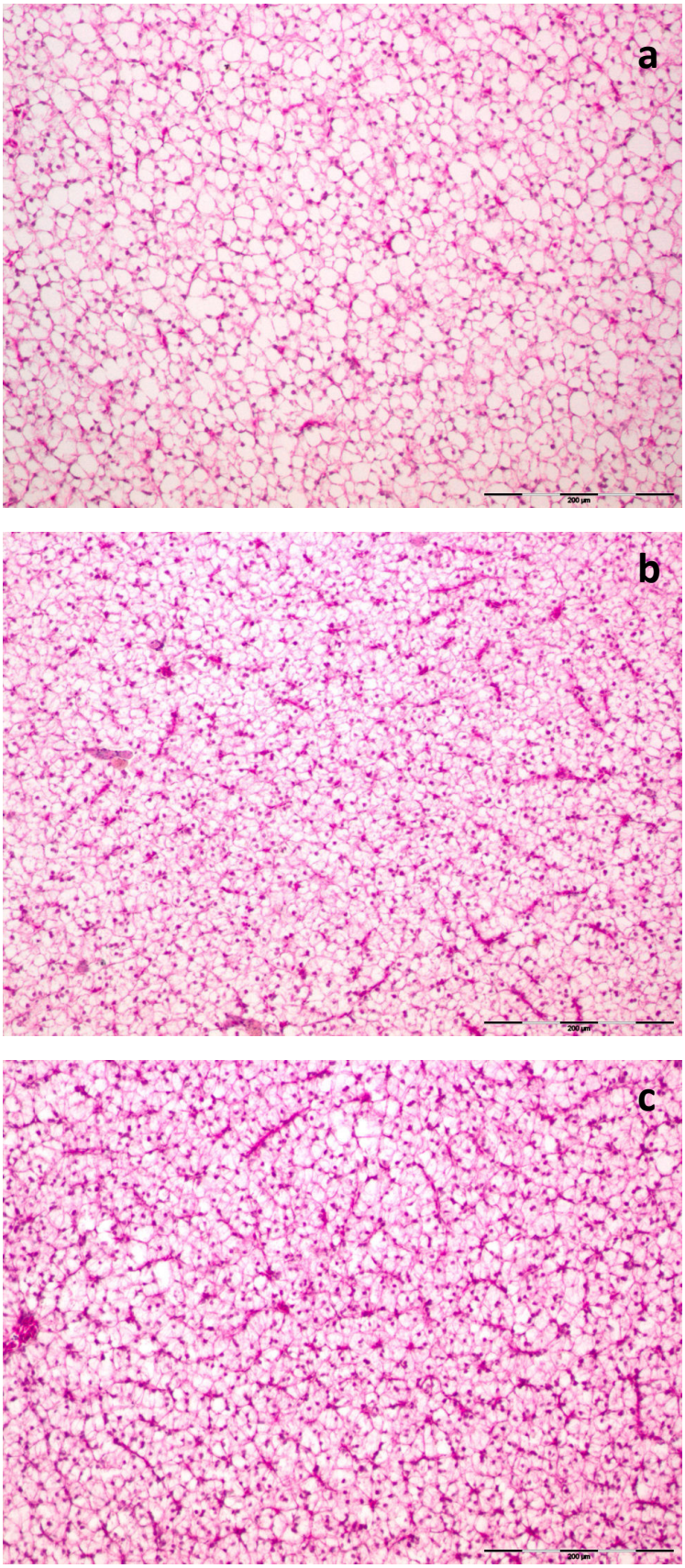


Figure 5.4. Liver micrographs (H&E; 400X) from fish fed (a) Control diet showing foci of swollen hepatocytes characterized by cytoplasm vacuolization and nuclei displaced to cellular periphery, (b) CLA05 diet with lower number of swollen hepatocytes and (c) CLA1 diet with regular morphology of the hepatocytes located around sinusoidal spaces.

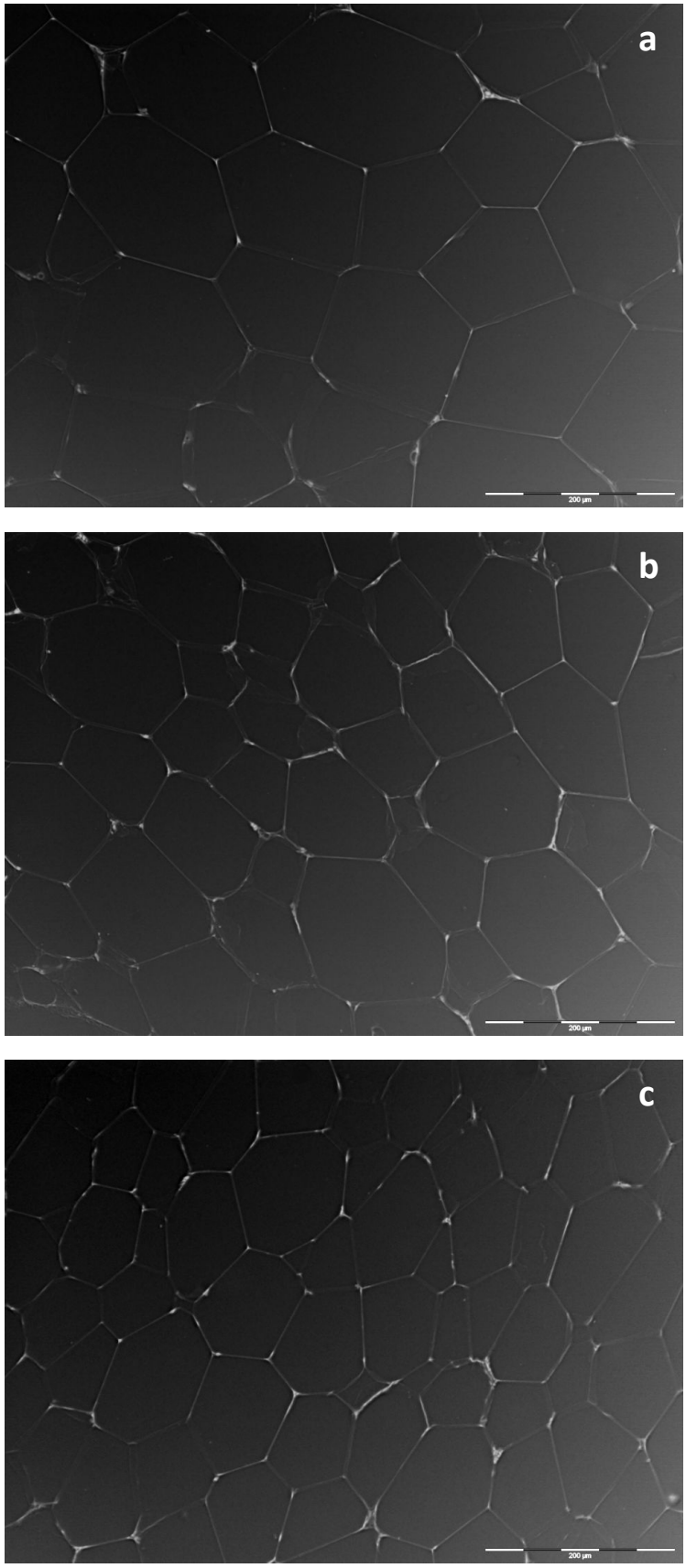


Figure 5.5. Micrographs of perivisceral adipose tissue (H&E; 400X) from fish fed (a) Control diet, (b) CLA05 diet and (c) CLA1 diet.

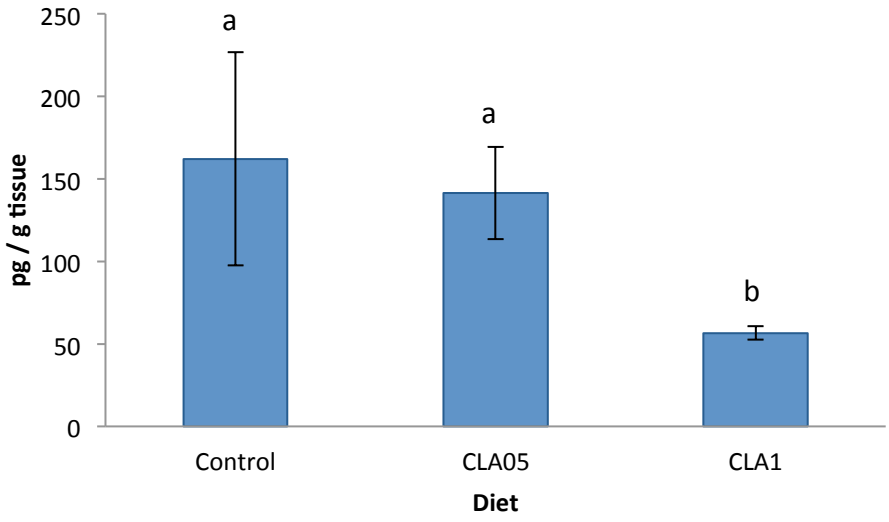


Figure 5.6. Concentration of PG in head kidney from European sea bass (n=15) fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks. Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different letters denotes significant differences among treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively.

5.4. Discussion

Growth performance was not affected by dietary CLA inclusion in fish diets during the experiment as seen in European sea bass juveniles (Valente *et al*., 2007a; Makol *et al*., 2009) and market size fish (Chapter 6), as well as in different market size fish species such as rainbow trout (Kennedy *et al*., 2007a; Ramos *et al*., 2008), carp (Schwarz *et al*., 2002) and Nile tilapia (dos Santos *et al*., 2011). Regarding muscle and liver proximate composition, no effect was noticed by dietary CLA inclusion as reported in yellow perch (Twibell *et al*., 2001), channel catfish (Twibell and Wilson, 2003), Atlantic salmon (Berge *et al*., 2004), rainbow trout (Bandarra *et al*., 2006; Valente *et al*., 2007b; Ramos *et al*., 2008), Atlantic cod (Kennedy *et al*., 2007a), yellow catfish (Tan *et al*., 2010) and European sea bass (Makol *et al*., 2009; Chapter 6) when fish were fed for up to 12 weeks.

In muscle, the increase in the saturated fraction and the slight reduction in the monounsaturated fraction in the fatty acid profile in fish fed 1% dietary CLA suggest

the inhibition of Δ-9 desaturase activity by dietary CLA as seen in pigs (Smith *et al*., 2002) since is the rate-limiting enzyme for converting saturated fatty acids to monounsaturated fatty acids (Cohen *et al*., 2002; Ntambi *et al*., 2002). Nevertheless, the increase in the saturated fraction was mainly due to the increase of stearic acid (18:0), which is not disadvantageous from a consumer point of view, since it has a neutral effect on lipoproteins (Wijendran *et al*., 2003). In liver, also a possible inhibition of Δ-9 desaturase activitiy could be suggested from the increase of stearic acid and the reduction of 18:1n-7 coupled with the reduction of palmitoleic acid (16:1n-7) which is synthetized from palmitic acid (16:0) by the action of Δ-9 desaturase. Similar results were obtained in European sea bass juveniles (Valente *et al*., 2007a), Atlantic salmon (Kennedy *et al*., 2005; Berge *et al*., 2007) and rainbow trout (Bandarra *et al*., 2007; Valente *et al*., 2007b). Both CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 were successfully incorporated into muscle and liver lipids as previously reported in European sea bass juveniles (Valente *et al*., 2007a; Makol *et al*., 2009) as well as in market size rainbow trout (Valente *et al*., 2007b; Ramos *et al*., 2008), carp (Schwarz *et al*., 2002) and Nile tilapia (dos Santos *et al*., 2011). As described in previous studies, the isomer *cis*-9, *trans*-11 was preferentially incorporated in fish tissues than *trans*-10, *cis*-12 isomer (Makol *et al*., 2009; Zhong *et al*., 2011), perhaps because the *trans*-10, *cis*-12 isomer is more efficiently oxidized (Churruca *et al*., 2009).

Concerning liver lipid metabolism, G6PD and ME enzymes activities were not affected by dietary CLA when fish were fed for 12 weeks, in agreement with the study of Valente *et al*. (2007b) for the same species, as well as when fish were fed for 20 weeks with CLA supplemented diets. Same results were obtained for lipolytic enzymes (ACO and L3HOAD) activities. These results are in concordance with the lack of effect of CLA on muscle and liver proximate composition and the perivisceral fat index found in this experiment, as well as with the no effect on total monounsaturated fatty acids found in liver which are the preferred substrate for fatty acid oxidation and are in agreement with a previous study in sea bream (Diez *et al*., 2007).

The slight reduction on perivisceral fat coupled with the reduction of adipocyte area in fish fed CLA05 diet could be related with the slight changes found in G6PD and ACO enzyme activities, where the first showed smaller levels and the second higher levels than the other experimental diets. This reduction in adipocyte area in fish fed 0.5% of CLA diet could also be related with the reduction of lipid uptake by adipocytes by the inhibition of lipoprotein lipase activity in adipose cells which is the key enzyme for fat uptake (Lin *et al*., 2001; Park *et al*., 2004; Park and Pariza, 2007), by the inhibition stearoyl-CoA desaturase (SCD) activity (Pariza *et al*., 2001; Park *et al*., 2004; Park and Pariza, 2007) or to a reduced preadipocyte differentiation into mature adipocytes (Simón *et al*., 2005; Park and Pariza, 2007). These results are in concordance with those found in *in vivo* studies in rats where adipocyte size was decreased and cell number increased by dietary CLA (Tsuboyama-Kasaoka *et al*., 2000; Noto *et al*., 2007), as well as when rats were fed the *trans*-10, *cis*-12 isomer alone (DeClercq *et al*., 2010) or the *cis*-9, *trans*-11 isomer (Lopes *et al*., 2008). *In vitro* studies showed that cultures of 3T3-L1 preadipocytes supplemented with CLA presented lower triglyceride content and smaller size (Evans *et al*., 2000) as well as when treated with trans-10, cis-12 isomer (Brown *et al*., 2001), and as proposed above, the possible inhibition of Δ-9 desaturase in muscle and liver could explain these results. Besides, there is evidence that some effects of CLA are mediated through transcription factors such as peroxisome-proliferator activated receptors (PPAR), which are ligand-dependent transcription factors that play critical roles in lipid homeostasis regulation in liver, muscle and adipose tissue. Particularly PPAR-γ, which is the nuclear receptor superfamily that controls lipid metabolism in adipose tissue (Peters *et al*., 2001; Brown *et al*., 2004; Domeneghini *et al*., 2006), it has been suggested to be reduced by CLA (Brown *et al*., 2004).

Regarding the area reduction found in hepatocytes in fish fed CLA diets, similar results were obtained previously in European sea bass juveniles (Makol *et al*., 2009), where hepatocytes showed a decreased cytoplasm lipid vacuolization suggesting lipid mobilization and hepatocyte turnover when fish were fed CLA diets. Besides, CLA has been reported to be a strong agonist for PPAR-α (Moya-Camarena *et al*., 1999a,b) the predominant form in liver. Further studies need to be conducted to

elucidate the relationship between CLA and PPAR in fish, where PPAR isotypes show a wider variety than in mammals.

The potential prostaglandins (PGs) production levels in head kidney were decreased in fish fed CLA diets being significant in fish fed 1% of CLA. Similarly, earlier studies in mammals found that CLA inhibites PG production (Liu and Belury, 1998; Kavanaugh *et al*., 1999; Wang *et al*., 2006; Stachowska *et al*., 2009). This could be related to either, an inhibition effect of CLA on COX-2 activity (Li *et al*., 2005; Zhang *et al*., 2005; Park and Pariza, 2007, Stachowska *et al*., 2009) or to a higher accumulation of CLA on the sn-2 position of phosphatidylinositol (PI) leaving less substrate disposable for derived ARA eicosanoid production, since CLA competes with ARA for this position (Watkins *et al*., 1997; Liu8 and Belury, 1998; Whigham *et al*., 2001, 2002; Eder *et al*, 2003; Ogborn *et al*., 2003; Park and Pariza, 2007; Stachowska *et al*., 2009).

In summary, the present study has shown that CLA could be included in market size European sea bass diets up to 1% for 20 weeks without compromising fish growth performance or proximate composition, improving liver morphology and reducing the potential of PGs production in head kidney. Besides, CLA isomers deposition levels in fish tissues were higher as the time of supplementation increase, obtaining the highest values when fish were fed CLA diets for 20 weeks.

Chapter 6

STUDY IV: Deposition of conjugated linoleic acid (CLA) in market size European sea bass (*Dicentrarchus labrax*) fillet and its effects on sensory and texture analyses

Abstract

Previous studies showed that conjugated linoleic acid (CLA) could be incorporated in diets for European sea bass juveniles (*Dicentrarchus labrax*) up to 4% without compromising growth performance and increasing isomer deposition in muscle tissue. CLA has antioxidative, anticarcinogenic, immunemodulative and diabetes modulation properties. The time course deposition of CLA and its effects on sensory and texture analyses has not been evaluated previously in this species. Four hundred and eighty six European sea bass (151.8 ± 2.26 g) were randomly allocated in 18 indoor 1 m^3 fibreglass tanks (4.1 kg/m^3) and assigned three diets containing graded amounts of CLA (0, 0.5 and 1%) which were administered for different periods during 20 weeks. Every 4 weeks fish were sampled for muscle proximate composition and fatty acid profile. At the end of the experiment fillet sensory and texture parameters were evaluated. Growth performance, feed conversion ratio and muscle proximate composition were not affected by dietary treatments. Muscle fatty acid profile was not affected by dietary treatments despite the increase of saturated fatty acids in fish fed diet with 1% of CLA for 16 weeks before harvest. Besides, the deposition levels of CLA isomers increased gradually depending on the CLA treatment, reaching its maximum level in fish fed 1% CLA diet for 20 weeks before harvest. CLA fillet

Keywords:
Conjugated linoleic acid
Dicentrarchus labrax
European sea bass
Fatty acids
Functional food
Sensory
Texture

accumulation levels were similar for those fish fed 1% CLA for 12 weeks and those fish fed 0.5% CLA diet for 20 weeks. Sensory analyses showed that fillet of fish fed 1% CLA diet were juicier ($P<0.05$) when compared to fish fed control diet, whereas fish fed 0.5% CLA diet had lower chewiness values than fish fed control diet. Texture parameters were not significantly affected by dietary CLA. These results suggest that market size European sea bass could successfully incorporate CLA isomers with positive effects on sensory analyses, reinforcing the potential of aquaculture fish as functional food.

6.1. Introduction

Aquaculture production has increased during the last decade. Improved food production systems and diet formulation helped to its development obtaining better market products. Several studies have reported different nutraceutical health properties of CLA, a group of positional and geometric isomers of linoleic acid (18:2n−6), in animal models such as body fat reduction (Whigham *et al*., 2007; Park and Pariza, 2009), anticarcinogenic effect (Ip *et al*., 1999; Kelley *et al*., 2007; Park and Pariza, 2009), prevention of cardiovascular diseases (Park and Pariza, 2009), inhibition of atherosclerosis (Lee *et al*., 1994; Kritchevsky *et al.*, 2004), reduced cholesterol and triacylglycerides levels (Lee *et al*., 2004; Bhattacharya *et al*., 2006), reduced inflammatory and improved immune responses (Cook *et al*., 1993; Yu *et al*., 2002; Bassaganya-Riera *et al*., 2003; Song *et al*., 2005; Bhattacharya *et al*., 2006) and bone mass (Park and Pariza, 2009). Human clinical studies reduced weight and adipose mass (Gaullier *et al*., 2007; Racine *et al*., 2010; Ing and Belury, 2011), reduced rheumatoid arthritis (Aryaeian *et al*., 2009), improved airway hyper-reactivity in mild asthmatics (MacRedmond *et al*., 2010), improved insulin sensitivity in sedentary individuals (Eyjolfson *et al*., 2004) and enhanced lipid metabolism (Noone *et al*., 2002).

Nowadays, the main sources of CLA include dietary supplements and few derived dairy products, even though the levels in these products are usually lower than 1% of total fatty acids (Manning *et al*., 2006; Schmid *et al*., 2006). CLA enrichment

trials of meat products like pork and broilers chicks have been conducted previously obtaining CLA levels lower or similar than those found in fish (Szymczyk *et al*., 2001; Lauridsen *et al*., 2005; Larsen *et al*., 2009; Cordero *et al*., 2010). In fish, the effects of CLA diet inclusion has been studied in different juvenile species, such as rainbow trout (*Oncorhynchus mykiss*) (Figueiredo-*Silva et al.*, 2005; Bandarra *et al*., 2006; Kennedy *et al*., 2007a, Atlantic salmon (*Salmo salar*) (Berge *et al*., 2004; Kennedy *et al*., 2005, 2006; Leaver *et al*., 2006), sea bream (*Sparus aurata*) (Diez *et al*., 2007), channel catfish (*Ictalurus punctatus*) (Manning *et al*., 2006), yellow perch (*Perca flavescens*) (Twibell *et al*., 2001), European sea bass (*Dicentrarchus labrax*) (Valente *et al.*, 2007a; Makol *et al*., 2009), hybrid striped bass (*Morone saxatilis* x *M. Chrysops*) (Twibell *et al*., 2000), Atlantic cod (*Gadus morhua*) (Kennedy *et al*., 2007b), yellow croacker (*Pseudosciaena crocea*) (Sang *et al*., 2007), tilapia (*Oreochromis niloticus*) (Choi *et al*., 1999; Yasmin *et al*., 2004) and rock fish (Choi *et al*., 1999). Unfortunately, only few of them comprised market size fish and none of them include texture analysis: rainbow trout (Kennedy *et al*., 2007a; Valente *et al*., 2007b; Ramos *et al*., 2008), carp (*Cyprinus carpio*) (Schwarz *et al*., 2002) and Nile tilapia (dos Santos *et al.,* 2011) and results obtained indicate the ability of fish to accumulate CLA in fillet with values similar or higher than those found in pork or chicken meat (Larsen *et al*., 2009; Cordero *et al*., 2010). However, the alteration of fillet proximate composition and fatty acid profile derive of CLA inclusion in diets may led in sensory alterations of the final product since muscle composition and fatty acids profile are the major responsible in texture and flavor of fish (Regost *et al*., 2004).

The objective of this study was to determine the effects of CLA supplementation on CLA accumulation along time previous to harvest as well as its effects on sensory and texture fillet properties of market size European sea bass.

6.2. Material and methods

6.2.1. Experimental diets

Three isonitrogenous and isoenergetic diets, based in a commercial formulation (Ecolife64), were designed in order to contain graded levels of CLA (CLA0=Control, CLA05=0.5% and CLA1=1%). CLA was supplied by BASF (Tarragona, Spain) as a supplement (50:50 mixture of each isomer as LUTA-CLA60®) and was added to the extruded diets in substitution of fish oil to maintain a constant energy content among dietary treatments. Diets were formulated and manufactured by a commercial feed producer (Biomar Iberia, S.A., Spain). Ingredients and proximate composition and fatty acid profiles are shown in Tables 6.1 and 6.2.

Table 6.1. Ingredients and proximate composition of diets with different levels of CLA (0%, 0.5% and 1%)

	Dietary CLA treatments		
	CLA0	**CLA05**	**CLA1**
Ingredients (%)			
Fish meal	41.00	41.00	41.00
Soyabean meal	25.00	25.00	25.00
Wheat	7.00	7.00	7.00
Corn gluten meal	4.50	4.50	4.50
Rapeseed meal	5.90	5.90	5.90
Vitamin and mineral	0.75	0.75	0.75
Fish oil[1]	15.85	15.12	14.38
CLA	0	0.73	1.47
Proximate composition (%)			
Crude protein	45.45	46.17	45.84
Crude fat	22.20	21.19	20.35
Ash	8.09	8.18	8.22
Moisture	3.66	3.44	3.82
Carbohydrates	24.25	24.46	25.59

Values are expressed as dry weight. Vitamin and mineral mix and CLA oil (LUTA-CLA 60®; containing 60% CLA methyl esters as a 50:50 mixture of c9, t11 and t10, c12 isomers), BASF (Spain), fish meal and fish oil (Sopropeche, France), soyabean meal (Cargill Inc., Spain), wheat (Hermanos Dueñas, Spain), corn gluten meal (SYRAL Iberia S.A.U., Spain), rapeseed meal (Esasa, Spain) [1] CLA was included at the expense of fish oil.

Table 6.2. Fatty acid composition (% of total identified fatty acids) of experimental diets

	Dietary CLA treatments		
Fatty acids	**CLA0**	**CLA05**	**CLA1**
14:0	7.76±0.05	7.70±0.03	7.68±0.06
14:1n-7	0.16±0.01	0.16±0.01	0.15±0.02
14:1n-5	0.12±0.01	0.09±0.02	0.09±0.03
15:0	0.51±0.02	0.59±0.06	0.52±0.04
16:0ISO	0.08±0.01	0.08±0.02	0.06±0.03
16:0	19.90±0.12	19.80±0.41	19.49±0.30
16:1n-7	9.57±0.25	9.28±0.31	8.96±0.39
16:1n-5	0.47±0.03	0.43±0.02	0.37±0.03
16:2n-6	1.55±0.01	1.60±0.03	1.48±0.04
16:2n-4	0.70±0.06	0.73±0.02	0.62±0.03
17:0	1.79±0.06	1.80±0.03	1.79±0.03
16:3n-4	0.11±0.02	0.10±0.02	0.11±0.01
16:3n-3	0.13±0.01	0.12±0.02	0.10±0.02
16:3n-1	0.05±0.01	0.05±0.00	0.05±0.01
16:4n-3	1.61±0.21	1.82±0.20	2.04±0.13
18:0	4.37±0.21	4.36±0.15	4.68±0.19
18:1n-9	13.03±0.09	13.05±0.02	13.04±0.04
18:1n-7	3.96±0.09	3.66±0.10	3.39±0.19
18:1n-5	0.20±0.02	0.18±0.03	0.17±0.02
18:2n-9	0.19±0.02	0.18±0.01	0.17±0.02
18:2n-6	4.02±0.10	4.14±0.09	4.24±0.06
18:2n-4	0.48±0.05	0.50±0.02	0.46±0.03
18:3n-6	0.24±0.04	0.26±0.03	0.27±0.02
18:3n-4	0.16±0.02	0.18±0.02	0.17±0.02
18:3n-3	0.83±0.06	0.84±0.03	0.90±0.05
18:3n-1	0.07±0.00	0.07±0.01	0.08±0.02
18:2 *c*9, *t*11	0.00±0.00	0.62±0.02	1.23±0.09
18:4n-3	1.65±0.13	1.76±0.19	1.99±0.11
18:2 *t*10, *c*12	0.00±0.00	0.74±0.03	1.40±0.10
18:4n-1	0.24±0.02	0.26±0.02	0.28±0.02
20:0	0.25±0.04	0.21±0.02	0.20±0.03
20:1n-9+n-7	1.05±0.05	0.95±0.04	0.88±0.04
20:1n-5	0.40±0.06	0.34±0.05	0.30±0.03
20:2n-9	0.20±0.05	0.17±0.03	0.15±0.04
20:2n-6	0.14±0.03	0.13±0.02	0.13±0.02
20:3n-6	0.20±0.02	0.15±0.03	0.15±0.02
20:4n-6	0.80±0.03	0.81±0.02	0.76±0.02
20:3n-3	0.06±0.01	0.06±0.00	0.06±0.00
20:4n-3	0.62±0.02	0.64±0.01	0.68±0.03
20:5n-3	12.71±0.26	12.12±0.15	11.73±0.29
22:1n-11	0.50±0.06	0.45±0.04	0.42±0.04
22:1n-9	0.22±0.02	0.18±0.06	0.18±0.05
22:4n-6	0.07±0.01	0.08±0.01	0.08±0.01
22:5n-6	0.25±0.02	0.26±0.01	0.29±0.02
22:5n-3	1.70±0.08	1.77±0.05	2.10±0.07
22:6n-3	6.87±0.26	6.49±0.34	5.91±0.21
Saturated	34.67±1.23	34.54±1.13	34.42±1.10
Monoenes	29.68±1.10	28.77±1.23	27.97±1.08
Σn-3	26.17±1.32	25.61±0.94	25.51±0.64
Σn-6	7.28±0.54	7.43±0.31	7.39±0.21
Σn-9	14.68±0.23	14.58±0.19	14.42±0.26
Σn-3 HUFA	21.95±0.91	21.07±0.46	20.47±0.39
Total CLA	0.00±0.00	1.36±0.09	2.63±0.10

CLA0, control diet; CLA05 and, diets with 0.5% and 1%,respectively; FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; CLA, conjugated linoleic acid.

6.2.2. Experimental conditions

European sea bass juveniles were maintained in stocking tanks and fed a commercial extruded diet (CLA0 diet) for 3 weeks until being well adapted to the environmental conditions. Afterwards, 486 fish were randomly distributed in 18 indoor 1 m^3, fibreglass tanks at initial stocking density of 4.1 kg/m^3 (27 fish/tank), supplied with filtered sea water (6 l/min water flow) and natural photoperiod (12L:12D).

Nine different treatment groups were established depending on the diet and on the feeding period of each diet. Control treatment (CLA0): control diet fed for 20 weeks before harvest, CLA05-20 treatment: CLA05 diet fed for 20 weeks before harvest, CLA05-16 treatment: CLA05 diet fed for 16 weeks before harvest, CLA05-12 treatment: CLA05 diet fed for 12 weeks before harvest, CLA05-8 treatment: CLA05 diet fed for 8 weeks before harvest, CLA1-20 treatment: CLA1 diet fed for 20 weeks before harvest, CLA1-16 treatment: CLA1 diet fed for 16 weeks before harvest, CLA1-12 treatment: CLA1 diet fed for 12 weeks before harvest and CLA1-8 treatment: CLA1 diet fed for 8 weeks before harvest. Each treatment was tested in duplicate.

Water temperature and dissolved oxygen along the feeding trial ranged 22-24°C and 6.34 ± 0.21 mg/l, respectively. Fish initial weight was 151.8 ± 2.26 g (mean ± SD). Fish were manually fed until apparent satiation, twice a day, 6 days a week for 20 weeks. After a fasting period of 24 hours, 10 fish from the initial stock and four fish per tank every 4 weeks were sampled for muscle proximate and fatty acid composition, growth parameters, hepatosomatic index (HSI), viscerosomatic index (VSI) and perivisceral fat index (PFI). At the end of the feeding period, 14 fish from treatments CLA0, CLA05-20 and CLA1-20 were randomly sampled, gutted and stored at 4ºC for 24h until sensory and texture tests were carried out. Feed conversion ratio (FCR) was defined as the amount of feed ingested divided by the generated biomass; specific growth rate (SGR) was defined as: [(ln final weight–ln initial weight)/number of days]x100; relative growth (%) defined as: [(final weight–initial weight)/initial weight)]x100 and PFI defined as: (visceral fat weight/body weight)x100; HSI defined as: (liver weight/body weight)×100 and VSI defined as: (viscera weight/body weight)×100.

6.2.3. Biochemical analyses

Fish tissues and diets biochemical composition was conducted following standard procedures (AOAC, 2000). Ash content was determined by combustion in a muffle furnace at 600°C for 12 h, moisture content was determined after drying at 105°C to constant weight, crude protein by acid digestion using Kjeldahl method (Nx6.25) and crude lipid was extracted following the method of Folch *et al*. (1957). Fatty acids from total lipids were prepared by base-catalysed transmethylation as described by Christie (1982) and separated by gas chromatography under the conditions described by Izquierdo *et al*. (1992), being quantified by flame ionizator detector (FID) and identified in comparison to external standards of CLA isomers (Sigma-Aldrich and Matreya, LLC). All analyses were conducted by triplicate.

6.2.4. Sensory analyses

Sensory tests were conducted on fish fillets cooked in aluminium boxes for 10 min in a steam oven (120°C). Immediately after cooking, fillets were offered to a panel of eleven selected and trained judges (ISO 1985, 1993). Tests were conducted in isolated and air-conditioned rooms with standardized light (ISO 1988). Judges were randomly offered closed food boxes labeled with codes containing fillets (3x4cm). Odour (intensity, marine and oily), appearance (colour, shininess and integrity), texture (firmness, juiciness, chewiness, adhesiveness and fatness) and flavour (intensity, marine, oily and aftertaste) were tested for samples of fish fed the different experimental diets and classified by trained judges in a continuous scale from 0 to 100 for each parameter. Detailed explanation of sensory parameters is shown in Table 6.3. Samples of fillets were also analyzed for biochemical and fatty acid composition and texture parameters.

Table 6.3. Sensory attributes for cooked European sea bass and attribute definitions.

Odour	
Intensity	Global odour intensity
Marine	Odour associated with seafood
Oily	Odour associated with fish oil
Appearance	
Colour	Intensity of white color in the uncut steak
Shininess	Intensity of shininess in the uncut steak
Integrity	Easiness to separate the miomers with the fork
Texture	
Firmness	Force required to deform the fillet between the tongue and palate
Juiciness	Amount of liquid released when the sample is chewed
Chewiness	Amount of chewing required before swallowing
Adhesiveness	Degree which the fillet clings to the teeth during chewing
Fatness	Degree of fat perception in the mouth during chewing
Flavour	
Intensity	Global flavour intensity
Marine	Flavour associated with seafood
Oily	Flavour associated with fish oil
Aftertaste	Degree of flavour persistence after swallowing

6.2.5. Texture analysis

Raw fillets from the left side of the same fish used in sensory analyses were tested for texture parameters. Skin was removed and four square pieces (2.5x2.5cm) were collected from each fillet above the lateral line. The texture analyser used was the TA-XT2 (Stable MicroSystems, England). A texture profile analysis (TPA) test was carried out using an aluminium compression plate with a 100 mm diameter. The compression rate was set to 0.8 mm/s and the strain to 80% penetration. Samples were compressed twice with a 60s interval between the two compressions (Tryggvadottir and Olafsdottir, 2000). Texture variables measured were fracture ability, hardness, springiness, cohesiveness, gumminess, chewiness, adhesiveness and resilience and were calculated as described by Ginés *et al.* (2004).

6.2.6. Statistical analyses

Means and Standard Deviations (SD) were calculated for each parameter measured. Statistical analyses followed methods outlined by Sokal and Rolf (1995). Data were submitted to a one-way analysis of variance (ANOVA) in order to analyze the effects of the different levels of CLA inclusion. When data did not pass a normality test, a log transformation was used to normalize data. When *F* values showed significance, individual means were compared using Tukey's test for multiple means comparison. Significant differences were considered for $P<0.05$.

6.3. Results

6.3.1. Growth performance

European sea bass fed experimental diets during 20 weeks did not show differences in growth performance, FCR, SGR or condition factor (K) among diets. Furthermore, HSI, VSI or PFI were not affected by dietary CLA inclusion or time of administration (Table 6.4).

6.3.2. Muscle proximate composition and fatty acid profile

By the end of the feeding trial, dietary CLA inclusion and time of administration did not modify muscle lipid, protein, moisture or ash proximate composition (Table 6.5). When compared to CLA0 diet, fish fed CLA1-16 diet showed an increase ($P<0.05$) in the total saturated fatty acids. Fish fed diet CLA1-20 had lower ($P<0.05$) levels of 18:0 when compared to fish fed CLA0, CLA05-12 and CLA05-16 diets. The monounsaturated fatty acids did not change among diets, but 18:1n-7 and 18:1n-5 fatty acids were reduced ($P<0.05$) in fish fed CLA05-12, CLA05-16 and CLA1-8 diets, and in fish fed CLA05-12 and CLA1-16 diets, respectively, when compared to CLA0, CLA05-20 and CLA1-50 diets. When compared to CLA0 diet, fish fed CLA05-12 and CLA1-16 diets presented lower ($P<0.05$) levels of 18:2n-9. Fish fed CLA05-12 diet also had lower

(P<0.05) levels of 18:2n-4 when compared to CLA0 and CLA05-20 diets. The only n-6 fatty acid modified was 22:4n-6 which was reduced (P<0.05) in fish fed CLA05-12, CLA05-16 and CLA1-12 when compared to CLA0, CLA05-20 and CLA1-20 diets. The deposition of CLA isomers and total CLA in muscle tissue was higher (P<0.05) in all CLA supplemented diets when compared to CLA0 diet. Besides, fish fed CLA1-20 diet had the highest (P<0.05) levels of CLA when compared to the other diets. Among diets containing CLA, deposition levels were higher (P<0.05) in fish fed CLA05-16 and CLA05-20 when compared to CLA05-8 and CLA05-12 diets. Whereas fish fed CLA1-12 diet had higher (P<0.05) levels of CLA than fish fed CLA05-8, CLA05-12, CLA05-16 and CLA1-8 diets, and fish fed CLA1-16 diet had higher (P<0.05) CLA levels compared to fish fed CLA05-8, CLA05-12, CLA05-16, CLA05-20, CLA1-8 and CLA1-12 diets (Table 6.6). Total CLA accumulation markedly depended on the level of CLA inclusion and the time of administration (Figure 6.1A), resulting in positive correlations between time and dose of CLA ($y=0.0004x^2+0.0273x+0.0079$, $R^2=0.9893$, P=0.002 in CLA05 diet; $y=0.0008x^2+0.0579x-0.009$, $R^2=0.9969$, P=0.002, P=0.000 in CLA1 diet). Individual CLA isomers were accumulated without difference among them (Figure 6.1B and 1C) resulting also in positive correlations between time of administration and dose supplemented ($y=0.0003x^2+0.0136x+0.004$, $R^2=0.9876$, P=0.002, $y=0.0003x^2+0.0118x+0.0048$, $R^2=0.9827$, P=0.003 for *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in CLA05 diet respectively and $y=0.0005x^2+0.0287x-0.0039$, $R^2=0.9982$, P=0.000; $y=0.0003x^2+0.0294x-0.0034$, $R^2=0.9969$, P=0.001 for *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in CLA1 diet, respectively).

Table 6.4. Biological and growth parameters (*mean ± SD*) of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments								
	CLA0	**CLA05-8**	**CLA05-12**	**CLA05-16**	**CLA05-20**	**CLA1-8**	**CLA1-12**	**CLA1-16**	**CLA1-20**
Feed intake (g)	8321.0±162.2	8070.8±213.8	7845.2±287.0	8354.8±342.5	8173.5±284.7	7977.3±283.8	8617.4±162.8	8154.5±56.3	8371.0±321.6
Initial weight (g)	151.1±0.9	150.9±1.4	150.5±0.8	154.1±1.7	152.9±2.5	151.9±2.6	152.1±3.1	151.9±2.6	151.3±1.0
Final weight (g)	354.5±29.7	375.1±75.3	328.9±33.5	362.9±52.7	374.4±48.0	372.5±50.7	357.7±533.9	387.7±51.4	363.0±65.1
Eviscerated weight (g)	311.6±28.9	333.2±69.0	285.9±35.9	320.2±46.7	333.7±43.8	332.6±45.2	310.9±29.3	341.6±41.3	299.4±45.0
Final length (cm)	29.1±0.5	29.4±1.7	28.4±1.1	29.2±1.4	29.8±0.6	30.1±1.3	29.4±1.0	29.9±1.3	29.3±1.2
K	1.7±0.06	1.8±0.06	1.8±0.02	1.7±0.04	1.8±0.03	1.7±0.04	1.8±0.05	1.8±0.04	1.7±0.05
SGR (%)	0.6±0.02	0.6±0.02	0.6±0.03	0.7±0.01	0.7±0.03	0.7±0.03	0.7±0.03	0.7±0.01	0.7±0.03
FCR	1.9±0.13	1.8±0.18	1.7±0.06	1.8±0.07	1.7±0.14	1.8±0.14	1.7±0.00	1.7±0.05	1.9±0.15
VSI (%)	12.2±1.5	11.2±1.6	13.1±5.5	11.7±1.1	10.9±1.2	10.7±1.3	13.0±3.2	11.7±3.1	12.7±2.0
HSI (%)	2.3±0.3	2.3±0.5	2.2±0.9	2.2±0.2	2.2±0.4	2.2±0.4	2.3±0.5	2.3±0.3	2.3±0.6
PFI (%)	6.0±1.8	5.4±1.8	5.2±1.2	5.4±0.7	5.0±1.3	5.1±1.1	5.5±2.3	5.7±1.7	6.4±1.6

Data are presented as means ± SD. Initial weight (n=486), final weight and length, relative growth, SGR, FCR and feed intake (n=54). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments ($P<0.05$). CLA, conjugated linoleic acid; FCR, feed conversion ratio; HSI, Hepatosomatic index; SGR, specific growth rate; VSI,Viscerosomatic index; PFI, perivisceral fat index; CLA0, control diet; CLA05 and CLA1 diets with 0.5% and 1% ofCLA inclusion, respectively. CLA0, control diet fed for 20 weeks before harvest; CLA05-20, CLA05 diet fed for 20 weeks before harvest; CLA05-16, CLA05 diet fed for 16 weeks before harvest; CLA05-12, CLA05 diet fed for 12 weeks before harvest; CLA05-8, CLA05 diet fed for 8 weeks before harvest; CLA1-20, CLA1 diet fed for 20 weeks before harvest; CLA1-16, CLA1 diet fed for 16 weeks before harvest; CLA1-12, CLA1 diet fed for 12 weeks before harvest and CLA1-8, CLA1 diet fed for 8 weeks before harvest.

Table 6.5. Muscle biochemical composition of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

		Biochemical composition			
		Lipids	**Proteins**	**Ash**	**Moisture**
Dietary CLA treatments	**CLA0**	20.49±1.81	74.50±3.78	1.44±0.11	72.86±1.27
	CLA05-8	15.94±3.16	78.31±4.20	1.42±0.01	72.82±0.75
	CLA05-12	21.43±0.48	73.23±4.91	1.41±0.06	71.58±0.20
	CLA05-16	19.42±6.16	75.18±2.95	1.46±0.16	72.02±2.16
	CLA05-20	24.64±3.93	72.25±0.62	1.45±0.04	69.63±2.49
	CLA1-8	15.12±4.99	78.43±2.12	1.54±0.10	73.19±1.08
	CLA1-12	17.84±1.20	76.40±2.16	1.45±0.12	73.19±1.08
	CLA1-16	19.08±2.49	75.21±3.36	1.49±0.09	72.10±0.80
	CLA1-20	19.12±3.87	75.40±2.52	1.47±0.04	71.85±1.58

Data are presented as means ± SD (n=9). Values are expressed as dry weight. Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively. CLA0, control diet fed for 20 weeks before harvest; CLA05-20, CLA05 diet fed for 20 weeks before harvest; CLA05-16, CLA05 diet fed for 16 weeks before harvest; CLA05-12, CLA05 diet fed for 12 weeks before harvest; CLA05-8, CLA05 diet fed for 8 weeks before harvest,;CLA1-20, CLA1 diet fed for 20 weeks before harvest; CLA1-16, CLA1 diet fed for 16 weeks before harvest; CLA1-12, CLA1 diet fed for 12 weeks before harvest and CLA1-8, CLA1 diet fed for 8 weeks before harvest.

6.3.3. Sensory analyses

No differences were found in proximate composition among diets of fish used in sensory analyses (Table 6.7). Fillet fatty acid profile of fish fed diets containing CLA showed an increased (P<0.05) deposition levels of CLA isomers and total CLA (Table 6.8). Sensory analyses showed that fish fed CLA1 diet had higher (P<0.05) juiciness than fish fed CLA0 diet. Besides, fish fed CLA05 diet had lower (P<0.05) chewiness than fish fed CLA0 diet. No differences among diets were found on the other parameters studied (Figure 6.2).

6.3.4. Texture analysis

No differences were found in proximate composition among diets of fish used in texture analyses (Table 6.9). Texture analyses showed that fish fed CLA diets had slightly higher values in fracture ability, cohesiveness and gumminess; whereas springiness and adhesiveness were slightly lower in the same diets, being springiness negatively correlated with dietary CLA levels ($y=-0.0105x+0.2183$; $R^2=0.9724$; $P=0.035$) (Table 6.9).

Table 6.6. Muscle fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments								
Fatty acids	**CLA0**	**CLA05-8**	**CLA05-12**	**CLA05-16**	**CLA05-20**	**CLA1-8**	**CLA1-12**	**CLA1-16**	**CLA1-20**
14:0	4.10±0.12	4.14±0.40	4.41±0.02	4.11±0.04	4.47±0.19	3.95±0.27	4.41±0.20	4.41±0.20	4.20±0.15
14:1n-7	0.08±0.00	0.09±0.01	0.09±0.00	0.09±0.00	0.09±0.01	0.08±0.01	0.09±0.00	0.09±0.01	0.09±0.00
14:1n-5	0.07±0.01	0.10±0.01	0.11±0.01	0.11±0.01	0.06±0.00	0.13±0.01	0.11±0.02	0.10±0.01	0.09±0.02
15:0	0.32±0.01	0.34±0.02	0.34±0.01	0.34±0.00	0.34±0.01	0.32±0.02	0.34±0.01	0.35±0.2	0.34±0.00
16:0ISO	0.05±0.00	0.05±0.00	0.06±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.06±0.02	0.05±0.00
16:0	18.06±0.26	18.81±0.22	18.65±0.2	18.25±0.5	18.61±1.0	18.37±0.8	18.35±0.4	19.17±0.2	18.76±0.1
16:1n-7	7.10±0.06	6.87±0.52	7.03±0.04	6.85±0.09	7.21±0.21	6.58±0.31	6.94±0.25	7.08±0.20	6.67±0.26
16:1n-5	0.26±0.01	0.24±0.01	0.24±0.00	0.24±0.01	0.26±0.00	0.23±0.02	0.24±0.01	0.25±0.01	0.25±0.01
16:2n-6	1.02±0.03	0.98±0.03	1.02±0.03	0.99±0.02	1.05±0.05	0.93±0.03	1.04±0.03	0.94±0.02	1.00±0.02
16:2n-4	0.44±0.01	0.43±0.07	0.45±0.01	0.41±0.03	0.47±0.01	0.58±0.04	0.54±0.04	0.45±0.04	0.49±0.01
17:0	1.12±0.03	1.11±0.09	1.14±0.00	1.11±0.02	1.12±0.07	1.04±0.04	1.10±0.08	1.11±0.05	1.03±0.05
16:3n-4	0.06±0.00	0.05±0.01	0.06±0.00	0.06±0.00	0.06±0.01	0.05±0.00	0.06±0.00	0.06±0.00	0.06±0.00
16:3n-3	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.00	0.08±0.01	0.08±0.00	0.08±0.00	0.08±0.00
16:3n-1	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00
16:4n-3	0.77±0.03	0.76±0.07	0.80±0.00	0.77±0.03	0.75±0.09	0.70±0.03	0.75±0.11	0.74±0.05	0.67±0.07
18:0	3.68±0.31	4.22±0.32	3.89±0.14	3.95±0.35	4.04±0.38	4.19±0.24	4.25±0.19	4.27±0.20	4.35±0.34
18:1n-9	17.72±0.28	16.88±0.76	17.69±0.5	17.26±1.0	17.78±0.9	16.83±0.5	17.11±0.6	17.15±0.9	17.20±1.3
18:1n-7	3.29±0.11	3.21±0.07	3.11±0.09	3.10±0.12	3.32±0.01	3.08±0.15	3.03±0.14	3.16±0.08	3.30±0.09
18:1n-5	0.27±0.04	0.22±0.04	0.18±0.04	0.23±0.02	0.26±0.02	0.23±0.01	0.21±0.03	0.19±0.04	0.27±0.03
18:2n-9	0.35±0.04	0.29±0.04	0.29±0.02	0.32±0.01	0.31±0.02	0.31±0.01	0.27±0.05	0.25±0.06	0.29±0.02
18:2n-6	7.34±0.94	6.78±0.65	7.53±0.06	7.13±1.6	7.74±0.30	7.25±0.93	7.58±1.59	7.17±0.61	7.02±0.70
18:2n-4	0.48±0.02	0.46±0.02	0.43±0.03	0.46±0.01	0.48±0.02	0.45±0.01	0.45±0.02	0.45±0.01	0.46±0.01
18:3n-6	0.25±0.01	0.24±0.01	0.23±0.00	0.25±0.01	0.25±0.01	0.24±0.01	0.24±0.01	0.23±0.01	0.24±0.01
18:3n-4	0.17±0.01	0.17±0.01	0.16±0.00	0.17±0.01	0.17±0.01	0.16±0.00	0.17±0.02	0.16±0.02	0.17±0.01
18:3n-3	1.16±0.06	0.99±0.07	1.13±0.03	1.06±0.13	1.08±0.05	1.05±0.10	1.08±0.11	1.00±0.05	1.08±0.05
18:3n-1	0.05±0.00	0.04±0.01	0.05±0.00	0.04±0.01	0.05±0.00	0.04±0.00	0.05±0.01	0.04±0.01	0.05±0.00
18:2 *c*9,*t*11	0.00±0.00^{a}	0.15±0.00^{b}	0.18±0.02^{b}	0.29±0.03^{cf}	0.38±0.02^{f}	0.24±0.04^{b}	0.43±0.05^{d}	0.59±0.07^{e}	0.77±0.05^{g}
18:4n-3	1.28±0.02	1.22±0.09	1.26±0.01	1.25±0.03	1.24±0.12	1.17±0.06	1.22±0.12	1.21±0.04	1.16±0.06
18:2 *t*10,*c*12	0.00±0.00^{a}	0.14±0.01^{b}	0.16±0.02^{b}	0.26±0.02^{cf}	0.35±0.02^{f}	0.23±0.04^{b}	0.41±0.05^{d}	0.53±0.05^{e}	0.69±0.04^{g}
18:4n-1	0.21±0.01	0.20±0.01	0.19±0.00	0.20±0.01	0.21±0.02	0.20±0.01	0.21±0.04	0.20±0.01	0.20±0.01
20:0	0.13±0.01	0.13±0.01	0.13±0.00	0.13±0.00	0.14±0.00	0.13±0.02	0.14±0.01	0.12±0.01	0.13±0.00
20:1n-9+n-7	1.11±0.09	1.07±0.03	1.18±0.09	1.16±0.09	1.14±0.03	1.08±0.09	1.14±0.11	1.10±0.02	1.27±0.17
20:1n-7	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.02±0.03	0.00±0.00	0.00±0.00
20:1n-5	0.23±0.02	0.21±0.01	0.22±0.00	0.21±0.00	0.24±0.01	0.21±0.01	0.22±0.01	0.22±0.01	0.23±0.01
20:2n-9	0.11±0.02	0.12±0.01	0.11±0.00	0.12±0.00	0.11±0.01	0.11±0.00	0.11±0.01	0.12±0.00	0.10±0.01
20:2n-6	0.42±0.03	0.42±0.03	0.43±0.01	0.45±0.05	0.42±0.01	0.42±0.05	0.42±0.03	0.42±0.02	0.41±0.03
20:3n-9	0.00±0.00	0.00±0.00	0.02±0.02	0.01±0.02	0.00±0.00	0.01±0.02	0.01±0.02	0.00±0.00	0.00±0.00
20:3n-6	0.12±0.01	0.06±0.00	0.09±0.03	0.08±0.04	0.10±.04	0.08±0.03	0.08±0.03	0.06±0.00	0.08±0.04
20:4n-6	0.89±0.06	1.02±0.07	0.88±0.02	0.92±0.12	0.82±0.03	1.02±0.09	0.91±0.03	0.90±0.08	0.90±0.11
20:3n-3	0.06±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.05±0.00	0.06±0.00
20:4n-3	0.57±0.02	0.55±0.01	0.55±0.01	0.58±0.03	0.57±0.05	0.55±0.02	0.58±0.06	0.56±0.01	0.58±0.00
20:5n-3	12.50±0.23	12.58±0.26	11.86±0.0	12.47±0.3	11.69±1.0	12.35±0.1	11.87±0.9	11.79±0.5	11.22±0.6
22:1n-11	0.24±0.06	0.21±0.02	0.30±0.09	0.28±0.08	0.25±0.01	0.24±0.07	0.28±0.09	0.22±0.01	0.37±0.15
22:1n-9	0.14±0.02	0.13±0.01	0.15±0.02	0.14±0.01	0.15±0.00	0.13±0.02	0.15±0.01	0.14±0.01	0.17±0.02
22:4n-6	0.08±0.00^{a}	0.06±0.01^{abc}	0.05±0.00^{b}	0.05±0.01^{b}	0.08±0.00^{a}	0.06±0.01^{a}	0.05±0.00^{b}	0.05±0.00^{b}	0.08±0.00^{a}
22:5n-6	0.31±0.01	0.32±0.02	0.30±0.01	0.31±0.02	0.28±0.02	0.31±0.01	0.30±0.01	0.30±0.02	0.30±0.03
22:5n-3	2.20±0.07	2.14±0.06	2.08±0.08	2.22±0.13	2.04±0.18	2.21±0.03	2.15±0.23	2.04±0.11	2.12±0.22
22:6n-3	11.05±0.53	11.64±0.96	10.64±0.6	11.36±1.0	9.58±0.88	12.21±0.5	10.68±0.5	10.38±0.9	10.96±1.0
Saturated	27.47±0.24	28.80±0.47^{ab}	28.61±0.3	27.94±0.8	28.77±1.4	28.05±1.2	28.63±0.3	29.49±0.3	29.26±0.1
Monoenes	30.54±0.07	29.23±0.60	30.30±0.3	29.67±1.1	30.77±1.0	28.83±0.6	29.54±0.2	29.70±1.0	29.89±1.9
Σn-3	29.66±0.71	30.02±0.99	28.45±0.7	29.84±1.4	27.10±2.2	30.38±0.5	28.47±1.7	27.86±1.6	27.53±2.2
Σn-6	10.43±0.90	9.87±0.62	10.56±0.1	10.17±1.0	10.75±0.3	10.32±0.9	10.62±1.6	10.07±.05	10.02±0.6
Σn-9	19.44±0.18	18.48±0.75	19.39±0.4	19.01±1.1	19.50±0.9	18.47±0.6	18.79±0.6	18.76±1.0	19.04±1.5
Σn-3 HUFA	26.37±0.77	26.96±1.13	25.18±0.6	26.68±1.5	23.94±2.0	27.37±0.5	25.34±1.6	24.82±1.6	24.54±2.2
Total CLA	0.00±0.00^{a}	0.29±0.01^{b}	0.35±0.04^{b}	0.55±0.05^{c}	0.72±0.03^{e}	0.46±0.07^{b}	0.84±0.10^{e}	1.12±0.12^{f}	1.45±0.09^{g}

Data are presented as means±SD (n=9). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively. CLA0, control diet fed for 20 weeks before harvest; CLA05-20, CLA05 diet fed for 20 weeks before harvest; CLA05-16, CLA05 diet fed for 16 weeks before harvest; CLA05-12, CLA05 diet fed for 12 weeks before harvest; CLA05-8, CLA05 diet fed for 8 weeks before harvest,;CLA1-20, CLA1 diet fed for 20 weeks before harvest; CLA1-16, CLA1 diet fed for 16 weeks before harvest; CLA1-12, CLA1 diet fed for 12 weeks before harvest and CLA1-8, CLA1 diet fed for 8 weeks before harvest. FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

Table 6.7. Muscle biochemical composition of European sea bass used for sensory and texture analyses fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments		
	CLA0	**CLA05**	**CLA1**
Lipids (%)	13.54±0.46	13.64±1.07	15.02±1.00
Protein (%)	80.45±0.90	82.14±0.67	80.43±1.84
Ash (%)	1.50±0.05	1.57±0.03	1.49±0.01
Moisture (%)	73.97±0.59	73.85±0.10	73.93±0.66

Data are expressed as dry weight. Data are presented as means ± SD (n=14). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively.

6.4. Discussion

Growth performance was not affected by dietary CLA inclusion in European sea bass diets during the experiment as seen in earlier studies in the same species fed 0.5-2% CLA for 12 weeks (Valente *et al*., 2007a; Makol *et al*., 2009) and with those found in different market size fish species such as rainbow trout fed 0.5-1% CLA for 8 and 12 weeks (Kennedy *et al*., 2007a Ramos *et al*., 2008), carp fed 1% CLA for 8 weeks (Schwarz *et al*., 2002) and Nile tilapia fed 5-10% CLA for 8 weeks (dos Santos *et al*., 2011) or those described in other fish species juveniles fed 0.5-5% CLA for up to 12 weeks (Twibell *et al*., 2000, 2001; Twibell and Wilson, 2003; Berge *et al*., 2004; Yasmin *et al*., 2004; Bandarra *et al*., 2006; Manning *et al*., 2006; Kennedy *et al*., 2007b; Jiang *et al*., 2010). However, in other studies growth performance was reduced in European sea bass fed 2-4% CLA for 12 weeks (Diez *et al*., 2007) and yellow catfish fed 0.5-2% CLA for 8 weeks (Tan *et al*., 2010). Regarding muscle proximate composition, no effect was noticed by dietary CLA inclusion or different period of supplementation as reported in yellow perch fed 0.5-1% CLA for 9 weeks (Twibell *et al*., 2001), channel catfish fed 0.5-1% CLA for 8 weeks (Twibell and Wilson, 2003), Atlantic salmon fed 2% CLA for 12 weeks (Berge *et al*., 2004), rainbow trout fed 0.5-2% CLA for 12 weeks (Bandarra *et al.*, 2006; Valente *et al*., 2007b; Ramos *et al*., 2008), Atlantic cod fed 0.5-1% CLA for 12 weeks (Kennedy *et al*., 2007b), yellow catfish fed 0.5-2% CLA for 8 weeks (Tan *et al*.,

Table 6.8. Muscle fatty acids profile (% of total identified fatty acids) of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks used for sensory and texture analyses.

	Dietary CLA treatments		
Fatty acids	**CLA0**	**CLA05-20**	**CLA1-20**
14:0	4.01±0.44	3.73±0.19	3.90±0.26
14:1n-7	0.09±0.01	0.08±0.00	0.08±0.00
14:1n-5	0.05±0.05	0.06±0.00	0.06±0.01
15:0	0.33±0.03	0.31±0.01	0.32±0.02
15:1n-5	0.01±0.01	0.00±0.00	0.01±0.01
16:0ISO	0.05±0.01	0.05±0.00	0.05±0.00
16:0	18.48±1.38	18.26±1.05	18.27±1.02
16:1n-7	6.79±0.51	6.23±0.25	6.31±0.35
16:1n-5	0.27±0.02	0.25±0.02	0.25±0.01
16:2n-6	1.00±0.07	0.94±0.03	0.95±0.03
16:2n-4	0.45±0.05	0.45±0.03	0.45±0.02
17:0	1.04±0.03	0.98±0.01	1.00±0.05
16:3n-4	0.05±0.00	0.05±0.00	0.05±0.00
16:3n-3	0.08±0.01	0.08±0.00	0.08±0.00
16:3n-1	0.03±0.00	0.03±0.00	0.03±0.00
16:4n-3	0.68±0.04	0.66±0.04	0.69±0.06
18:0	4.00±0.25	4.52±0.22	4.49±0.20
18:1n-9	17.13±0.38	16.33±0.63	16.43±0.74
18:1n-7	3.27±0.24	3.15±0.06	3.07±0.15
18:1n-5	0.28±0.02	0.26±0.00	0.23±0.05
18:2n-9	0.33±0.02	0.30±0.01	0.26±0.07
18:2n-6	6.99±0.32	6.81±0.19	6.96±0.03
18:2n-4	0.46±0.02	0.45±0.02	0.43±0.04
18:3n-6	0.24±0.01	0.23±0.00	0.23±0.01
18:3n-4	0.16±0.00	0.16±0.00	0.15±0.01
18:3n-3	1.01±0.07	0.98±0.04	1.00±0.04
18:3n-1	0.05±0.00	0.03±0.03	0.05±0.00
18:2 *c*9,*t*11	0.00±0.00[a]	0.38±0.07[b]	0.71±0.04[c]
18:4n-3	1.16±0.07	1.16±0.04	1.19±0.07
18:2 *t*10,*c*12	0.00±0.00[a]	0.34±0.06[b]	0.63±0.03[c]
18:4n-1	0.19±0.01	0.20±0.01	0.19±0.02
20:0	0.13±0.01	0.11±0.01	0.12±0.00
20:1n-9+n-7	1.17±0.18	1.07±0.10	1.07±0.05
20:1n-5	0.22±0.02	0.14±0.12	0.21±0.01
20:2n-9	0.11±0.01	0.10±0.00	0.10±0.00
20:2n-6	0.40±0.03	0.41±0.01	0.39±0.01
20:3n-9	0.00±0.00	0.01±0.02	0.00±0.00
20:3n-6	0.12±0.00	0.08±0.03	0.11±0.00
20:4n-6	1.03±0.02	1.05±0.01	1.00±0.09
20:3n-3	0.05±0.00	0.05±0.00	0.05±0.00
20:4n-3	0.54±0.02	0.55±0.01	0.55±0.02
20:5n-3	12.22±1.34	12.55±0.77	12.40±0.91
22:1n-11	0.32±0.13	0.24±0.09	0.23±0.06
22:1n-9	0.15±0.03	0.13±0.02	0.13±0.01
22:4n-6	0.08±0.00	0.08±0.00	0.08±0.00
22:5n-6	0.35±0.03	0.35±0.01	0.34±0.04
22:5n-3	2.10±0.25	2.22±0.17	2.09±0.16
22:6n-3	12.32±1.80	13.32±1.21	12.60±1.64
Saturated	28.04±2.13	27.90±1.48	28.16±1.41
Monoenes	29.75±1.45	28.03±0.95	28.07±1.22
Σn-3	30.16±3.48	31.56±2.27	30.64±2.74
Σn-6	10.21±0.36	9.96±0.19	10.07±0.07
Σn-9	18.90±0.57	17.95±0.73	17.99±0.79
Σn-3 HUFA	27.23±3.36	28.69±2.16	27.69±2.66
Total CLA	0.00±0.00[a]	0.72±0.13[b]	1.35±0.06[c]

Data are presented as means ± SD (n=14). Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments ($P<0.05$). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively. CLA0, control diet fed for 20 weeks before harvest; CLA05-20, CLA05 diet fed for 20 weeks before harvest; CLA1-20, CLA1 diet fed for 20 weeks before harvest. FA, fatty acid; HUFA, highly unsaturated fatty acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid, ARA, arachidonic acid; CLA, conjugated linoleic acid.

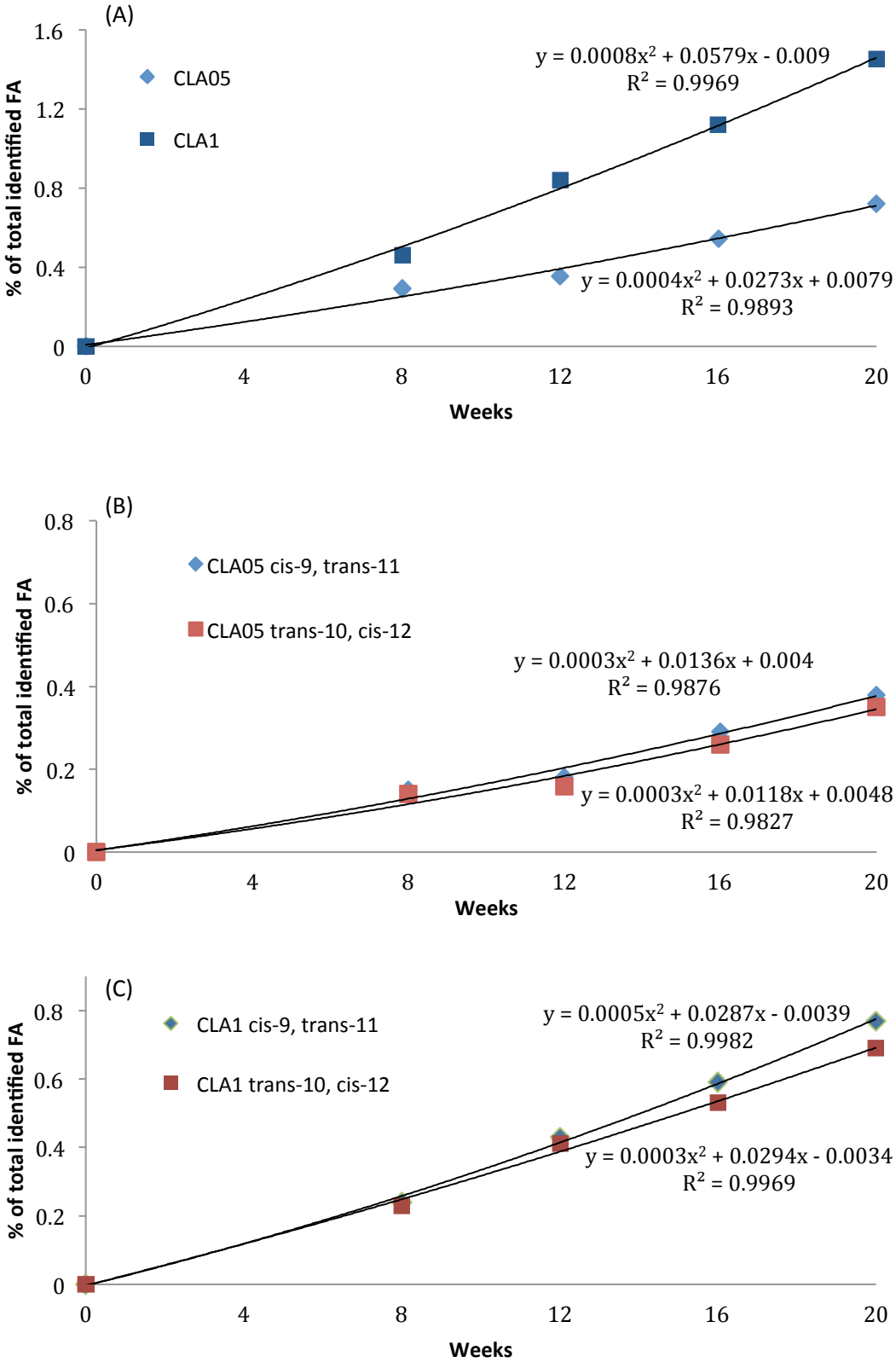


Figure 6.1. Time course accumulation and correlations of total CLA (A), CLA isomers in CLA05 diet (B) and CLA isomers in CLA1 diet (C).

Table 6.9. Instrumental texture measurements of raw fillets of European sea bass fed graded dietary levels of CLA (0, 0.5 and 1%) for 20 weeks

	Dietary CLA treatments		
	CLA0	**CLA05**	**CLA1**
Fracture ability	1671.44±742.39	1998.09±402.78	1792.88±594.90
Hardness	12101.30±4693.44	11756.12±2947.32	12106.15±4026.87
Springiness	0.21±0.04	0.20±0.03	0.19±0.03
Cohesiveness	4.66±1.20	4.92±0.89	4.87±0.85
Gumminess	2452.77±1408.57	2564.75±1110.48	2687.03±1317.27
Chewiness	515.67±357.04	502.94±240.77	520.72±289.79
Adhesiviness	-126.65±61.71	-113.26±45.76	-113.84±45.43
Resilience	0.10±0.03	0.10±0.02	0.10±0.03

Significance of differences between means was determined by one-way ANOVA followed by Tukey's multiple comparison post hoc test as described in the Materials and methods. Different superscript letters within rows indicates significant differences between treatments (P<0.05, n=11). CLA0, control diet; CLA05 and CLA1, diets with 0.5% and 1% of CLA inclusion, respectively.

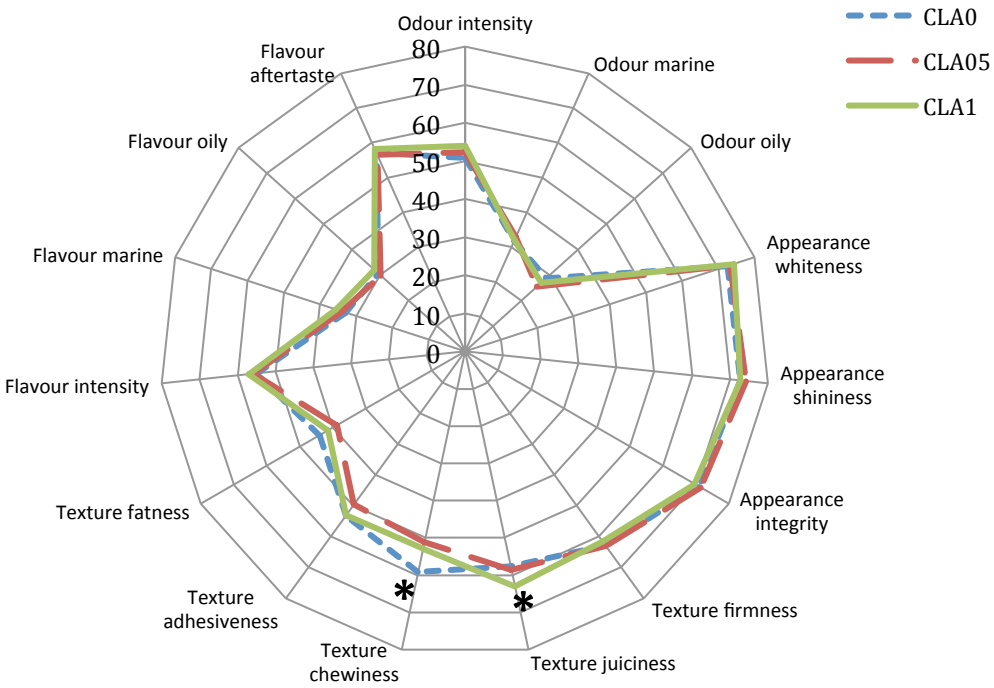


Figure 6.2. Scores of principal sensory parameters evaluated of sea bass fillets fed experimental diets during 20 weeks. Odour (intensity, marine and oily), appearance (whiteness, shininess and integrity), texture (firmness, juiciness, chewiness, adhesiveness and fatness) and flavour (intensity, marine, oily and aftertaste). Values are presented as percent of variation from fish fed dietary CLA. Symbol denotes significant differences ($P < 0.05$, $n = 11$).

2010) and European sea bass fed 0.5-2% for 12 weeks (Makol *et al*., 2009). However, a reduction in muscle total lipids was observed in large yellow croacker fed 2-4% for 8 weeks (Sang *et al*., 2007) and Nile tilapia fed 5-10% for 8 weeks (dos Santos *et al*., 2011), and an increase in Atlantic salmon fed 1-2% CLA for 12 weeks (Kennedy *et al*., 2005). These results suggest that CLA effects in fish may depend on species, fish size, time of administration, CLA dose or even chemical form of CLA included in diets.

The total percentage of the main fatty acid groups were not as strongly affected by dietary CLA inclusion as they were in European sea bass juveniles fed 0.5-2% CLA for 12 weeks (Valente *et al*., 2007a; Makol *et al*., 2009). Similar results were obtained in market size rainbow trout fed 0.5-1% CLA for 12 weeks (Valente *et al*., 2007b; Ramos *et al*., 2008), suggesting the effect of the developmental stage of fish on lipid metabolism. There was only an increase in the saturated fraction in fish fed 1% of dietary CLA sixteen weeks before harvest, mainly due to the slight increase of palmitic acid (16:0) and stearic acid (18:0). Both CLA isomers, *cis*-9, *trans*-11 and *trans*-10, *cis*-12 were successfully incorporated into muscle lipids as previously reported in European sea bass juveniles fed 0.5-2% CLA for 12 weeks (Makol *et al*., 2009) as well as in market size rainbow trout fed 0.5-1% CLA for 12 weeks (Valente *et al*., 2007b; Ramos *et al*., 2008). Eight weeks of 1% CLA supplementation led to an increase of both isomers similar to those fish fed with 0.5% CLA up to 16 weeks (0.46 % of total fatty acids; 0.15 mg/g muscle). On the contrary, feeding fish with 1% of CLA for 12 weeks were enough to obtain CLA deposition levels similar to those fish fed 0.5% CLA for 20 weeks (0.84 % of total fatty acids; 0.38 mg/g muscle). Longer feeding times with 1% CLA diets led to higher CLA deposition levels up to 1.45% of total fatty acids (0.68 mg/g muscle). Results are slightly different to those found previously in European sea bass juveniles fed 0.5-2% CLA for 12 weeks (Makol *et al*., 2009) if expressed in percentage (1.83 % of total fatty acids), but became similar if expressed as fillet CLA content (0.73 mg/g muscle) due to the different muscle lipid content when comparing juveniles and adult fish. Similar results were found in rainbow trout fed 0.5-1% CLA for 12 weeks (Kennedy *et al*., 2007a; Ramos *et al*., 2008) where smaller fish size reflected lower CLA levels in muscle when expressed as percentage but became similar when expressed as

mg CLA/g muscle. In humans, dietary CLA intake is estimated about 95-440 mg/day differing by country (Schmid *et al*., 2006), but higher levels (2.4-3.5 g/day) seems to be needed to promote human health benefits (Terpstra, 2004; Racine *et al*., 2010). By eating 200 g of fish fillet from fish fed 1% of CLA for 20 weeks before harvest, consumers will ingest extra 136 mg of CLA, which is almost the 50% of the total human daily intake in some western populations (Ramos *et al*., 2008) and then contributing to increase the total daily CLA ingestion.

No differences were found in proximate composition among diets of fish used in sensory and texture analyses. Sensory analyses showed that fish fed 1% CLA diet were juicier than fish fed the control diet, which could be related with the slight increase in lipid content found in muscle. Besides, fish fed control diet had higher chewiness than fish fed 0.5% CLA diet what may be related by the slight lower adhesiveness and fatness found in the same diet. These results suggest that fish fed CLA diets would be well accepted by consumers since fish fillets enriched with CLA did not have different odour or flavor when compared to fish fed control diet. Few studies have been conducted in sensory and texture analyses in fish fed CLA diets. In rainbow trout, Schabbel *et al*. (2004) and Ramos *et al.* (2008) did not find differences in sensory analyses when fish were fed diets containing CLA up to 3% and 1%, respectively for 12 weeks. However, Valente *et al.* (2007b) found in the same species slight differences between fish fed CLA diets up to 1 % for 12 weeks and the control group, these results were controversial since half of the panelists considered these differences positive and the other half negative.

No differences were found in texture analysis of raw fillets among treatments, but there was a negative correlation between CLA inclusion level and springiness. Furthermore, fish fed CLA diets had slightly higher values in fracture ability, cohesiveness and gumminess, which maybe could be related with a lower proteolytic degradation rate of muscle tissue in fish fed CLA diets or to a better intramuscular collagen conservation in CLA diets as collagen is the main constituent of the connective tissue. Further analyses should be done to elucidate the effects of CLA on proteolytic degradation and collagen conservation. These results are in agreement with those

found in Nile tilapia filet texture, either cooked or raw, where it was not affected by dietary CLA when included up to 10% for 8 weeks (dos Santos *et al*., 2010). In pork meat, texture was also not affected by dietary CLA inclusion (Tischendorf *et al*., 2002; Lauridsen *et al.*, 2005; Lorenzen *et al*., 2007; Intarapichet *et al.*, 2008), whereas in broilers breast meat was harder and drier (Du and Ahn, 2002). Then, it seems again that dietary CLA effects are species related.

In summary, results obtained in this study clearly show that CLA can be included in fish diets up to 1% and administered for up to 20 weeks before harvest without compromising fish growth performance, feed efficiency and muscle proximate composition. Both CLA isomers included in fish diets were successfully incorporated into muscle lipids, obtaining a functional food and being CLA concentrations on fish fillet dependent on both, the level of CLA inclusion and time of supplementation. Besides, dietary CLA have positive effects on sensory analyses and no significant effects on texture. Further studies are recommended in order to determine if muscle CLA is retained when dietary CLA is restricted before harvesting.

In conclusion, it is possible to enrich European sea bass fillets with the same levels of CLA in different ways, by increasing CLA inclusion level in fish diets so it will occur in lesser time, or reducing CLA inclusion level for a longer time. Besides, it is possible to achieve higher CLA fillet levels with higher concentrations (1% of CLA) and longer times up to 20 weeks before harvest.

Chapter 7

Conclusions

1. Dietary CLA can be incorporated in European sea bass diets up to 4% without compromising its culture performance neither in juveniles nor in market size fish.

2. Dietary CLA inclusion of even up to 4% in European sea bass diets did not affect negatively nutrient digestibility.

3. CLA was accumulated in all tissues in a dose-dependent manner in relation to the inclusion level supplemented, regardless of the culture density and fish size.

4. Increase in dietary CLA up to 4% was correlated with a reduction in perivisceral fat deposition, this effect being more pronounced when fish is reared at lower densities (2 Kg/m^3).

5. CLA inclusion at 0.5% in market size European sea bass diets resulted in a reduction in adipocytes area and an increase in adipocyte number, in relation to the tendency to reduced perivisceral fat deposition.

6. Dietary CLA inclusion up to 2% resulted in lower lipid vacuolization and reduction in hepatocytes area regardless of the culture density and fish size.

7. The activity of the lipolytic enzyme Acyl-CoA oxidase increased in liver of European sea bass with the inclusion of CLA at 2 and 4%.

8. CLA inclusion in European sea bass juveniles diets up to 2% and reared at low densities (2 Kg/m^3) reduced muscle saturated and monounsaturated fatty acids and increased n-3 and n-3 HUFA fatty acids. However, increase in either fish size or density masked this effect, and only the saturated fatty acids were reduced in market size fish fed 1% CLA.

9. Dietary CLA inclusion positively affected immune system in juvenile sea bass, increasing the serum lysozyme activity in lower density reared fish (up to 1% CLA) and the phagocytic activity of head kidney leucocytes in higher density reared fish (4 % CLA). No effects were observed in market size fish.

10. CLA supplementation for 20 weeks tend to improve sensory properties of European sea bass fillet, increasing increased fillet juiciness at 1% CLA and reducing fillet chewiness at 0.5% CLA.

Chapter 8

Resumen

8.1. INTRODUCCIÓN

8.1.1. La producción acuícola de lubina (*Dicentrarchus labrax*)

Según el último informe APROMAR (2011), cerca del 50% del total de alimentos acuáticos consumidos a nivel mundial provienen de la acuicultura y se prevee que alcanzará valores cercanos al 65% en 2030. En 2009, la producción total de acuicultura para consumo humano alcanzó 73 MMT en contraste con las 65 MMT del sector pesquero. La producción acuícola ha aumentado de forma sorprendente desde 1950 donde se produjeron 0,6 MMT contrastando con las 73 MMT en 2009, representando un valor de mercado aproximado de 88120 millones de euros. Incluso, se percibe que el crecimiento exponencial del sector se ha minimizado durante la última década, disminuyendo la tasa de crecimiento anual de un 9% en los años 80 y 90, a un 6% durante la primera década del siglo XXI. Hecho especialmente acentuado en la región Europea. A pesar de estos datos, en 2009 la producción total de peces de acuicultura en la Comunidad Europea (UE) alcanzó las 629401 toneladas, aumentando un 0,9% desde 2008, lo que significó aproximadamente 2398 millones de euros. La producción acuícola española representa el 10,2% del total de la producción de la UE, alcanzando un valor de mercado de 286 millones de euros.

Respecto a la producción de peces marinos en el sur de Europa y las regiones Mediterráneas, la dorada (*Sparus aurata*), lubina (*Dicentrarchus labrax*) y rodaballo (*Psetta maxima*) son las principales especies cultivadas. Aunque actualmente el cultivo de corvina (*Argyrosomus regius*) es común en diversos países Mediterráneos. La producción de lubina alcanzó 118931 toneladas en 2010, siendo Grecia (39,6%), Turquía (29,5%) y España (10,5%) los productores principales. En 2010, la producción de lubina en España alcanzó las 12495 toneladas, lo que significó un descenso del 9,7% respecto a 2009, debido principalmente al aumento observado durante 2008-2009 del 40,7%. A pesar de ello, el crecimiento anual de la producción de lubina en España sigue siendo del 10% durante los últimos 4 años y todo apunta a que seguirá creciendo. Dentro de España, las Islas Canarias son la principal comunidad productora de lubina con un 30% del total de la producción española; dividiéndose el resto entre Andalucía, Murcia, Valencia y Cataluña.

Desafortunadamente, la tendencia actual de incrementar los contenidos energéticos en las dietas para peces, así como la inclusión de fuentes de proteínas o grasas de origen vegetal en sustitución de harinas y aceites de pescado en esas dietas (Caballero *et al*., 1999, 2004) ha derivado en un incremento de la deposición de grasa perivisceral, hepática y subcutánea que puede afectar a la calidad del producto final, así como una reducción de la optimización de la producción acuícola, con la consecuente preocupación de los acuicultores (Izquierdo *et al*., 2005). Una de las especies más propensa al exceso de acumulación de grasa es la lubina, donde la acumulación de grasa puede deberse a una respuesta fisiológica más que patológica (Izquierdo *et al*., 2003). Por lo que atendiendo a este hecho, así como a que el ácido linoléico conjugado (CLA) se ha demostrado que reduce la deposición de grasa en roedores y otros mamíferos (Park *et al*., 1999, 2005; Park y Pariza, 2007), se consideró que la lubina era un buen objetivo para su estudio en esta Tesis.

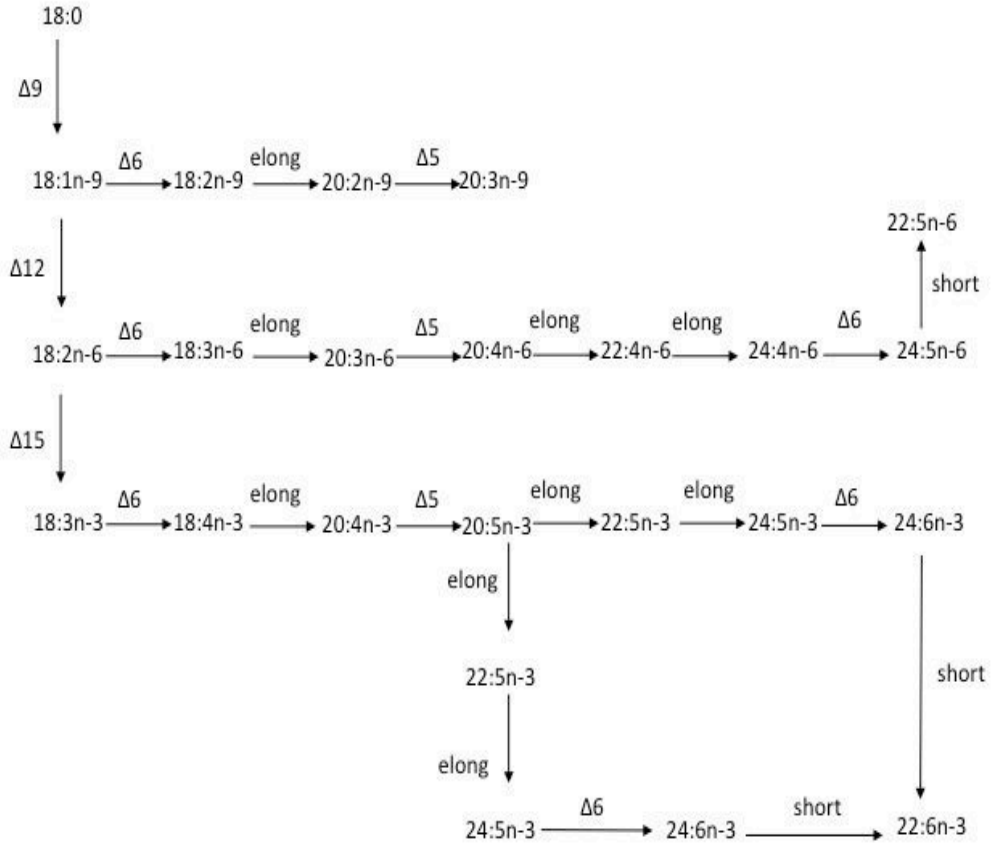
8.1.2. Lípidos y ácidos grasos

Los lípidos constituyen un amplio y diverso grupo de aceites y grasas que son compuestos orgánicos, solubles en solventes orgánicos (hidrocarburos, cloroformo, benceno, éteres y alcoholes) y apenas solubles en agua. Incluyen un amplio rango de compuestos tales como ácidos grasos y sus derivados, carotenoides, terpenos, esteroides y ácidos biliares.

Los ácidos grasos son los principales constituyentes de los lípidos, teniendo diferentes funciones fisiológicas y estructurales como componentes de membrana, enzimática, energética y hormonal (McMurphie, 1988), entre otras. Un ácido graso se denomina como saturado cuando no presenta dobles enlaces entre carbonos. Mientras que los ácidos grasos que contienen un doble enlace se denominan monoinsaturados (MUFA), y los que presentan dos o más dobles enlaces son poliinsaturados (PUFA). Los ácidos grasos altamente insaturados (HUFA) son aquellos PUFA con 20 o más carbonos y tres o más dobles enlaces. La geometría de los dobles enlaces en los PUFA puede ser cis (Z) y/o trans (E), dependiendo de si los átomos de hidrógeno están en el mismo plano o en el opuesto, respectivamente.

En peces, los principales PUFA son el ácido araquidónico (ARA, 20:4n-6), ácido eicosapentaenoico (EPA, 20:5n-3), ácido docosahexaenoico (DHA, 22:6n-3) y el ácido α-linolénico (LNA, 18:3n-3), siendo los peces la principal fuente dietética de n-3 HUFA (Sargent and Tacon, 1999). Los peces marinos y continentales tienen diferentes requerimientos de ácidos grasos esenciales (EFA) debido a sus diferentes capacidades de elongación y desaturación de ácidos grasos. EFA son aquellos ácidos grasos que deben ser suministrados en la dieta de los peces debido a que son incapaces de sintetizarlos *de novo* a partir de otros ácidos grasos. Las especies continentales presentan suficientes actividades enzimáticas de desaturación y elongación (Δ5 y Δ6) para producir ARA, EPA y DHA a partir de sus precursores como el ácido linoléico (18:2n-6) y el LNA cuando estos son administrados en la dieta; mientras que en los peces marinos estas actividades enzimáticas son muy limitadas, y por lo tanto, estos ácidos grasos han de ser suministrados a través de la dieta (Fig. 1). Por lo tanto, los EFA en especies continentales son LN y LNA, mientras que especies marinas son ARA, EPA y DHA (Sargent *et al*., 1995; Izquierdo, 1996, 2005; Bell, 1998).

Figura 8.1. Elongación y desaturación de los ácidos grasos. Adaptado de Vagner y Santigosa (2011).



8.1.3. Funciones de los lípidos en peces

Las principales funciones de los lípidos son como moléculas de acumulación de energía, componentes estructurales de membranas celulares, precursores de eicosanoides y ligandos de factores de transcripción. Los lípidos se acumulan principalmente en como triacylglicéridos (TAG), que consisten en una molécula de glicerol y tres ácidos grasos esterificados. Los ácidos grasos se liberan a partir TAG para la obtención de energía a través del proceso de β–oxidación cuando se requiere energía (Sargent *et al*., 1989; Frøyland *et al*., 2000) o como fuente estructural de membranas celulares. Los PUFA son constituyentes importantes de los fosfolípidos confiriéndoles diferentes propiedades a las membranas tales como disminuir su fluidez, mientras que los saturados y los MUFA proporcionan rigidez adicional (Tocher, 2003).

Los lípidos, y especialmente los ácidos grasos, son la principal fuente de energía para el crecimiento, la reproducción y la natación de los peces marinos, siendo los ácidos grasos saturados y los MUFA los primeros en utilizarse para la obtención de energía, conservando los PUFA para funciones estructurales. Esta especificidad en la oxidación de los ácidos grasos en peces determina la composición de los TAG del tejido adiposo de los peces, siendo importante para el bienestar de los peces y del consumidor.

Los ácidos grasos acumulados como fosfolípidos (PL) tienen función estructural en las membranas celulares, siendo más estables que los ácidos grasos acumulados como TAG. Como se ha comentado anteriormente, la fluidez y la permeabilidad de la membrana depende de su composición de ácidos grasos. Los PUFA son incorporados en los PL o en los TAG dependiendo de su grado de insaturación, por lo que el LNA y el LN con normalmente esterificados como TAG, mientas que el ARA, EPA y DHA son normalmente esterificados como PL (Henderson y Tocher, 1987). En peces, los altos niveles de n-3 HUFA que se encuentran en sus membranas son vitales para mantener su estructura y función (Tocher, 2003).

Los PUFA de cadena larga actúan como precursores de eicosanoides, siendo el ácido dihomo-γ-linolénico (DHGLA, 20:3n-6), ARA y EPA los principales precursores de

eicosanoides como las prostaglandinas (PG), leucotrienos (LT), tromboxanos (TX) y lipoxinas (LX), los cuales son metabolitos bioactivos de ácidos grasos que pueden modular las funciones del sistema inmune (Gershwin *et al*., 1985; Uhing *et al*., 1990; Tocher, 2003). Mientras que el ARA es el mayor precursor de eicosanoides, especialmente de las PG de la serie-2 (Horrobin, 1983), el ácido DHGLA y el EPA lo son de las PG de las series 1 y 2, respectivamente (Crawford, 1983). Además, el ratio ARA/EPA puede determinar la acción de los eicosanoides en las membranas celulares, por lo que la ingesta por medio de la dieta de n-3 y n-6 PUFA pueden afectar a dichas acciones (Tocher, 2003).

Los ácidos grasos, principalmente los PUFA y sus derivados también están involucrados en la regulación de las actividades de diversos factores de transcripción que controlan genes responsables del metabolismo lipídico (Jump, 2004). Uno de esos factores de transcripción son los receptores activadores del proliferador de peroxisomas (PPARs), que son receptores de hormonas nucleares que regulan la expresión de genes involucrados en el equilibrio homeostático en mamíferos (Desvergne y Wahli, 1999; Hihi *et al*., 2002). Se conocen tres isoformas de PPARs en mamíferos (PPARα, PPARβ/δ and PPARγ), cada uno de los cuales se expresa en diferentes tejidos; en peces hay escasos estudios realizados pero todo indica que las mismas isoformas están presentes en sus tejidos (Boukouvala *et al*., 2004; Leaver *et al*., 2005).

8.1.4. El sistema inmune en peces y los lípidos

La cooperación entre diferentes células del sistema inmune, a través de procesos asociados a las membranas y a través de diversos mediadores lipídicos, es esencial para obtener una respuesta inmune óptima (FAO, 1994). Diversos estudios con animales, cultivos tisulares, así como en humanos demuestran que tanto el nivel y el grado de saturación de los lípidos dietéticos influencian la respuesta inflamatoria e inmunológica, incluso el origen de la respuesta inmune depende del tipo de ácido graso, edad, poder antioxidante (Waagbø *et al*., 2003; Puangkaew *et al.*, 2004), estado de salud del sujeto (FAO, 1994) y requerimientos de EFA de la especie. Por ejemplo, en animales de laboratorio, una dieta con niveles altos de lípidos inhibe la función de los

linfocitos, directamente relacionado con el nivel y el tipo de grasa (Calder et al., 2002; Montero e Izquierdo, 2010), mientras que los SFA parece que tienen un efecto leve sobre la proliferación de linfocitos, producción de citoquinas o sobre la actividad de la células natural killer (NK) (Yacoob *et al*., 1994a, b; Montero e Izquierdo, 2010). La familia de los n-3 PUFA reduce la proliferación de linfocitos, actividad de las células NK a través de la producción de eicosanoides, quemotaxis de los neutrófilos y la secreción de diversas citoquinas, tal como IL-1, IL-2 y TNF (Endre et al., 1989, 1993; Yacoob and Calder, 1995, 2007; De Pablo and Álvarez, 2000; Wallace et al., 2001). De hecho, la modulación de citoquinas por parte de estos ácidos grasos parece ser la responsable de la reducción de la proliferación de linfocitos tanto en animales como en humanos, debido a los efectos inhibitorios de los n-3 PUFA sobre la expresión de la molécula CD25 que constituye el receptor de IL-2 (Soyland *et al*., 1994; De Pablo y Álvarez, 2000). Los mecanismos responsables de la modificación de la síntesis de citoquinas no están esclarecidos todavía, pero una posible explicación puede ser la reducción de la producción de mRNA de citoquinas debido a los PUFA (Robinson *et al*., 1996; De Pablo y Álvarez, 2000). Por el contrario, la serie n-6 son capaces de estimular la producción de citoquinas pro-inflamatorias como el TNF-alpha, IL-1 e IL-6, y consecuentemente aumentar la vasodilatación o la permeabilidad vascular (Calder, 2002; Montero e Izquierdo, 2010).

En peces, de forma general, se han estudiado tres mecanismos principales mediante los cuales los FA dietéticos pueden afectar al sistema inmune. El primero afecta a la composición lipídica de la membrana celular a través de cambios en la composición de FA de los fosfolípidos (PLs) de membrana (Balfry y Higgs, 2001), influenciando diversas respuestas inmunes basadas en las interacciones de membrana de los leucocitos, tales como fagocitosis, antígeno-anticuerpo o la producción de citoquinas. El segundo mecanismo alterado por los FA dietéticos engloba cambios en las señales de transducción, posiblemente debido a efectos sobre la proteína quinasa C (Balfry y Higgs, 2001) y debido al efecto de la suplementación de aceite de pescado sobre el aumento de la sensibilidad del INF-γ de los macrófagos de mamíferos (Erickson y Hubard, 1993). La tercera vía por la que los FA dietéticos pueden afectar al sistema inmune es a través de la producción de eicosanoides a partir de ARA, EPA y

DHA no esterificados. A pesar de ello, diversos estudios realizados en cultivos celulares de mamíferos han resaltado los efectos de los lípidos dietéticos sobre la apoptosis, peroxidación lipídica y la regulación de la expresión genética (Para revisión ver De Pablo y Álvarez, 2000) y su implicación en la inmunomodulación.

8.1.5. Lípidos y calidad del filete

La calidad de los alimentos marinos puede caracterizarse tanto por sus propiedades físicas como la frescura del producto y su aspecto, así como por sus propiedades nutricionales y sensoriales (Rosenlund et al., 2010).

En peces cultivados, la alimentación con dietas artificiales provee un amplio rango de nutrientes, lo que no sólo determina la tasa de crecimiento sino también la composición del filete, y en particular su contenido lipídico, que puede ser modificado tanto cuantitativa como cualitativamente (Izquierdo *et al*., 2003). Cualitativamente proveyendo altos niveles de n-3 LC-PUFA que se asocian normalmente a los beneficios para la salud de los alimentos de origen marino consumidos (Kris-Etherton *et al*., 2002; Calder, 2004; Wang *et al*., 2006; Rosenlund *et al*., 2010) o por la inclusión de otros lípidos con propiedades beneficiosas para la salud humana como puede ser el CLA, resultando en nuevos alimentos funcionales. Cuantitativamente, un aumento en los niveles de grasa de las dietas puede resultar en aumento del porcentaje lipídico del filete producido, lo que podría influir en la calidad física y organoléptica del filete (Lopparelli *et al*., 2004). Particularmente, la deposición de grasa intramuscular parece limitar los interrelaciones entre el colágeno y las fibras musculares, reduciendo su fortaleza mecánica (Fasolato *et al*., 2005) y reduciendo la textura, que se correlaciona por el consumidor con un filete más graso, y con una reducción de la aceptación y valor de mercado (Torrissen *et al*., 2001).

8.1.6. Metabolismo de los ácidos grasos

8.1.6.1. Lipogénesis

Lipogénesis es el término utilizado para describir las reacciones para la formación de nuevos lípidos endógenos (Tocher, 2003), y se considera un proceso idéntico al de mamíferos (Sheridan, 1994). El proceso clave de la lipogénesis está catalizado por el complejo de la ácido graso sintetasa (FAS) que requiere NADPH durante los pasos secuenciales de condensación (Henderson y Sargent, 1985), obteniendo ácidos grasos saturados (ácido palmítico, 16:0 y ácido esteárico, 18:0) tanto en mamíferos como en peces (Sargent *et al*., 1989), que pueden ser desaturados por la stearoyl-CoA desaturasa (SCD) para formar ácido palmitoleico (16:1n-7) y ácido oleico (18:1n-9) para su posterior desaturación y elongación, particularmente en condiciones de deficiencia de EFA (Tocher, 2003). La enzima glucosa-6-fosfato dehidrogenasa (G6PD) y la enzima málico (ME) son los principales productores de poder reductor en forma de NADPH, siendo la primera de ellas la que más NADPH produce (Dias *et al*., 1999), pero el contenido celular de ambas dehidrogenasas varía dependiendo de las condiciones alimenticias y hormonales (Tocher, 2003). Cabe destacar que el uso de dietas ricas en lípidos en acuicultura, favorece que los peces no bio-sinteticen ácidos grasos *de novo* de forma significativa. Realmente, los peces tienden a acumular amplios depósitos lipídicos en forma de TAG, cuando la energía suministrada excede el gasto energético, en lugares específicos (principalmente en tejido adiposo) que son movilizados cuando los requerimientos energéticos del organismo supera la energía suministrada por la dieta (Tocher, 2003).

8.1.6.2. Lipolisis

La oxidación de ácidos grasos en una fuente importante de energía en los peces que puede ocurrir en el hígado, músculo rojo y blanco y en el corazón (Frøyland *et al*., 1998, 2000). La β-oxidación de ácidos grasos puede producirse en dos órganos distintos, las mitocondrias y los peroxisomas, tanto en mamíferos como en peces (Mannaerts *et al*., 1979; Reddy y Mannaerts, 1994). En peces, la β-oxidación en peroxisomas puede suponer hasta un 50% de la β-oxidación total (Crocket y Sidell, 1993a, b; Nanton *et al*., 2003), pero es la mitad de eficiente produciendo energía que

la β-oxidación mitocondrial (Mannaerts y van Veldhoven, 1996; Stubhaug *et al*., 2007) ya que en la β-oxidación en peroxisomas la mitad de la energía se pierde en forma de calor. A pesar de ello, los peroxisomas son más abundantes que las mitocondrias, por lo que los peces parecen utilizar esta vía debido a la capacidad total de β-oxidación (Stubhaug *et al*., 2007). La enzima limitante en la β-oxidación mitocondrial es la carnitina palmitoyl transferasa-1 (CPT-I) (McGarry y Foster, 1979; Frøyland *et al*., 1998), mientras que la acyl-CoA (ACO) cataliza el proceso limitante de la β-oxidación en peroxisomas (Inestrosa *et al*., 1979; Fraser *et al*., 1997). Existen evidencias que los ácidos grasos saturados (SFA) y los MUFA pueden ser directamente catabolizados en peces mediante la β-oxidación mitocondrial (Sargent *et al*., 1989), mientras que la β-oxidación de los PUFA depende de las moléculas de PUFA, siendo más difícil que puedan ser directamente β-oxidadas en las mitocondrias, por lo que en peces tal vez ocurra como en mamíferos, donde la β-oxidación en peroxisomas parece tener una función principal como sistema de desintoxicación, de acortamiento de cadena y de β-oxidación de ácidos grasos de cadena muy larga (Jakobs y Wanders, 1991; Mannaerts y van Veldhoven, 1993).

8.1.7. Ácido linoleico conjugado (CLA)

8.1.7.1 Introducción

El ácido linoleico conjugado (CLA) engloba a un grupo genérico de isómeros geométricos y posicionales del ácido linoleico con dobles enlaces. A pesar de que hasta la fecha se han identificado 58 isómeros presentes en los alimentos, con los dobles enlaces en diversas posiciones (7, 9; 8, 10; 9, 11; 10, 12; 11, 13) (Kramer et al., 1998; Pariza *et al*., 2001; Park and Pariza, 2007), la investigación se ha centrado básicamente en dos isómeros, el *cis*-9, *trans*-11 y el *trans*-10, *cis*-12, que son los isómeros considerados bioactivos (Pariza *et al*., 2001) (Fig. 2). El CLA presente de forma natural en los productos lácticos, leche y alimentos como la ternera consiste principalmente en el isómero *cis*-9, *trans*-11 (80-90%), mientras que el resto de isómeros están presentes en bajas proporciones (Chin *et al*., 1994; Khnanal and Dhiman, 2004). El isómero *cis*-9, *trans*-11 se produce por la biohidrogenación del ácido linoleico a ácido esteárico mediante bacterias presentes en el rumen (*Butyrivibrio fibrisolvens*) (Kepler

et al., 1966), pero también mediante la Δ-9 desaturación del ácido trans-11 vaccénico (18:1) en tejidos de mamíferos (Corl *et al*., 2003; Kay *et al*., 2004; Mosley *et al*., 2006a, b), mientras que el isómero *trans*-10, *cis*-12 utilizado en estudios de investigación se considera sintético porque se encuentra en cantidades muy pequeñas (3-5%) en las fuentes naturales (Park and Pariza, 1998; Khnanal y Dhiman, 2004; Dhiman *et al*., 2005). Consecuentemente, la mayor parte de los estudios experimentales se están realizando utilizando los isómeros *cis*-9, *trans*-11 y *trans*-10, *cis*-12 individualmente o juntos como CLA, donde los isómeros están presentes en una proporción 50:50, producidos a partir de aceites ricos en ácido linoleico, el propio ácido linoleico por isomerización alcalina o hidrogenación parcial (Banni, 2002). En la actualidad, las principales fuentes de CLA incluyen suplementos dietéticos como cápsulas que contienen cantidades de hasta 1014 mg por cápsula, y unos pocos productos derivados de la leche, aunque los niveles de esos productos son normalmente inferiores al 1% del total de ácidos grasos (Manning *et al*., 2006; Schmid *et al*., 2006). En humanos, la ingesta a través de la dieta de CLA se estima alrededor de 95-440 mg diario, difiriendo entre países (Schmid *et al*., 2006), pero parece que se necesitan niveles más altos (2,4-3,5 g diario) para obtener beneficios sobre la salud (Tersptra, 2004). Generalmente, el 60% de la ingesta humana de CLA proviene de productos lácticos, mientras que otro 37% proviene de productos cárnicos, siendo la proporción entre los isómeros *cis*-9, *trans*-11 y *trans*-10, *cis*-12 desde 30:1 a 70:1, respectivamente (Wahle *et al*., 2004; Haro *et al*., 2006).

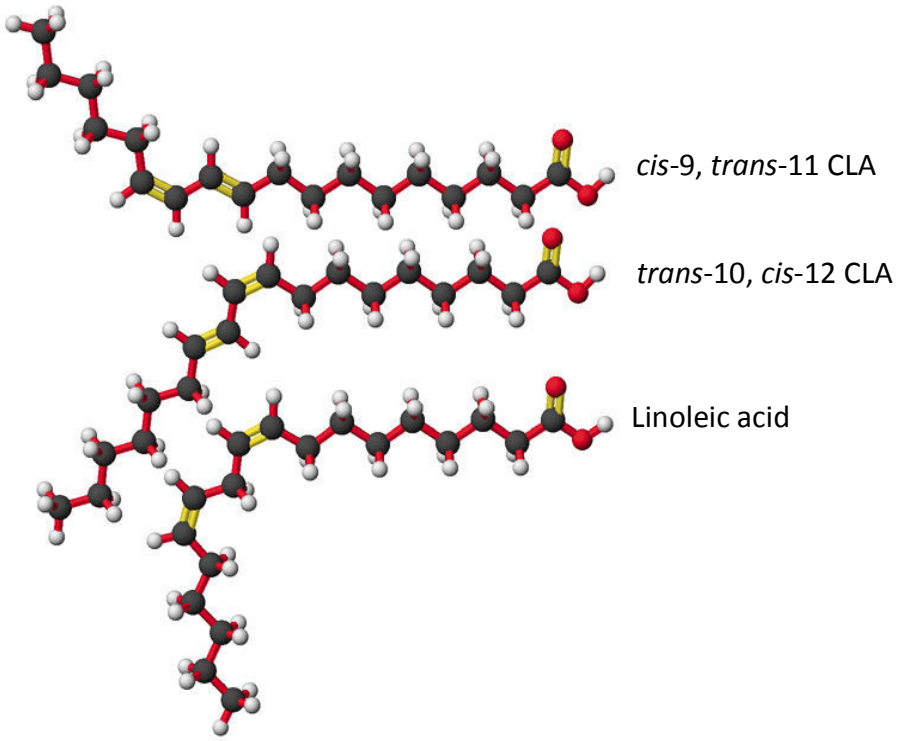


Figura 8.2. Estructura de los isómeros de CLA, *cis*-9, *trans*-11 y *trans*-10, *cis*-12 y el ácido linoleico (Modificado de Bauman *et al*., 1999)

Incrementar la calidad de los productos derivados de animales en el mercado es uno de los objetivos del sector agrícola como la acuicultura. Por lo tanto, se ha promovido el enriquecimiento de alimentos con componentes que tengan efectos beneficiosos en la salud humana, como puede ser el CLA. La forma de conseguirlo consiste mediante la inclusión de CLA en las dietas para animales para aumentar los niveles de CLA que se encuentran de forma natural en sus tejidos, y así obtener los efectos beneficiosos del CLA sobre los propios animales, teniendo la inclusión CLA en dietas para acuicultura el mismo objetivo que en animales terrestres.

8.1.7.2. CLA y la composición corporal en humanos

Durante la última década, los efectos del CLA en humanos se han estado evaluando. Estos estudios muestran los efectos del CLA en humanos, principalmente en cuanto a la composición corporal, sistema inmune y propiedades anticancerígenas, obteniendo a veces resultados contradictorios (ver Tabla 8.1). En cuanto a la composición corporal, diversos estudios mostraron una reducción de la grasa corporal y una reducción del diámetro sagital abdominal cuando el CLA se incluyó en la dieta (Blankson *et al*., 2000; Riserus *et al*., 2001; Smedman and Vessby, 2001; Thom *et al*., 2001; Kreider *et al*., 2002; Gaullier *et al*., 2004, 2005), así como una reducción del tamaño de los adipocitos (Evans *et al*., 2002) y un incremento de la masa magra corporal junto con un aumento de la tasa metabólica en reposo (Kamphuis *et al*., 2003; Gaullier *et al*., 2004). Por otro lado, en otros estudios no se obtuvieron efectos (Kreider et al., 2002; Riserus et al., 2004; Bhattacharya *et al*., 2006). Estudios relacionados con el sistema inmune obtuvieron, en la mayoría de casos que el CLA mejoró la respuesta inmune promoviendo la formación de anticuerpos (Sugano *et al*., 1998), activando el factor nuclear kB (Chung *et al*., 2005; De Roos *et al*., 2005; Poirier *et al*., 2005) o por el aumento de los niveles de IgA e IgM (Song *et al*., 2005). Por el contrario, no se obtuvo ningún efecto en la producción de citoquinas y eicosanoides en otros estudios (Kelley *et al*., 2001; Albers *et al*., 2003; Nugent *et al*., 2005). Estudios realizados en células mamarias cancerígenas y las propiedades anticancerígenas del CLA mostraron también resultados contradictorios. Durgam y Fernandes (1997) obtuvieron una reducción de las células cancerígenas, mientras que Rissanen *et al*. (1997) y McCann *et al*. (2004) no obtuvieron ningún efecto. La misma tendencia se obtuvo de los efectos del CLA sobre la diabetes y la resistencia a la insulina, donde Medina *et al*. (2000) mostraron un aumento de los niveles de insulina en suero y Chung *et al*. (2005) obtuvieron que el CLA producía resistencia a la insulina, y en salud ósea, donde diversos estudios mostraron un incremento de la masa ósea (Brownbill *et al*., 2005; Gaullier *et al*., 2005), mientras que otros no estudios no obtuvieron ningún efecto (Gaullier *et al*., 2004; Doyle *et al*., 2005).

8.1.7.3. CLA y la composición corporal en animales experimentales

Un amplio número de estudios han demostrado diversas propiedades del CLA en modelos animales. En la mayoría de los estudios, se han señalado los efectos sobre el crecimiento y composición corporal, sus propiedades anticancerígenas, efecto inmunomodulador y modulador de la diabetes, mientras que en otros, no se detectó ningún efecto (ver Tabla 8.2). Por ejemplo, el crecimiento se incrementó en roedores y cerdos (Chin *et al*., 1994; Bee, 2000; Dugan *et al*., 2004; Valeille *et al*., 2004; Storkson *et al*., 2005), así como una mejor eficiencia alimenticia en los mismos modelos (Li y Watkins, 1998; Dugan *et al*., 2004). En la mayoría de estudios, se observó una reducción de la grasa corporal en diferentes especies de mamíferos (Park *et al*., 1997, 1999; Cook *et al*., 1998; West *et al*., 1998, 2000; DeLany y West, 2000; DeLany *et al*., 1999; Azain *et al*., 2000; Tsuboyama-Kasaoka *et al*., 2000; Whigham *et al*., 2000; Ryder *et al*., 2001; Akahoshi *et al*., 2002; Takahashi *et al*., 2002; Azain, 2003; Yamasaki *et al*., 2003; Wang y Jones, 2004), así como, en otros estudios, un aumento de la masa magra unida a esta reducción de grasa corporal (Mirand *et al*., 2004; Park *et al*., 2005; Bhattacharya *et al*., 2006).

Tabla 8.1. Efectos del CLA en humanos.

Función	Efectos	Referencias
Composición corporal	↓ Grasa corporal	Blankson *et al*., 2000
		Smedman and Vessby, 2001
		Thom *et al*., 2001
		Kreider *et al*., 2002
		Gaullier *et al*., 2004, 2005
	↑ Tasa metabólica en reposo y masa magra corporal	Kamphuis *et al*., 2003
		Gaullier *et al*., 2004
	↓ Diámetro abdominal sagital	Riserus *et al*., 2001
	Sin efecto en grasa corporal	Kreider *et al*., 2002
		Riserus *et al*., 2004
		Bhattacharya *et al*., 2006
	↓ Tamaño adipocitos	Evans *et al*., 2002
Sistema inmunne	Favorece la formación de anticuerpos	Sugano *et al*., 1998
	Activación del factor nuclear kB	Chung *et al*., 2005
		De Roos *et al*., 2005
		Poirier *et al*., 2005
	↑ Respuesta inmune (IgA e IgM)	Song *et al*., 2005
	No effecto sobre citoquinas y eicosanoides	Kelley *et al*., 2001
		Albers et al., 2003
		Nugent et al., 2005
Anticancerígenas	↓ Células cancerígenas mamarias	Durgam and Fernandes, 1997
	No effecto sobre cancer de pecho	Rissanen *et al*., 2003
		McCann *et al*., 2004
Diabetes y Resistencia a la insulina	↑ Niveles insulin en suero	Medina *et al*., 2000
	Produce resistancia insulina	Chung *et al*., 2005
Salud ósea	↑ Masa ósea	Brownbill *et al*., 2005
		Gaullier *et al*., 2005
	No efecto	Gaullier *et al*., 2004
		Doyle *et al*., 2005

Se han obtenido diversos estudios sobre el efecto del CLA y el sistema inmune. Akahoshi *et al*. en 2002 y 2004 encontraron un efecto inhibidor del CLA sobre las citoquinas en ratones; Sugano *et al*. (1998) y Yamasaki *et al*. (2000) encontraron que el CLA favoreció la formación de IgA, IgM e IgG, pero no de IgE. También una mejora de la inmunidad celular se observó en cerdos y ratones (Sugano *et al*., 2001; Bassaganya-Riera *et al*., 2001), así como una reducción de eicosanoides y producción de histamina en roedores y en estudios *in vitro* (Sugano *et al*., 1998; Whigham *et al*., 2001; Wahle *et al*., 2004). Además, se observó una reducción de los efectos catabólicos del sistema inmune posterior a la inyección de endotoxinas en roedores y pollos, así como un aumento de la capacidad fagocítica (Cook *et al*., 1993; Miller *et al*., 1994; Sisk *et al*., 2001). El efecto anticancerígeno del CLA en roedores también ha sido demostrado. Se ha observado la inhibición de la carcinogénesis químicamente inducida y de la proliferación de células cancerígenas (Ha *et al*., 1987, 1999; Visonneau *et al*., 1997; Hubbard *et al*., 2000; Ip *et al*., 2002), así como la reducción o inhibición de la angiogénesis (Masso-Welch *et al*., 2002; Toomey *et al*., 2003). El CLA afectó a la diabetes y a la resistencia a la insulina, mientras que otros estudios mostraron una reducción de los niveles de insulina en ratas (Houseknecht *et al.*, 1998; Ryder *et al*., 2001; Belury *et al*., 2002; Evans *et al*., 2002; Kritchevsky *et al*., 2004); en ratones se observó resistencia a la insulina (Tsuboyama *et al*., 2000; Poirier *et al*., 2005) y una mejora a la tolerancia a la glucosa (Houseknecht *et al*., 1998; Henriksen *et al*., 2003). Se han realizado escasos estudios sobre las propiedades antiaterogénicas del CLA, pero se ha demostrado una reducción de la ateroesclerosis en conejos y hámsters (Lee *et al*., 1994; Nicolosi *et al*., 1997; Kritchevsky *et al*., 2004). En todos estos estudios, la ingestión de CLA redujo el LDL colesterol y el total, así como los TAG en sangre, así como una reducción del efecto de la ateroesclerosis en las aortas.

8.1.7.4. CLA en peces

Basado en los efectos de la suplementación de CLA en la dieta en animales experimentales, se espera que aparezcan efectos similares en peces cuando son alimentados con dietas enriquecidas con CLA. Hasta el comienzo de esta tesis, se habían realizado diversos estudios en peces. Algunos de ellos mostraron que el CLA no afectó significativamente a los parámetros de crecimiento o eficiencia alimenticia como en trucha arcoíris (*Oncorhynchus mykiss*) (Figueiredo-Silva *et al*., 2005; Bandarra *et al*., 2006), salmón Atlántico (*Salmo salar*) (Berge *et al*., 2004; Kennedy *et al*., 2005, 2006; Leaver *et al*., 2006), pez gato (*Ictalurus punctatus*) (Twibell and Wilson, 2003), perca amarilla (*Perca flavescens*) (Twibell *et al*., 2001), carpa (*Cyprinus carpio*) (Schwarz et al., 2002), tilapia (*Oreochromis niloticus*) (Yasmin *et al*., 2004). Mientras, que otros estudios mostraron una reducción del crecimiento en híbrido de lubina americana (*Morone saxatilis* x *M. chrysops*) (Twibell *et al*., 2000) y pez gato (Manning *et al*., 2006), una mejora de la eficiencia alimenticia se observó en híbrido de lubina americana (Twibell *et al*., 2000). Estos resultados sugieren que el efecto del CLA en peces depende de la especie y de las condiciones experimentales. Además, los coeficientes de digestibilidad aparente (ADC) o retención de nutrientes no se vieron afectados por el CLA en la dieta en trucha arcoíris (Figueiredo-Silva *et al*., 2005) (ver Tabla 8.3 para información detallada).

Tabla 8.2. Efectos del CLA en animales experimentales.

Función	Efectos	Referencias
Crecimiento	↑ Crecimiento en roedores y cerdos	Chin *et al*., 1994 Bee, 2000 Dugan *et al*., 2004 Valeille *et al*., 2004 Storkson *et al*., 2005
	↑ Eficiencia alimenticia en roedores y cerdos	Li and Watkins, 1998 Dugan *et al*., 2004
Composición corporal	↓ Deposición grasa corporal en roedores, pollos, cerdos y perros	Park *et al*., 1997, 1999 Cook *et al*., 1998 West *et al*., 1998, 2000 DeLany and West, 2000 DeLany *et al*., 1999 Azain *et al.*, 2000 Tsuboyama-Kasaoka *et al.*, 2000 Whigham *et al.*, 2000 Ryder *et al.*, 2001 Akahoshi *et al.*, 2002 Takahashi *et al.*, 2002 Azain, 2003 Yamasaki *et al.*, 2003 Wang and Jones, 2004
	↓ Masa grasa y ↑ masa magra	Mirand *et al*., 2004 Park *et al*., 2005 Bhattacharya *et al*., 2006
Sistema inmune	Inhibe citoquinas pro-inflamatorias (TNF-α) en ratones	Akahoshi *et al*., 2002, 2004
	↑ Formación anticuerpos en ratones y cerdos	Sugano *et al*., 1998 Bassaganya-Riera *et al*., 2001
	↓ Efectos catabólicos de la estimulación del sistema inmune en roedores y pollos e incrementa la fagocitosis	Cook *et al*., 1993 Miller *et al*., 1994 Sisk *et al*., 2001
	↓ Producción eicosanoides e histamina en roedores y cultivos celulares	Sugano *et al*., 1998 Whigham *et al*., 2001 Wahle *et al*., 2004
	↑ IgA, IgB e IgM en ratas	Sugano *et al*., 1998 Yamasaki *et al*., 2000

Tabla 8.2. Efectos del CLA en animales experimentales (Cont.).

Función	Efectos	Referencias
Anticancerígenos	Inhibe la carcinogénesis químicamente inducida en roedores	Ha *et al*., 1987, 1990 Ip *et al*., 2002
	Inhibe la proliferación células cancerígenas y aumenta apoptosis en roedores	Visonneau *et al*., 1997 Hubbard *et al*., 2000
	↓ Angiogénesis en tumores mamarios implantados en ratas	Masso-Welch *et al*., 2002
	Inhibe angiogénesis en ratones	Toomey *et al*., 2003
Diabetes y resistencia a la insulina	↓ Diabetes en ratas	Houseknecht *et al*., 1998 Kritchevsky *et al*., 2004
	Mejora tolerancia de la glucosa en ratas	Houseknecht *et al*., 1998 Henriksen *et al*., 2003
	Induce resistencia a la insulina en ratones	Tsuboyama *et al*., 2000 Poirier *et al*., 2005
	↓ Niveles de insulina	Ryder *et al*., 2001 Belury *et al*., 2002 Evans *et al*., 2002
Antiaterogénicas	↓ Ateroesclerosis en conejos, hámsters y ratones	Lee *et al*., 1994 Nicolosi *et al*., 1997 Kritchevsky *et al*., 2004

Se han observado reducciones del contenido de grasa corporal y un aumento de la masa magra en animales terrestres cuando el CLA se incluyó en la dieta. Pero en los estudios realizados en peces, se han obtenido efectos contradictorios. Por ejemplo, no se observaron efectos en composición proximal en trucha arcoíris (Figueiredo-Silva *et al*., 2005; Bandarra *et al*., 2006), salmón Atlántico (Berge *et al*., 2004), pez gato (Twibell and Wilson, 2003) y tilapia (Yasmin *et al*., 2004), mientras que se produjo un aumento del contenido lipídico en hígado en salmón Atlántico (Kennedy *et al*., 2005), se obtuvo un descenso del contenido lipídico de hígado y pez entero en la misma especies (Leaver *et al*., 2006). En perca amarilla, el contenido lipídico en hígado se redujo cuando se incluyó CLA en la dieta (Twibell *et al*., 2001).

La composición de FA en los tejidos de los peces depende del tejido analizado, pero en general, la fracción de SFA aumentó, principalmente debido los ácidos palmítico y esteárico, y la fracción de MUFA disminuyó, debido principalmente a los ácidos palmitoleico y oleico tanto en músculo como en hígado (Twibell *et al*., 2000; Berge *et al*., 2004; Bandarra *et al*., 2006).

Los efectos sobre los HUFA son contradictorios y dependen de la especie estudiada. Se observó una reducción de EPA y DHA en músculo de híbrido de lubina americana (Twibell *et al*., 2000), salmón Atlántico (Kennedy *et al*., 2005) y trucha arcoíris (Bandarra *et al*., 2006). En perca amarilla, estos n-3 HUFA no se vieron afectados por el CLA incluido en la dieta en tejido muscular, mientras que sí se redujeron en hígado (Twibell *et al*., 2001). En salmón Atlántico no se detectó ningún efecto sobre estos ácidos grasos (Berge *et al*., 2004; Kennedy *et al*., 2005; Leaver *et al*., 2006). Además de estos efectos obtenidos por la inclusión de CLA en las dietas sobre los perfiles de FA de los tejidos de los peces, el CLA se acumuló en todos los tejidos de los peces analizados (músculo, hígado y grasa perivisceral) mostrando una correlación positiva con el contenido de CLA en la dieta en todos los estudios publicados en peces hasta el comienzo de esta tesis (Twibell *et al*., 2000, 2001; Twibell and Wilson, 2003; Berge *et al*., 2004; Kennedy *et al*., 2005; Bandarra *et al*., 2006; Leaver *et al*., 2006; Manning *et al*., 2006), siendo la deposición de CLA en los tejidos mayor que en otras especies de vertebrados (Dugan *et al*., 2001; Azain, 2003) (ver Tabla 8.4 para información detallada).

Tabla 8.3. Efectos de la suplementación de CLA en dietas para peces sobre los parámetros de crecimiento.

Especie	Dosis CLA	Resultados	Referencias
Salmón Atlántico (*Salmo salar*)	0, 0,5, 1 y 2% en juveniles	No efecto en parámetros de crecimiento	Berge *et al*., 2004
	0, 1 y 2% in smolts	No efecto en parámetros de crecimiento	Kennedy *et al*., 2005, 2006
	0, 2 y 4% en post-smolts	No efecto eficiencia conversión alimenticia o crecimiento	Leaver *et al*., 2006
Pez gato (*Ictalurus punctatus*)	0, 0,5 y 1% en juveniles	No efecto en peso ganado, eficiencia alimenticia o ingesta	Twibell and Wilson, 2003
	0, 0,5 y 1% en juveniles	No efecto en ingesta y FCR ↓ Peso corporal ganado con 0,5% CLA y 2,25% aceite de maiz	Manning *et al*., 2006
Híbrido de lubina americana (*Morone saxatilis* x *M. chrysops*)	0, 1% CLA en juveniles	↓ Ingesta y peso ganado ↑ Eficiencia alimenticia y HSI	Twibell *et al*., 2000
Trucha arcoiris (*Oncorhynchus mykiss*)	0, 0,5, 0,75, 1 y 2% en juveniles	No efecto en parámetros de crecimiento, FCR y utilización de nutrientes o energía	Figueiredo-Silva *et al*., 2005
	0, 0,5, 0,75, 1 and 2% in juveniles	No efecto en parámetros de crecimiento, FCR, HSI y VSI	Bandarra *et al*., 2006
Pez roca	0, 1 and 10%	↓ Crecimiento con 10% CLA	Choi *et al*., 1999

Tabla 8.3. Efectos de la suplementación de CLA en dietas para peces sobre los parámetros de crecimiento (Cont.)

Especie	Dosis CLA	Resultados	Referencias
Tilapia (*Oreochromis niloticus*)	0, 1 and 10%	↑ Crecimiento con 1% ↓ Crecimiento con 10%	Choi *et al*., 1999
	0 and 5% en juveniles	No efecto en parámetros de crecimiento	Yasmin *et al*., 2004
Perca amarilla (*Perca flavescens*)	0, 0,5 and 1% in juveniles	No efecto en parámetros de crecimiento	Twibell *et al*., 2001

Tan sólo hay tres estudios publicados hasta la fecha acerca del efecto del CLA en dietas para peces sobre el metabolismo de los peces (centrándose en enzimas lipogénicas y lipolíticas) y han obtenido resultados inconcluyentes. En trucha arcoíris, no se observó ningún efecto sobre las enzimas G6PD, ME y FAS en hígado (Figueiredo-Silva *et al*., 2005), así como en las enzimas ME y FAS en salmón Atlántico (Leaver *et al*., 2006). La enzima CPT-I en hígado y músculo blanco tampoco se vieron afectadas, mientras que en músculo rojo, la actividad enzimática se redujo por el CLA en la dieta de salmón Atlántico (Leaver *et al*., 2006). Por el contrario, la actividad de la CPT-I aumentó en músculo blanco y rojo para la misma especie (Kennedy *et al*., 2006) (ver Tabla 8.5 para información detallada).

Tabla 8.4. Efectos de la suplementación de CLA en dietas para peces sobre las propiedades bioquímicas

Especie	Dosis CLA	Resultados	Referencias
Salmón Atlántico (*Salmo salar*)	0, 0,5, 1 y 2% en juveniles	No efecto en composición proximal ↑ Deposición total de n–3	Berge *et al*., 2004
	0, 1 and 2% en juveniles	Clara tendencia a aumentar lípidos totales y TAG en hígado y músculo ↑ Niveles CLA en músculo e hígado ↓ SFA y MUFA en hígado ↓ HUFA en músculo	Kennedy *et al*., 2005
	0, 1 y 2% en juveniles	↑ HUFA en hígado	Kennedy *et al*., 2006
	0, 2 y 4% en juveniles	↓ Contenido lipídico ↑ Contenido proteico	Leaver *et al*., 2006
Pez gato (*Ictalurus punctatus*)	0, 0,5 y 1% en juveniles	No efecto en composición proximal	Twibell and Wilson, 2003
	0, 0,5 y 1% en juveniles	↓ N-3HUFA en músculo	Manning *et al*., 2006
Híbrido de lubina americana (*Morone saxatilis* x *M. chrysops*)	0, 1% CLA en juveniles	↓ Lípidos totales en hígado ↓ PFI ↓ EPA y DHA en músculo ↑ EPA y DHA en hígado	Twibell *et al*., 2000

Tabla 8.4. Efectos de la suplementación de CLA en dietas para peces sobre las propiedades bioquímicas (Cont.)

Especie	Dosis CLA	Resultados	Referencias
Trucha arcoiris (*Oncorhynchus mykiss*)	0, 0,5, 0,75, 1 y 2% en juveniles	No efecto en composición proximal	Figueiredo-Silva *et al*., 2005
	0, 0,5, 0,75, 1 y 2% en juveniles	No efecto en composición proximal ↑ SFA y PUFA en músculo y vísceras ↓ MUFA en en músculo y vísceras	Bandarra *et al*., 2006
Tilapia (*Oreochromis niloticus*)	0 y 5% en juveniles	No efecto en composición proximal	Yasmin *et al*., 2004
Yellow Perch (*Perca flavescens*)	0, 0.5 and 1% en juveniles	No efecto en composición proximal del músculo ↓ Contenido lípidos en hígado	Twibell *et al*., 2001

Tabla 8.5. Efecto de la suplementación de CLA en dietas para peces sobre las enzimas lipogénicas y lipolíticas.

Especie	Dosis CLA	Resultados	Referencias
Trucha arcoíris (*Oncorhynchus mykiss*)	0, 0,5, 0,75, 1 y 2% en juveniles	No efecto de las actividades de G6PD, ME y FAS en hígado	Figueiredo-Silva *et al*., 2005
Salmón Atlántico (*Salmo salar*)	0, 1 y 2% en juveniles	↑ CPT-I en músculo (rojo y blanco)	Kennedy *et al*., 2006
	0, 2 y 4% en juveniles	No efecto en ME y CPT-I en hígado No efecto en CPT-I en músculo (rojo y blanco)	Leaver *et al*., 2006

Tan sólo se ha realizado un estudio del efecto del CLA en la dieta sobre las propiedades sensoriales, sin haberse obtenido diferencias en ninguno de los atributos sensoriales estudiados en trucha arcoíris cuando se alimentó con dietas con hasta un 3% de CLA (Schabbel *et al*., 2004). Por lo que más estudios son necesarios para determinar si la inclusión de CLA puede afectar a las propiedades sensoriales de los peces.

En resumen, se ha observado que los efectos del CLA están relacionados con la especie estudiada, que es dosis-dependiente y que también depende del tamaño de los peces y las condiciones experimentales de cultivo. Además, la forma química en la que se suplementa el CLA (como ácido graso libre, metil éster u otra forma) puede influenciar los efectos del CLA sobre la fisiología de los peces.

8.2. OBJETIVOS

Considerando que el exceso en la deposición de grasa es una de las limitaciones que presenta el cultivo de lubina, y en base al beneficio potencial de la inclusión dietética CLA en dietas para vertebrados superiores respecto a la reducción en las tasas de deposición de grasa y como potenciador del valor nutricional del alimento producido, el objetivo principal de esta tesis fue investigar los efectos de la suplementación dietética de CLA en el cultivo de la lubina. Para ello, se plantearon los siguientes objetivos secundarios:

1. Determinar los efectos de la suplementación dietética de varios niveles de CLA en la producción de cultivo de la lubina, prestando especial atención al efecto sobre el crecimiento.

2. Estudiar el efecto de la suplementación dietética de varios niveles de CLA en la deposición de grasa así como en la composición lipídica y de ácidos grasos de los diferentes tejidos de la lubina.

3. Investigar los efectos de la suplementación dietética con CLA en el músculo, hígado y grasa perivisceral de la lubina.

4. Proporcionar un mejor entendimiento sobre los efectos de la suplementación con CLA en el metabolismo de la lubina, así como los mecanismos potencialmente implicados en este proceso como pueden ser la digestibilidad de nutrientes y la actividad de las enzimas lipolíticas y lipogénicas.

5. Estudiar los efectos de la inclusión de CLA en dietas para lubina en la salud en términos de parámetros del sistema inmune.

6. Determinar los efectos de la suplementación dietética de CLA en dietas para lubina sobre la acumulación de CLA en los tejidos y sus efectos sobre la calidad del filete producido en términos de parámetros de textura y sensoriales.

8.3. MATERIALES Y MÉTODOS

8.3.1. Dietas

En función del experimento realizado, se formularon diferentes dietas isolipídicas e isoproteicas comerciales con niveles crecientes de CLA (CLA0=Control, CLA05=0.5%, CLA1=1%, CLA2=2% and CLA4=4%). El CLA incluido en las dietas fue suministrado por BASF (50:50 mezcla de cada isómero) y se incorporó en las dietas en substitución de aceite de pescado con objeto de mantener el contenido energético constante entre los diferentes tratamientos. La formulación, composición proximal y composición en ácidos grasos de cada dieta están indicadas en los capítulos correspondientes a cada experimento.

8.3.2. Condiciones experimentales

Los juveniles de lubina empleados en los experimentos realizados se obtuvieron de una granja local (ADSA, San Bartolomé de Tirajana, Islas Canarias, España) de la que fueron transportados al Instituto Canario de Ciencias Marinas (ICCM, Las Palmas, España) dónde fueron aclimatados en tanques de 1000 l a las nuevas condiciones de cultivo (3 semanas). Una vez aclimatados, se distribuyeron de forma aleatoria y homogénea en los tanques experimentales correspondientes a cada experimento (1000, 500 y 120 l; Fig. 2.1a, 2.1b y 2.1c, respectivamente), y fueron alimentados con las dietas correspondientes a cada uno de ellos.

A lo largo de toda la experimentación se siguieron condiciones de cultivo estandarizadas. El suministro de agua marina (37 ppm salinidad) se realizó de forma continua y en sistema abierto. La iluminación fue natural indirecta con un fotoperiodo natural de aproximadamente 12L:12O, correspondiente a la época del año. La temperatura y el oxígeno disuelto se midieron diariamente (Oxy Guard-H*y*y beta; Zeigler Bros, Gardners, E.E.U.U.).

Los diseños experimentales y protocolos de muestreo seguidos durante los diferentes estudios se resumen en los esquemas representados en las figuras 2.2, 2.3, 2.4 y 2.5 correspondiendo a los experimentos I, II, III y IV, respectivamente.

Figura 8.3. Tanques usados a lo largo de la experimentación. (a) 1000 l; (b) 500 l; (c) Tanques de digestibilidad con un sistema de recolección de heces descrito por por Cho *et al.* (1985) y modificado por Robaina *et al.* (1995).

Fig. 8.4 Representación esquemática del diseño experimental y protocolo de muestreo seguido a lo largo del Experimento I

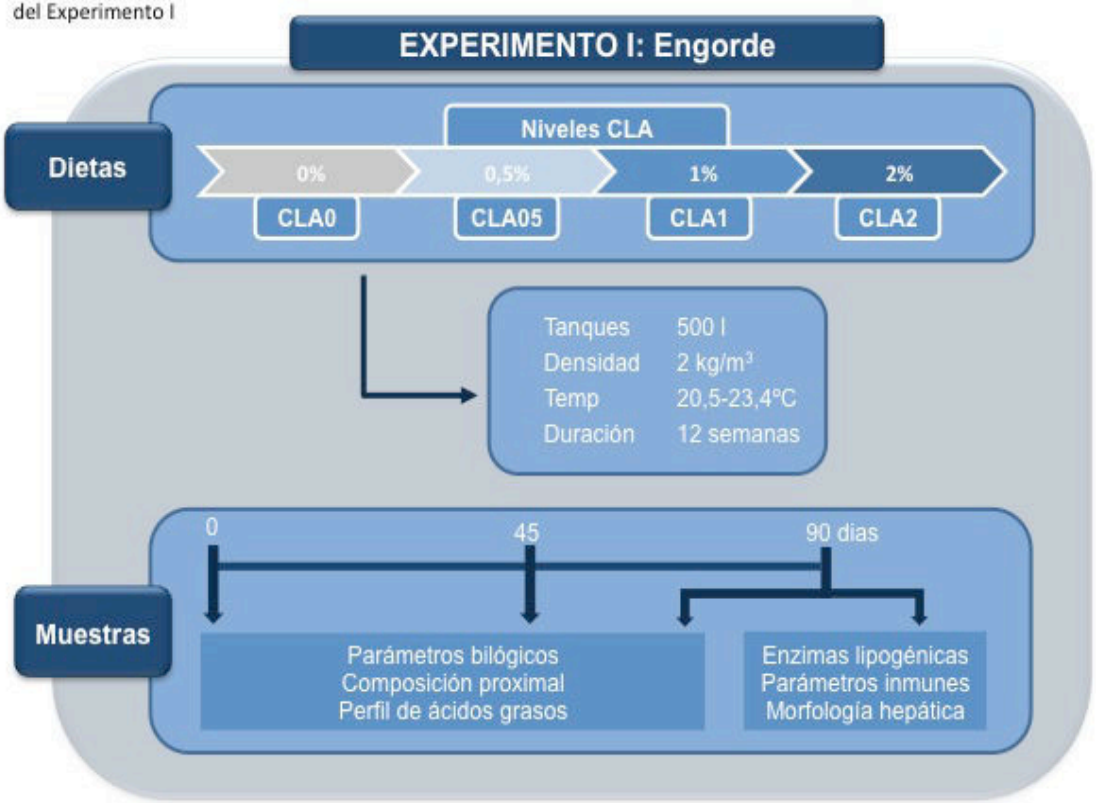


Fig. 8.5. Representación esquemática del diseño experimental y protocolo de muestreo seguido a lo largo del Experimento II

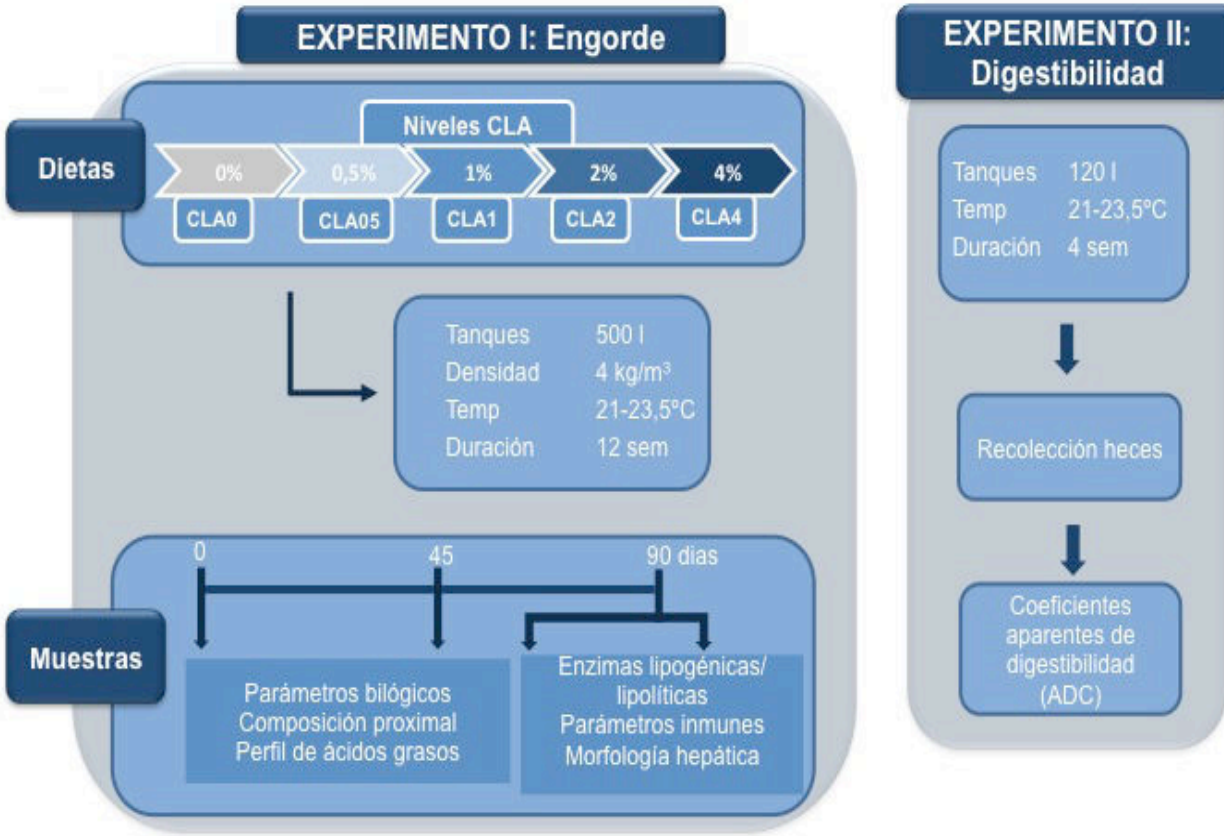


Fig. 8.6. Representación esquemática del diseño experimental y protocolo de muestreo seguido a lo largo del Experimento III

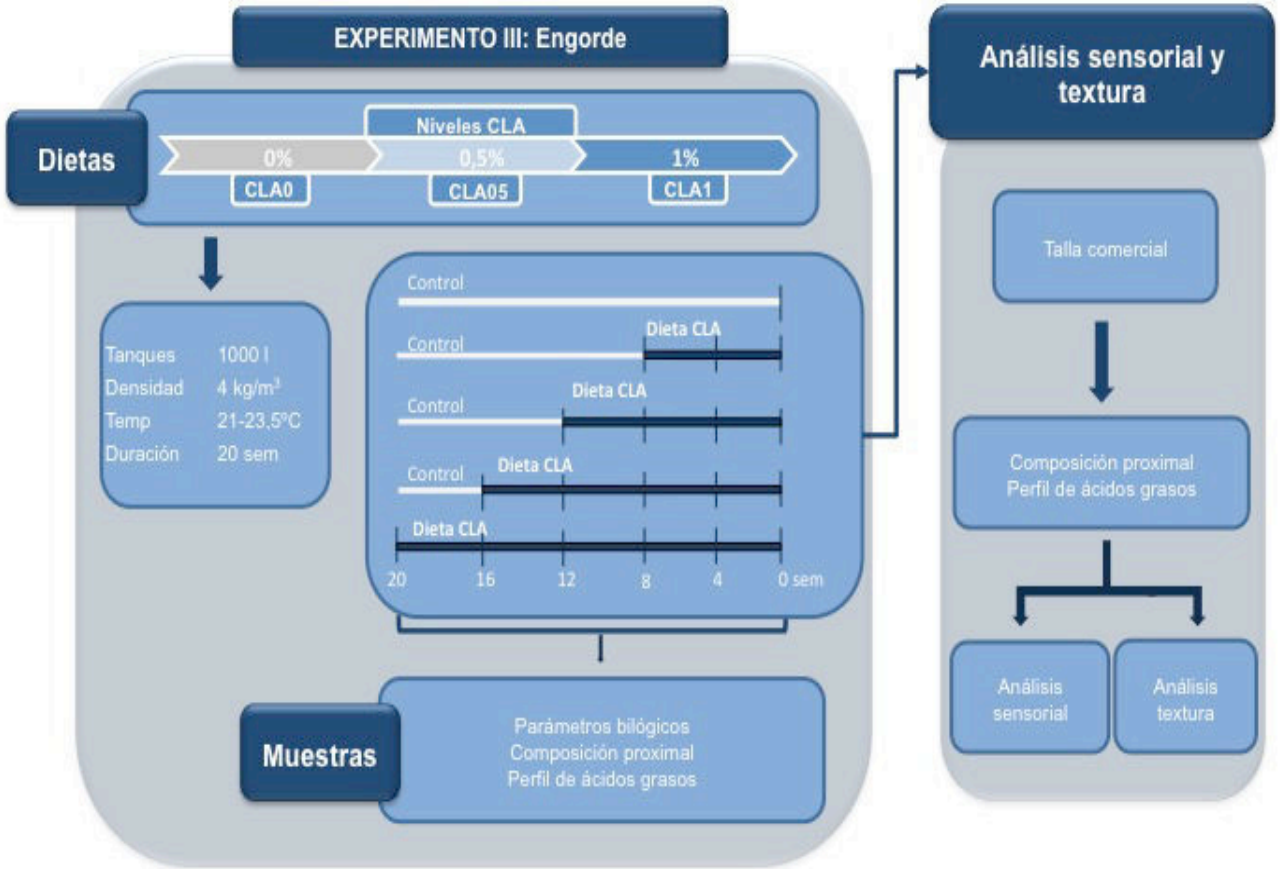
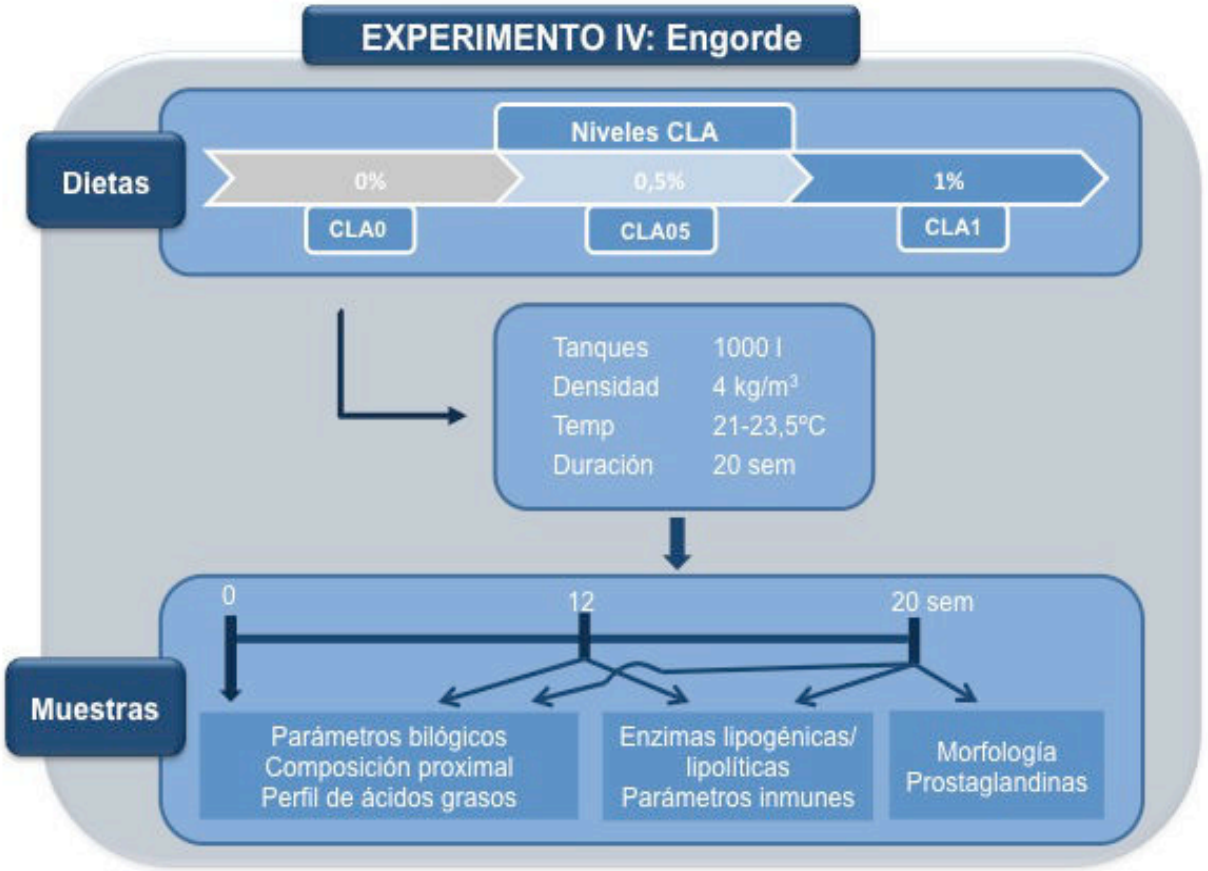


Fig. 8.7. Representación esquemática del diseño experimental y protocolo de muestreo seguido a lo largo del Experimento IV



8.3.3. Parámetros biológicos

8.3.3.1. Crecimiento relativo

Con objeto de evaluar las posibles diferencias potenciales en la eficiencia del alimento entre los diferentes niveles de CLA incluidos en las dietas se evaluó el crecimiento relativo. Éste se define como la relación entre la biomasa ganada (g) respecto al peso inicial (g).

Crecimiento relativo = [(Peso final – Peso inicial) / Peso inicial] x 100

8.3.3.2. Tasa de crecimiento específico o SGR

Este parámetro relaciona el peso ganado respecto a la duración del experimento, tal que:

SGR= [(ln Peso final –ln Peso inicial) / n° días] x 100

8.3.3.3. Tasa de conversión alimenticia o FCR

Se evaluó el índice de conversión o FCR de cada dieta o lote experimental, con objeto de determinar la eficiencia del alimento ingerido en relación al crecimiento generado. Se define como la relación entre el alimento ingerido (g) por biomasa generada (g peso húmedo).

FCR = Alimento ingerido / Biomasa generada

8.3.4. Análisis bioquímicos

8.3.4.1. Lípidos totales

Los lípidos procedentes de las muestras previamente pesadas fueron extraídos siguiendo el método descrito por Folch *et al.* (1957). Los lípidos extraídos fueron disueltos en cloroformo y conservados a -80°C en atmósfera de nitrógeno, para evitar su oxidación.

8.3.4.2. Perfiles de ácidos grasos

Los ésteres metílicos de los ácidos grasos fueron obtenidos mediante una transmetilación básica-catalizada en una mezcla de tolueno y metóxido de sodio 0,5M en metanol anhidro según el método descrito por Christie (1982). Los ésteres metílicos de los ácidos grasos obtenidos fueron extraídos con hexano y purificados por cromatografía de absorción con cartuchos NH_2 Sep-Pack (Waters S.A., Massachussets, USA), separados por cromatografía de gases (GC-14A, Shimadzu, Japan; columna de una longitud de 30m y un diámetro interno de 0,32 mm Sílice con Supelco-10, Supelco, Bellefonte, USA) según las condiciones descritas por Izquierdo *et al.*, (1992), y cuantificados con un detector de ionización de llama e identificados por la comparación con estándares externos conocidos de isómeros de CLA (Sigma-Aldrich and Matreya, LLC.) y aceites de pescado (EPA 28, Nippai, Ltd Tokyo, Japan). Todos los análisis se realizaron por triplicado.

8.3.4.3. Proteína bruta

El contenido proteico de las muestras se según la técnica Kjendahl (AOAC, 2000) para el análisis de piensos y peces. Las muestras fueron digeridas en ácido surfúrico al 37% en presencia de un catalizador de cobre. Después de su digestión, las muestras se destilaron en ácido bórico y el nitrógeno total liberado se cuantificó por valoración con ácido clorhídrico 0,1N, convirtiendo el valor obtenido en proteína bruta mediante su multiplicación por el factor empírico 6,25.

8.3.4.4. Humedad

La humedad contenida en las muestras se determinó siguiendo el método aceptado por la Asociación Química Analítica Oficial de Estados Unidos (AOAC, 2000) por desecación de una cantidad de muestra conocida hasta peso constante en un horno a 110°C.

8.3.4.5. Cenizas

El contenido en cenizas de las muestras se analizó siguiendo el método aceptado por la Asociación Química Analítica Oficial de Estados Unidos (AOAC, 2000) mediante incineración en un Horno Mufla a 600 °C por 12 horas hasta peso constante.

8.3.5. Análisis de digestibilidad

Para realizar los análisis de digestibilidad las heces fueron recogidas de los tanques de digestibilidad diariamente mediante el sistema de recolección de heces descrito por Cho *et al*. (1985) y modificado por Robaina *et al.* (1995). Inmediatamente después de su recolección, estas fueron congeladas y almacenadas a -20ºC hasta su análisis, previo al cual fueron liofilizadas. Los coeficientes aparentes de digestibilidad (ADC) para lípidos y proteínas se calcularon mediante la fórmula descrita por Maynard y Loosli (1969). El marcador interno utilizado en estos estudios fueron las cenizas insolubles en ácido y se determinaron mediante el método descrito por Atkinson *et al*. (1984). Cada dieta fue evaluada por triplicado.

$$\mathbf{ADC\ (\%) = 100 - [(100 \times (W / W_1) \times (W_2 / W_3))]}$$

Donde: W es el % de marcador en las dietas

W_1 es el % de marcador en las heces

W_2 es el % de nutriente en las heces

W_3 es el % de nutriente en la dieta.

8.3.6. Análisis enzimáticos

8.3.6.1. Enzimas lipogénicas (G6PD y ME)

Las muestras de hígado y músculo fueron homogenizadas en 3 volúmenes de solución tampón (20 mM Tris-HCl, 0,25 M sacarosa, 2 mM EDTA, pH 7.4) y los homogeneizados se centrifugaron a 20.000 g durante 40 min a 4°C. Las actividades enzimáticas de la glucosa-6-fosfato dehidrogenasa (G6PD, EC 1.1.1.49) y la enzima málica (ME, EC 1.1.1.40) se analizaron siguiendo los métodos descritos por Dias *et al.* (1998). El contenido de proteína soluble de las muestras se determinó mediante el método Bradford (1976) usando albúmina de suero bovino como estándar. Se prestó especial atención a que las tasas iniciales de actividad fueran estables en la solución tampón durante el tiempo y a la temperatura a la que los ensayos fueron conducidos (Álvarez *et al.*, 1998). La actividad enzimática (UI), definidas como micro moles de sustrato convertido a producto a la temperatura ensayada por minuto, se expresaron por mg de proteína soluble (actividad específica). Todos los análisis se realizaron por triplicado.

8.3.6.2. Enzimas lipolíticas (ACO y L3HOAD)

La actividad de la enzima Acyl-CoA oxidasa (ACO, EC 1.3.3.6) fue evaluada en la fracción peroxisómica enriquecida de hígado previa homogenización en tres volúmenes de solución tampón (20 mM Tris-HCl, 0,25M sacarosa, 2 mM EDTA, pH 7.4). Los homogeneizados se centrifugaron a 7.000 g durante 10 min a 4°C y el sobrenadante resultante se recogió y se mantuvo en hielo para su posterior procesado. La fracción celular resultante se lavó con 500 µl de solución tampón y se centrifugó en las mismas condiciones descritas anteriormente. El sobrenadante resultante se combinó con el anterior y se centrifugaron a 18.000 g durante 30 min a 4ºC. La fracción celular resultante se resuspendió en 600 µl de solución tampón y se sonicó en un baño de sonicación durante 30 min. Seguidamente se procedió a una centrifugación a 18.000 g durante 45 min a 4ºC y los sobrenadantes resultantes se recogieron para su posterior análisis. La actividad de la enzima ACO se evaluó espectrofotométricamente mediante la determinación de la producción de H_2O_2 junto con la oxidación de 2´,7´- diacetato de

diclorofluoresceína (LDCF) a 502nm. Las concentraciones del mix de reacción fueron las siguientes: 2,6 mM LDCF, 1M aminotriazol, 5mg/ml peroxidasa de rábano tipo II, 5% tritón X-100, 1 M Tris-HCl pH 8,5, 15 mM Flavina adenina dinucleótido, 50mg/ml albúmina de suero bovino (BSA). La reacción se inició mediante la adición de 1mM Palmitoyl-CoA.

La actividad de la enzima L-3-hydroxyacyl-CoA dehydrogenasa (L3HOAD, EC 1.1.135) fue evaluada en la fracción mitocondrial de hígado siguiendo el método descrito por Menoyo et al. (2004) y centrifugada según el método descrito por Harper y Saggerson (1975). La actividad de la enzima L3HOAD se evaluó mediante el protocolo descrito por Bradshaw y Noyes (1975) para fraciones mitocondriales rotas por sonicación en una solución de Triton X-100 al 1%. El contenido de proteína soluble de los homogenizados de hígado se determinó mediante el método Bradford (1976) usando albúmina de suero bovino como estándar. Se prestó especial atención a que las tasas iniciales de actividad fueran estables en la solución tampón durante el tiempo y a la temperatura a la que los ensayos fueron conducidos (Álvarez *et al.*, 1998). La actividad enzimática (UI), definidas como micro moles de sustrato convertido a producto a la temperatura ensayada por minuto, se expresaron por mg de proteína soluble (actividad específica). Todos los análisis se realizaron por triplicado.

8.3.7. Análisis histológicos

Las muestras de músculo, hígado y grasa perivisceral fueron fijadas en formol tamponado al 10%, incluidas en parafina, cortadas a 5 µm y teñidas con hematoxilina y eosina (H&E) (Martoja and Martoja-Pierson, 1970). Las imágenes fueron tomadas a una magnificación final de x400 con una microscopio Nikon Microphot-FXA equipado con una cámara Olympus DP50. Las áreas de los hepatocitos y adipocitos así como sus longitudes máximas y minímas se analizaron mediante un software de análisis de imágenes (Image Pro Plus®).

8.3.8. Análisis inmunológicos

8.3.8.1. Obtención de sangre y preparación de las muestras

La sangre se obtuvo por punción caudal con jeringuillas de 1 ml de capacidad. Con el objetivo de minimizar los efectos estresantes derivados del manejo, el tiempo de manipulación no superó el minuto. Para la evaluación del brote respiratorio de los neutrófilos circulantes en sangre (NBT) la sangre fue transferida a un tubo Eppendorf empapado con heparina. Para la obtención de suero por centrifugación, la sangre se transfirió a tubos Eppendorf donde se dejó sedimentar por 2h previo a su centrifugación. Las muestras se almacenaron a -80°C hasta su análisis

8.3.8.2. Potencial del brote respiratorio de los neutrófilos circulantes (NBT)

Se evaluó el brote respiratorio de los neutrófilos circulantes mediante el método descrito por Siwicki *et al.* (1993). Esta técnica tiene como objetivo medir la producción de aniones superóxido producidos por los neutrófilos circulantes en sangre a través de la reducción del NBT (Nitroazul de tetrazolio) a formazán en presencia de radicales oxígeno.

8.3.8.3. Lisozima

La actividad de la lisozima en suero se determinó mediante el método turbidimétrico descrito por Anderson and Siwiki (1994) usando lisozima de clara de huevo disuelto en solución de fosfatos tamponada con estándar.

8.3.8.4. Vía alternativa del complemento

La actividad de la vía alternativa del complemento se determinó según la técnica descrita por Sunyer y Tort (1995) para dorada usando células sanguíneas rojas de conejo (RBC). La dilución de suero causante de la lisis del 50% células sanguíneas se denomina ACH50 y los resultados se presentan como ACH50/ml.

8.3.8.5. Actividad bactericida

La actividad bactericida frente a la bacteria *Vibrio anguillarum* de las muestras de suero se determinó según el método descrito por Torrecillas et al., (2011a). Dónde la concentración de *V. anguillarum* fue ajustada a 10^8 ufc/ml y el descenso de absorbancia por minuto evaluado.

8.3.8.6. Capacidad fagocítica de los leucocitos aislados del riñón anterior

Se extrajeron los riñones anteriores de los peces, los cuales fueron macerados en un medio de cultivo (MEM) y filtrados a través de una membrana de nylon. La solución celular resultante se colocó sobre un gradiente de Percoll 34%/51% (v/v) con un 10% de solución balanceada de Hanks (HBSS) y fue centrifugada a 800g por 30 min. Se recogieron células depositadas en la interfase, se lavaron por centrifugación a 800g por 5 min con objeto de eliminar el posible Percoll residual. La fracción celular resultante se diluyó en 1 ml de solución MEM suplementada y se determinó su viabilidad y concentración. Seguidamente, se ajustó la concentración de leucocitos a la deseada y se incubó durante una hora frente a la bolas de látex (1 µm, Fluka) siguiendo el protocolo descrito por Esteban y Messenger (1997) para *V. anguillarum*. Finalmente la actividad fagocítica se determinó según el método descrito por Blazer (1991). Se consideró actividad positiva únicamente cuando se encontró la partícula de látex en el interior del citoplasma.

8.3.8.7. Análisis de prostaglandinas

Una vez diseccionados los tejidos, se pesaron y se digirieron en 4 ml de HBSS sin Ca^{2+} y suplementada al 2% con colagenasa durante 30 min en agitación orbital continua ($100U \cdot min^{-1}$). Después de la digestión, las muestras fueron filtradas a través de una membrana de nylon y lavadas con 4 ml de HBSS sin Ca^{2+}. La suspensión celular obtenida se lavó por centrifugación y el pellet resultante se suspendió en 1 ml de HBSS sin Ca^{2+}. Las células fueron estimuladas durante 30 min con 50 µM Ca^{+} PMA (Phorbol 12-myristate 13-acetate, Sigma Chemicals, CA, USA) *y* 10 µM A321 (Sigma Chemicals, CA,

USA) y la reacción se paró con 50 µl ácido fórmico 2M. Las muestras fueron almacenadas a -80°C hasta su purificación.

Para la purificación de las fracciones estimuladas (pool de 3 peces/tanque) en primer lugar se precipitaron los posibles restos/desechos por centrifugación. Los sobrenadantes fueron extraídos usando minicolumnas "Sep-pack" de sílice de octadecyl (C18) (Millipore, Watford, Reino Unido) según el método descrito por Powell (1982) y detallado por Bell *et al.* (1994). Se aplicó 1 ml del sobrenadante a la columna, la cual previamente fue prelavada con 5 ml de metanol y 10 ml de agua MiliQ. Seguidamente a la aplicación de la muestra la columna, ésta fue lavada sucesivamente con 10 ml de agua MiliQ, 5 ml de etanol al 15% (v/v) y 5 ml de hexano:cloroformo (65:35; v/v) antes de la elución de los prostanoides con 10 ml de acetato etílico. La solución resultante se evaporó a sequedad bajo nitrógeno, se suspendió de nuevo en 100 µl de metanol y se almacenó a -80°C hasta su uso.

Para este análisis se utilizó un kit específico de análisis de prostaglandinas (Prostaglandin E2 EIA Kit- Monoclonal; Cayman Chemical Co., MI, USA). Este análisis se basa en la competición entre la PGE_2 y un conjugado de PGE_2-acetilcolinaesterasa para una cantidad limitada de anticuerpo monoclonal PGE_2.

8.3.9. Análisis sensorial

Los peces fueron mantenidos en ayuno durante las 24 h previas a su sacrificio el cual fue llevado a cabo en hielo. Una vez sacrificados se eviscerаron, filetearon y se mantuvieron a 4°C hasta su análisis. La cocción de los filetes se llevó a cabo en recipientes de aluminio (3x4 cm) durante 10 min en un horno de vapor a 120°C. Inmediatamente después de ser cocinados, se ofrecieron aleatoriamente y en los recipientes cerrados y marcados con un código a un panel de 8 catadores entrenados (ISO 1985, ISO 1993). La evaluación se llevó a cabo en habitaciones aisladas, climatizadas y previstas de una luz estandarizada (ISO 1988). Los parámetros evaluados fueron los siguientes: Olor (intensidad, marino y aceitoso), apariencia (color, brillo e integridad), textura (firmeza, jugosidad, masticabilidad, adhesividad y contenido graso) y sabor

(intensidad, marino, aceitoso y sabor residual) y se evaluaron en una escala continua de 0 a 100 para cada parámetro evaluado.

8.3.10. Análisis de textura

Los filetes de la parte izquierda de los mismos peces usados en el análisis sensorial fueron evaluados para los diferentes parámetros de textura. La piel se eliminó de las muestras y se cortaron 4 piezas cuadradas (2,5 x 2,5 cm) de cada filete de la parte superior de la línea lateral. Las muestras se mantuvieron en hielo hasta su análisis con el texturómetro TA-XT2 (Stable MicroSystems, England). El perfil de textura se obtuvo mediante compresión con una superficie de aluminio de 100 mm de diámetro. La tasa de compresión utilizada se estableció en 0,8 mm/s a una capacidad de penetración del 80%. Las muestras fueron comprimidas dos veces con un intervalo de 60 s entre las dos compresiones (Tryggvadottir y Olafsdottir, 2000). Los parámetros de textura evaluados fueron: capacidad de fractura, dureza, jugosidad, cohesividad, gomosidad, adhesividad y resistencia y fueron calculadas según las recomendaciones de Ginés *et al.* (2004).

8.3.11. Análisis estadísticos

Se calcularon las medias y desviaciones estándar para cada parámetro evaluado. Los métodos estadísticos aplicados siguieron los criterios de Sokal y Rolf (1995). Los datos se sometieron a un análisis de la varianza unidireccional (ANOVA) con objeto de analizar los efectos de los diferentes niveles de inclusión de CLA. En los casos en los que las varianzas no cumplían una distribución normal, se normalizaron los datos mediante una transformación logarítmica. Cuando los valores F mostraron diferencias significativas, las medias individuales se compararon mediante un Test de Tukey para la comparación multiple de medias. Se consideró como diferencia significativa cuando $P<0.05$. Los análisis fueron llevados a cabo mediante el uso del software Statgraphics (Statgraphics Plus 5.1 for Windows, Statpoint Technologies Inc., Warrenton, VA, USA).

8.4. RESUMENES DE LOS EXPERIMENTOS

8.4.1. CAPÍTULO 3: EFECTO DEL ÁCIDO LINOLEICO CONJUGADO (CLA) SOBRE LA UTILIZACIÓN DIETÉTICA DE LOS LÍPIDOS, LA MORFOLOGÍA INTESTINAL Y ALGUNOS PARÁMETROS INMUNES EN JUVENILES DE LUBINA (*DICENTRARCHUS LABRAX*)

El incremento energético en dietas para peces ha derivado en una mayor deposición de grasa, especialmente el lubina, hecho que afecta especialmente a los productores de peces. La inclusión de ácido linoléico conjugado (CLA) en otras especies de vertebrados puede derivar en una menor deposición de grasa. Con objeto de determinar si la inclusión de CLA en dietas para lubina afecta a la deposición de grasas, al metabolismo y composición lipídica, así como a la morfología de diferentes tejidos, al crecimiento y a determinados parámetros inmunes, se alimentó a juveniles de lubina con niveles crecientes de CLA (0, 0,5, 1 y 2%). El crecimiento y la tasa de conversión alimenticia no fueron afectados por la inclusión de CLA, aunque la ingesta fue reducida ($P<0.05$) tras alimentar con el nivel de inclusión más alto de inclusión (2% CLA). Paralelamente, la suplementación con CLA al 2% redujo ($P<0.05$) la grasa perivisceral principalmente marcado por una reducción de ácidos grasos monoinsaturados ($P<0.05$). La inclusión de CLA en dietas para juveniles de lubina no afectó a la composición proximal de los tejidos analizados, pero redujo ($P<0.05$) la fracción de ácidos grasos saturados y monoinsaturados e incrementó ($P<0.05$) la fracción de ácidos grasos de las serie n-3 así como los ácidos grasos altamente insaturados en músculo. La concentración de CLA en peces suplementados incrementó ($P<0.05$) en músculo, hígado y grasa perivisceral. Se observó una reducción progresiva del nivel de vacuolización citoplasmática en los hepatocitos así como una morfología más regular en los peces alimentados con niveles crecientes de CLA, junto con un incremento progresivo en la actividad de la enzima málica (1% CLA). Finalmente, la inclusión de CLA hasta 1% aumentó ($P<0.05$) la actividad del lisozima y mostró una correlación positiva con la actividad de la vía alternativa del complemento en suero.

8.4.2. CAPÍTULO 4: EFECTO DE LA SUPLEMENTACIÓN DIETÉTICA CON DIFERENTES NIVELES DE ÁCIDO LINOLEICO CONJUGADO (CLA) SOBRE EL METABOLISMO LIPÍDICO Y EL SISTEMA INMUNE DE LA LUBINA (*DICENTRARCHUS LABRAX*)

En el presente estudio, se evaluaron los efectos derivados de la suplementación dietética con diferentes niveles de ácido linoléico conjugado (CLA) sobre el crecimiento, la digestibilidad de nutrientes, el metabolismo lipídico en hígado, la morfología hepática así como sobre el sistema inmune. Para ello, setecientas cincuenta lubinas con un peso medio de 39.98±0.47 g, fueron distribuidas aleatoriamente en 15 tanques de 500 l de capacidad (4kg/m^3) y alimentadas durante 12 semanas con cinco dietas que contenían diferentes niveles de CLA (0, 0.5, 1, 2 and 4%).

La inclusión de CLA en las dietas no afectó al crecimiento así como a la digestibilidad de nutrientes. El contenido lipídico y proteico del músculo de los peces alimentados con CLA se vio reducido y aumentado, respectivamente, en los peces alimentados con los niveles más altos de este ácido graso. Los isómeros de CLA se acumularon de forma gradual dependiendo del nivel de inclusión en la dieta en todos los tejidos evaluados. La actividad de las enzimas G6PD, ME y L3HOAD no se vio afectada por la inclusión de CLA en las dietas, todo y que la actividad de la enzima ACO se vio incrementada en los peces alimentados con las dietas que incluían CLA al 2 y 4 %. La inclusión de CLA hasta un 2% en dietas para lubina redujo el área de los hepatocitos así como su longitud mínima. Finalmente, la capacidad fagocítica de los leucocitos del riñón anterior se vio estimulada en los peces alimentados con la dieta que incluía un 4 % de CLA.

Basándonos en estos resultados podemos concluir que el CLA tiende a reducir la acumulación de grasa perivisceral y el contenido lipídico en músculo, siendo el nivel de inclusión al 2% CLA, el nivel óptimo a utilizar con objeto de promover la movilización hepática de lípidos vía incremento de la lipolisis, y únicamente el nivel de inclusión al 4% tiene un efecto potenciador del sistema inmune.

8.4.3. CAPÍTULO 5: EFECTO DE LA SUPLEMENTACIÓN DIETÉTICA A LARGO PLAZO CON ÁCIDO LINOLEICO CONJUGADO (CLA) SOBRE EL CRECIMIENTO Y EL METABOLISMO LIPÍDICO EN LUBINA (*DICENTRARCHUS LABRAX*)

El objetivo del presente experimento fue evaluar el efecto de la suplementación dietética con acido linoléico conjugado (CLA) sobre el crecimiento, la morfología hepática en lubina (*Dicentrarchus labrax*). Para ello, doscientos tres ejemplares de lubina (152.4 ± 1.96 g) se distribuyeron aleatoriamente en 9 tanques de 1m^3 de capacidad (4.1 kg/m^3) y fueron alimentados durante 20 semanas con una de las tres dietas experimentales que contenían niveles crecientes de CLA (0, 0,5 and 1%). La alimentación con CLA no afectó al crecimiento ni a la composición proximal de los diferentes tejidos evaluados. El perfil de ácidos grasos de músculo e hígado se vio alterada por la inclusión de CLA. En el músculo, la inclusión de CLA al 1% aumentó la fracción de ácidos grasos saturados pero no tuvo efecto sobre esta fracción en hígado. La actividad de las enzimas lipogénicas y lipolíticas evaluadas en hígado no se vio afectada por la inclusión de CLA en las dietas. El área de los hepatocitos se redujo en los peces alimentados con 1% CLA, así como el área de los adipocitos en los peces alimentados con 0,5% y 1% CLA dónde además el número de adipocitos por unidad de área se incrementó. La capacidad de producción de eicosanoides del riñón anterior se redujo por la inclusión de CLA al 1%, correlacionándose además negativamente con la inclusión de CLA en las dietas suministradas. El presente estudio sugiere que el CLA puede ser incorporado hasta un 1% en dietas para lubina a partir de 150 g de peso por 20 semanas, siendo este acumulado en sus tejidos y sin afectar el crecimiento o la actividad enzimática, todo y que mejora la morfología hepática así como la de la grasa perivisceral.

8.4.4. CAPÍTULO 6: DEPOSICIÓN DEL ÁCIDO LINOLEICO CONJUGADO (CLA) EN EL MÚSCULO DE LUBINA *(DICENTRARCHUS LABRAX*) DE TALLA COMERCIAL Y SUS EFECTOS SOBRE LOS PARÁMETROS DE TEXTURA Y SENSORIALES DEL FILETE OBTENIDO.

Estudios anteriores muestran que el ácido linoléico conjugado (CLA) puede ser incorporado en dietas para juveniles de lubina (*Dicentrarchus labrax*) hasta un 4% sin comprometer el crecimiento e incrementando la deposición de los isómeros del CLA en el músculo. EL CLA tiene propiedades antioxidantes, anticancerígenas, immunomoduladoras así como un efecto modulador de la diabetes en humanos. La deposición de CLA en el filete producido y sus efectos sobre la textura y propiedades sensoriales de éste, no han sido evaluados anteriormente en esta especie. Para ello, cuatrocientos ochenta y seis ejemplares de lubina (151.8 ± 2.26 g) se distribuyeron aleatoriamente en 18 tanques de 1 m^3 de capacidad (4.1 kg/m^3) y fueron alimentados durante 20 semanas con una de las tres dietas experimentales que contenían niveles crecientes de CLA (0, 0,5 and 1%). Cada cuatro semanas se muestrearon los peces con objeto de evaluar la composición proximal y el perfil de ácidos grasos del músculo. Al final del experimento se evaluaron los parámetros sensoriales y de textura del filete producido. El crecimiento, la tasa de conversión alimenticia así como la composición proximal de músculo no se vieron afectadas por la suplementación con CLA. El perfil de ácidos grasos en el músculo no se vio afectado por la inclusión de CLA, a pesar del incremento en la fracción de ácidos grasos saturados en los peces alimentados con CLA al 1% por 16 semanas previo al despesque. Por otro lado, la concentración de los isómeros del CLA aumentó gradualmente y de manera dependiente al nivel de inclusión de este ácido graso en las dietas, alcanzando el máximo nivel de acumulación en peces alimentados con CLA al 1% por 20 semanas antes del despesque. La acumulación de CLA en el filete producido fue similar en los peces alimentados con CLA al 1% por 12 semanas y en los peces alimentados con CLA al 0,5% por 20 semanas antes del despesque. Los análisis sensoriales reflejan que los filetes de los peces alimentados con CLA al 1% fueron más jugosos ($P<0.05$) comparados con los filetes de los peces alimentados con la dieta control, mientras que los filetes de los peces alimentados con CLA al 0,5% presentaron menor masticabilidad que los de los peces alimentados con la dieta control.

Los parámetros de textura en el filete producido no se vieron afectados por la inclusión de CLA en las dietas. Estos resultados sugieren que la lubina de talla comercial puede incorporar satisfactoriamente los isómeros de CLA en el filete producido con efectos positivos sobre los atributos sensoriales de éste, y de esta manera el potencial de los peces cultivados como alimento funcional se ve reforzado.

8.5. CONCLUSIONES

1. El CLA puede ser incorporado en dietas para lubina hasta un 4% sin comprometer el crecimiento de los peces, tanto en juveniles como en peces de talla comercial.

2. La inclusión dietética de CLA hasta un 4% en dietas para lubina no afecta negativamente a la digestibilidad de nutrientes.

3. El CLA suministrado fue acumulado en todos los tejidos ensayados de una forma dosis dependiente en relación al nivel de inclusión de CLA utilizado, independientemente de la densidad de cultivo empleada así como de la talla de pescado ensayada.

4. El incremento en los niveles de inclusión de CLA en dietas para lubina has un 4 % fue correlacionado con una reducción en la deposición de grasa perivisceral, siendo este efecto más marcado cuando los peces se cultivaron a bajas densidades (2 Kg/m^3).

5. La inclusión de CLA al 0,5% en dietas para lubina de talla comercial se tradujo en una reducción del área de los adipocitos así como en un incremento en el número de estos por unidad de área, en relación a la tendencia de una menor deposición de grasa perivisceral encontrada.

6. La inclusión de CLA hasta un 2% en dietas para lubina resultó en un menor grado de vacuolización lipídica de los hepatocitos así como en una reducción del tamaño de estos, independientemente de la densidad de cultivo o talla de pescado utilizada.

7. Los niveles de inclusión de CLA al 2 y 4 % aumentaron la actividad de la enzima lipolítca Acyl-CoA oxidasa en el hígado de la lubina.

8. La inclusión de CLA en dietas para juveniles de lubina hasta un 2% y cultivadas a bajas densidades (2 Kg/m^3) redujo los ácidos grasos saturados y monoinsaturados en músculo e incrementó las fracciones de las series n-3 y n-3 HUFA. Todo y que, cuando se incrementaron la densidad de cultivo o la talla de lubina estudiada, sólo se vieron reducidos los ácidos grasos saturados en talla comercial cuando el CLA se suministró al 1%.

9. La inclusión dietética de CLA afectó positivamente al sistema inmune de juveniles de lubina, aumentando la actividad de la lisozima en suero en peces cultivados a bajas densidades (hasta un 1%) y aumentando la actividad fagocítica de los leucocitos de riñón anterior cuando se aplicaron densidades de cultivo más elevadas (4% CLA). No se detectaron efectos sobre el sistema inmune en peces de talla comercial.

10. La suplementación de CLA por 20 semanas tiende a mejorar las propiedades sensoriales del filete de lubina, aumentando su jugosidad cuando se suplementa la 1% y reduciendo su masticabilidad cuando es suplementado al 0.5%.

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