UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

PhD Thesis

Effect of feeding gilthead seabream (Sparus aurata) with different levels of n-3 and n-6 lipids from vegetable oils on fish health and resistance to stress

Rachid Ganga

Grupo de Investigación en Acuicultura

Las Palmas de Gran Canaria, Spain
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IUSA
Instituto Universitario Sanidad Animal Seguridad Alimentaria

Anexo I

Dª BEGOÑA ACOSTA HERNÁNDEZ, 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 26 de noviembre de 2009 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "Effect of feeding gilthead seabream (*Sparus aurata*) with different levels of n-3 and n-6 lipids from vegetable oils on fish health and resistance to stress", presentada por el doctorando D Rachid Ganga y dirigida por la Doctora Dª Marisol Izquierdo López y el Doctor D. Gordon Bell.

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 veintiseis de noviembre de dos mil nueve.

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Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

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Título de la Tesis

"Effect of feeding gilthead seabream (*Sparus aurata*) with different levels of n-3 and n-6 lipids from vegetable oils on fish health and resistance to stress",

Tesis Doctoral presentada por **D Rachid Ganga**

Dirigida por **la Doctora Dª Marisol Izquierdo López y el Doctor D. Gordon Bell.**

El/la Director/a **El/la Doctorando/a,**

Las Palmas de Gran Canaria, a 26 de noviembre de 2009

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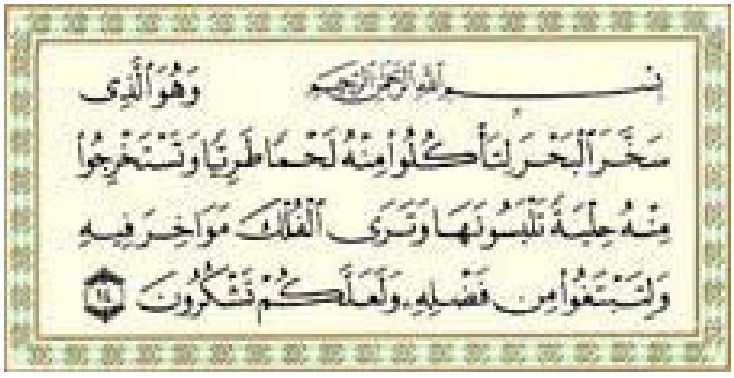

“ En el nombre de Alá, el Compasivo, el Misericordioso, Él es Quien ha sujetado el mar para que comáis de él carne fresca y obtengáis de él adornos que poneros. Y ves que las naves lo surcan. Para que busquéis Su favor. Quizás, así, seáis agradecidos”

16 - Las abejas (Al nahl), verso 14. Revelado antes de Higrah (Sagrado CORAN)

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LIST OF ABBREVIATIONS

ACTH Adrenocorticotrophic acid

ARA Arachidonic acid (20:4n-6)

ATP Adenosine triphosphate

cAMP Adenosine 3', 5'-cyclic monophosphate

CE Cholesterol ester

COX Cyclooxygenase

CR Cathecolamine receptor

CRF Cortisol releasing factor

CRH Corticotropic releasing hormone

DHA Docosahexaenoic acid (22:6n-3)

DHGLA Dihomogammalinolenic acid (20:3n-6)

DPA Docosapentaenoic acid

EFA Essential fatty acid

EPA Eicosapentaenoic acid

FA Fatty acid

FAME Fatty acid methyester

FAO Food Agriculture Organization

FCR Food conversion ratio

FIFO Fish in fish out

FM Fish meal

FO Fish oil

GAS General adaptation syndrome

GC Gas chromatography

GLA Gamalinolenic acid

GR Glucocorticoid receptor

HPI Hipothalamus-Pituitary-Interrenal Cell

HSC Hypothalamus-Sympathetic nerves-Chromaffin

HUFA Highly unsaturated fatty acid

IFFO International Fishmeal and Fish Oil Organisation

INDO Indomethacin

LA Linoleic acid

LNA Linolenic acid

LO Linseed oil

LOX Lipoxygenase

LPH Lipotropic hormone

LT Leukotriene

LX Lipoxines

MDA Malonaldehyde

MSH Melanocyte stimulating hormone

NADPH Nocotinamide adenine dinucleotide phosphate

NDGA Nordihydroguaiaretic acid

NK Natural killer

NSB Non specific binding

OA Oleic acid

PBL Peripheral blood leucocytes

PC Phosphatidyl choline

PE Phosphatidyl ethanolamine

PG prostaglandin

PI Phosphatidyl inositol

PKA Protein kinase A

PPARs Peroxisome proliferators-activated receptors

PUFA Polyunsaturated fatty acid

RIA Radioimmunoassay

SAM Symoathetic-Adrenal-Medullar

SBM Soybean meal

SO Soybean oil

TBARS Thiobarbituric acid reactive substances

TFA Total fatty acids

TX Thromboxane

VM Vegetable meal

VO Vegetable oil

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إهـــــــداء

إلـى أمي العـزيزة ...
وإلـى روح أبي الطاهـرة

Chapter 1

General Introduction

1- Lipids as a constituent of fish diets

1.1- Lipids and fatty acids

Lipids are a large and diverse group of naturally occurring organic compounds that have in common a ready solubility in organic solvents (hydrocarbons, chloroform, benzene, ethers and alcohols) and general insolubility in water. There is a great structural variety among the lipids, having as a basic form a hydrocarbon chain with a carboxylic group at one end and a terminal methyl group at the other (n or *w* carbon). The acyl chain could be saturated or unsaturated and can be esterified to other molecules; then, lipids include a diverse range of compounds, like fatty acids and their derivatives, carotenoids, terpenes, steroids and bile acids.

Lipids constitute a heterogeneous group of compounds, and different classifications are used to distinguish them. For chromatography purposes, lipids are divided in two broad classes including complex lipids and simple lipids. The latter include nearly all the commercially important fats and oils of animal and plant origin such as, triacylglycerols, diacylglycerols, 2-monoacyl-sn-glycerols, sterols, waxes, tocopherols and free fatty acids. Complex lipids including glycerophospholipids, glycoglycerolipids, sphingomyelin, glycosphingolipids and eicosanoids. Otherwise, according to their degree of polarity, lipids are separated into polar or neutral lipids. Polar lipids including glycerophospholipids (currently named phospholipids), glucoglycerolipids, sphingomyelin and glycosphingolipids, whereas neutral lipids contain fatty acids and their derivative glycerolipids, sterols, waxes and tocopherols, among others.

Fatty acids are the basic building block of the lipids, certain are essential components of the diet, as they cannot be synthesised by animals and have specific physiological and structural functions. Polyunsaturated fatty acids (PUFA) are fatty acids containing more than 16 carbon atoms with two or more double bonds. The PUFA most frequently found in nature are: docosahexaenoic acid (DHA; 22:6n-3), docosapentaenoic acid (DPA; 22:5n-3 or n-6), eicosapentaenoic acid (EPA; 20:5n-3), arachidonic acid (ARA; 20:4n-6), γ-linolenic acid (GLA; 18:3n-6), α-linolenic acid (LNA; 18:3n-3) and linoleic acid (LA; 18:2n-6). These are important dietary nutrients for mammals including humans (Simopoulos, 2000). Highly unsaturated fatty acids (HUFA), such as DHA, DPA, EPA and ARA are those PUFA of 20 and more carbon atoms in their aliphatic chain with 3 or more unsaturated bonds, and fish are the major

dietary source of n-3 HUFA (Ackman, 1980; Sargent and Tacon, 1999). ARA generally accounts for only 1-2% of the total fatty acids in fish phosphoglycerides, with the notable exception of the phosphatidylinositols where ARA can be the major PUFA (Izquierdo, 1996; Sargent *et al*., 1993), thus, fish have high nutritional requirements for ARA, EPA and DHA (Sargent *et al*., 1997).

Fish naturally consume diets rich in (n-3) PUFA, however, the tissue fatty acid compositions of cultivated fish is generally determined both by the type of dietary lipid ingested and the ability of the individual fish species to modify that dietary input via both pathways of catabolism and conversion including desaturation and elongation (Henderson and Tocher, 1987; Sargent *et al*., 1989; Torstensen *et al*., 2000; Bell *et al*., 2001, 2002). It is generally understood that marine fish are incapable of these conversions (Tocher and Ghioni, 1999) and require preformed EPA, ARA and DHA in their diets.

1.2- Lipid functions in fish

In common with other vertebrate so far studied including humans, fish have obligatory dietary requirements for both (n-6) and (n-3) PUFA. In order to provide the fish with adequate equilibrated diets, it is necessary to know the physiological and metabolic functions of the different dietary nutrients. Furthermore, lipids have multiple structural and physiological functions in fish, since fatty acids are the main cell membrane constituents.

Furthermore, some fatty acids are also important precursors for a range of highly biologically active mediators of fish metabolism and physiology. Thus, fatty acids can act as second messengers required for translation of external signals, as they are produced rapidly as a consequence of the binding of specific agonists to plasma membrane receptors. Within cells, fatty acids can act to amplify or otherwise modify signals to influence the activities of such enzymes as protein kinases, phospholipases, and many more. They are involved in regulating gene expression, mainly targeting genes that encode proteins with roles in fatty acid transport or metabolism via effects on transcription factors, i.e. peroxisome proliferators-activated receptors (PPARs) in the nuclei of cells, such effects can be highly specific to particular fatty acids. Indeed, phospholipids play multiple roles in cells other than establishing permeability barriers, they provide a matrix for the assembly and function of a wide variety of enzymes, they participate in the synthesis of macromolecules, and they act as molecular signals to

influence metabolic events.

The instability of biomembranes, including mechanical fragility and osmotic leakiness is affected by its fatty acids composition. Therefore, environment may change membrane fluidity, which acts as a sensitive receptor initiating cellular regulation (Beney and Gervais, 2001). It is important that membrane fluidity is also affected by the content of HUFA in constituent phospholipids, which due to their three dimensional structure and lower hydrogen-hydrogen interactions, increase protein insertion. For example, the fatty acid composition of phospholipids from membranes determines the level of its fluidity (Bell *et al*., 1986), and considering that fish is a poikilotherm organism, its membrane fatty acid content is crucial to adapt to the environmental temperature changes. Furthermore, changes in the fatty acid composition of the cell membranes could directly affect the activities of membrane-bound enzymes and receptors, perturbing consequently different physiological processes (growth, reproduction, immune system…etc).

The essential fatty acid (EFA) requirements of freshwater and marine fish species have been extensively studied over the past 20 years and are known to vary both quantitatively and qualitatively (Sargent *et al*., 1989, 1995, 2002). In freshwater fish, EFA requirements can be met by supplying LA and/or LNA, although better performances can be achieved by supplying the "bioactive" forms of the n-3 HUFA, mainly EPA and DHA (Kanazawa, 1985). Dietary lipids provide essential PUFA necessary for growth and development of cells and tissues (Sargent *et al*., 1995) and are also a major source of energy (Sargent *et al*., 1989) yielding 9 Kcal/gr, promoting their use to partially spare protein in aquaculture feeds and enhancing diet profitability (Vergara *et al*., 1996; Morrow *et al*., 2004). In addition, fish phosphoglycerides are naturally rich in DHA and EPA and it can be assumed that these fatty acids fulfil the same structural role in biomembrane phosphoglycerides that ARA does in higher terrestrial mammals (Sargent, 1995).

In fish, some reports show positive effects of n-3 FA on immune response, as the increased activity of head kidney macrophages has been associated with higher levels of dietary n-3 fatty acids in catfish (Blazer, 1991; Sheldon and Blazer, 1991). Ashton et al. (1994) have found that head kidney supernatants derived from rainbow trout fed a diet enriched with n-3 fatty acids had greater migration stimulating ability than supernatants from fish fed an n-6 fatty acid enriched diet. Thereby, adequate amount of n-3 fatty acids was found to be essential for the correct function of immune system.

While long-chain PUFAs possess a wide range of cellular functions, one of their most important is to supply precursors for the production of eicosanoids, which are bioactive fatty acid metabolites that can modulate many immune functions (Gershwin *et al*., 1985; Uhing *et al*., 1990). Eicosanoids are produced from C_{20} PUFA, especially dihomo-gamma-linolenic-acid (20:3n-6; DHGLA), ARA and EPA, by the action of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes to yield a wide range of bioactive compounds, including prostaglandins (PG) and thromboxanes (TX) produced by the action of COX. LOX yields a range of monohydroxy fatty acids (e.g. 5 (S)-hydroxy-eicosatetraenoic acid; 5-HETE derived from ARA), while di- and tri-hydroxy fatty acids, such as leukotrienes (LT), lipoxins (LX), are also formed via epoxy intermediates (Samuelson, 1983).

ARA is the major eicosanoid precursor in mammalian cells, giving rise to, among other products, 2-series prostaglandins (Horrobin, 1983). Other C_{20} PUFAs, such DHGLA and EPA are also substrates for eicosanoid production and yield prostaglandins of the 1- and 3-series, respectively. Although DHGLA and EPA are generally poorer substrates for prostaglandin synthetase than ARA (Crawford, 1983), they both compete for the enzyme binding site and can reduce the production of ARA-derived prostaglandins (Willis, 1981; Bell *et al*., 1994)

Eicosanoids have diverse pathophysiological actions including immune response and inflammatory processes. Thus, they are involved in the regulation of the immune system by their direct effects on cells such as macrophages and lymphocytes or their indirect effects via cytokines (Rowley, 1995). PG, especially PGE_2 are important in modulating the function of immune cells and while PGE_2 is required for normal immune function, overproduction can be immunosuppressive (Kinsella *et al*., 1990). Furthermore, the lipoxygenase product LTB_4 is a powerful modulator of immune cell function, augmenting proliferation of T and B cells, stimulating the release of cytokines from monocytes and T cells, acting as a potent chemoattractant and inducing natural killer (NK) cell activity (Kinsella *et al*., 1990; Claesson *et al*., 1992). LOX derivatives are also shown to regulate and mediate the interactions between innate and adaptive immunity of the host defence (Hedi and Norbert, 2004). Recent studies indicate that some pathogens disable invertebrate immunity by inhibiting eicosanoid actions in cellular immunity, indicating the key role of eicosanoids on immune defence (Dean *et al*., 2002; Stanley and Miller, 2006). Dietary n-3HUFA levels influence patterns of eicosanoid production in fish tissues, possibly as a consequence of changes in the

EPA/ARA ratio in tissues (Bell *et al*., 1993).

1.3- Lipids sources in aquafeeds

Aquaculture has traditionally used products from industrial fisheries, namely fish meal (FM) and fish oil (FO), to convert relatively cheap protein and oil into high value products, a practice that is both scientifically and commercially sound (Bell *et al*., 2002). However, the rapid development of aquaculture, which is expanding at over 10% per year (Tidwell and Allan, 2002), together with the FO uses for domestic animals feeds and pharmacological uses have greatly increased its cost and affected its availability. Consequently, demand for these products is rapidly increasing and current estimates suggest that aquaculture feeds will consume approximately 90% and 60-70% of the world FO and FM supplies respectively by 2010 (Figure 1.1) (Allodi, 2007) and it is expected that by 2012 the FO needs for aquaculture will exceed the total production. Therefore, availability of FM and FO for aquafeeds is limited (Anon, 2002) during stabilised periods of wild fisheries catches, and endangered when wild catches decrease as a consequence of natural phenomena such as "El Niño" (Anon, 2002; Naylor, 2004). Moreover, the continuous use of FM and FO may place added pressure on natural resources and convert aquaculture to a net user rather than in a net contributor to world fish supply, with disastrous consequences for the ecosystem (Staniford, 2002; Milewski, 2002; New, 2002; Allan, 2004). Elsewhere, one of the long continued debates in aquaculture is the use of FM and FO in feeds and the amount of wild fish it takes to produce farmed fish, called Fish In-Fish Out (FIFO) ratios ranging from 3:1 to 10:1 (International Fishmeal and Fish Oil Organisation: IFFO, 2008); and the most recent review published by Tacon & Metian (2008) gave the FIFO for salmon as 4.9:1, meaning that it takes 4.9 tonnes of wild fish to produce 1 tonne of salmon. Moreover, the FO prices doubled during the last 5 years (Figure 1.1), and they continue to rise making it more cost-effective to divert increasing proportions of these ingredients to human consumption. Therefore, the restrictions in cost and availability imposed on FM and FO have lead, during the last 15 years, to the gradual substitution of these ingredients by alternative sustainable lipid and protein sources

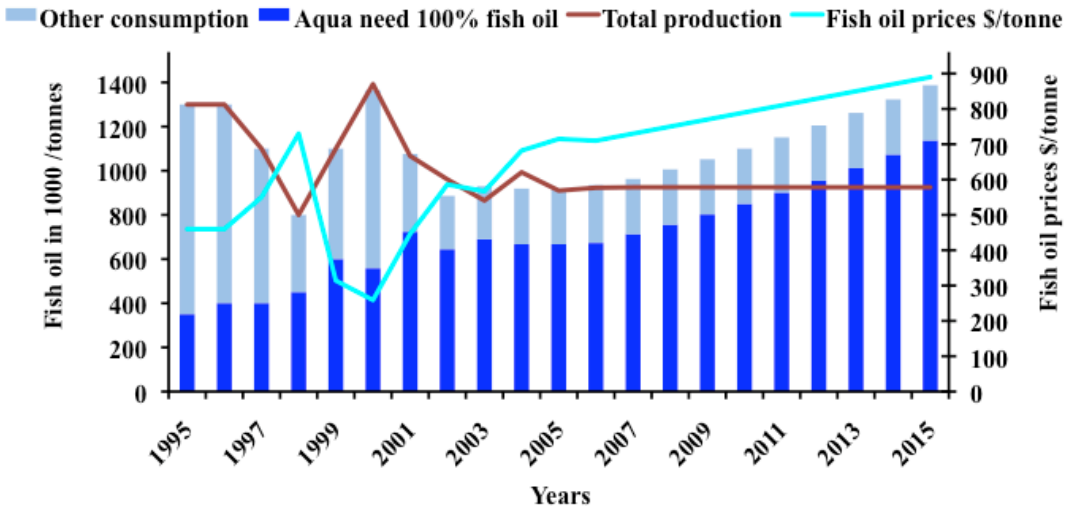

Figure 1.1: The evolution and expectation of Fish Oil uses and needs from 1995 to 2015. Based on data from Allodi 2007.

This graph shows clearly the significant increase of FO use for aquaculture during the last 15 years and its projection to increase in the future 5 years. The noticeable drop in FO production in 1998 was due to "El Niño" phenomena. Thus, FO uses for aquafeeds increased from less than 30% to more than 80% of total FO production annually and it is expected that the all FO needs for aquafeeds could exceed the total FO production by 2011. This pressure on the limited wild resources caused, FO prices to double during the last decade (Figure 1.1) as the prices rose from 400 US $/tonne in 2000 to more 750 US $/tonne today, and it is expected that it will continue to increase up to 900 US $/tonne in 2015 (Tacon and Metian, 2008). In consequence, alternatives to FO are urgently required, with vegetable oils as the prime candidates (Bell and Waagbø, 2008)

Marine fish are mostly adapted to a carnivorous/piscivorous diet that is naturally rich in n-3 HUFAs, developing a relative deficiency in the enzymes (desaturases and elongases) responsible for the conversion pathway from LA and LNA to their HUFA end products (Tocher and Ghioni, 1999; Ghioni *et al*., 1999; Zheng *et al*., 2009). As with vertebrates, PUFA are essential in the diet of fish, but there are special requirements for HUFAs such EPA and DHA, rather than shorter chain PUFA found in VO (Tocher, 2003a,b).

At present, more than 100 million tonnes of vegetable oils are produced per year including palm, soybean, linseed, rapeseed, olive and sunflower oils, with palm and

soybean showing by far the biggest productions (Figure 1.2) (O'Mara, 1998). Vegetable oils (VO) and vegetable meals (VM) frequently show more predictable productions and prices than FO and FM, which can be an advantage in providing consistent aquafeed production.

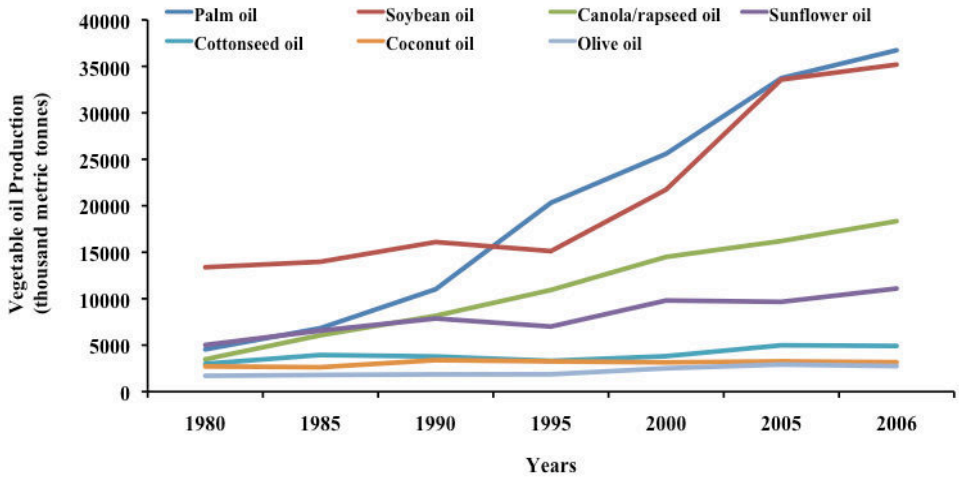

Figure 1.2: Evolution of different plants oils production, data compiled from Tacon et al., 2006.

Thus, the less sustainable FM and FO could be used for commodities or as strategic ingredients, their inclusion in aquafeeds being dependant on the fish species and life stage requirements as well as relative ingredient prices. Consequently VOs are considered presently as the only viable, cost competitive alternative lipid source for aquaculture diets and a number have been used as partial and complete replacement for FO (Bell *et al*., 2005).

Sustainable protein source alternatives to FM include various vegetable meals with crude protein contents of 20 to 50% (Hertrampf and Piedad-Pascual, 2000), with soybean meal (SBM) currently representing the predominant choice. However, more carnivorous fish have a limited ability to tolerate high SBM contents in their diets (Kaushik, 1990; Torstensen *et al*., 2008) due to the poorer digestibility, lower availability of some essential amino acids and inferior palatability of SBM in comparison to FM as well as the presence of anti-nutritional factors. Regarding FO substitution, several studies have pointed out the successful inclusion of different blends of VOs in diets for different species without compromising their growth or feed utilisation, the level of inclusion depending on the species (Bell *et al*., 2001; Caballero *et al*., 2002, Montero *et al*., 2003; Izquierdo *et al*., 2003, 2005; Torstensen *et al*., 2005; Mourente and Bell, 2006). For instance, it has been shown that it is possible to replace

up to 60% of the FO by VOs in diets for seabream without compromising growth, survival, feed utilization or fillet organoleptic properties, when fish are fed either for a medium (3 months, Izquierdo *et al*., 2000, 2003) or long period (8 months) (Menoyo *et al*., 2004; Izquierdo *et al*., 2005). However, the effect of those or higher substitution levels on fish health and stress resistance have not been clearly determined.

1.4- Vegetable oils and fish health

Inclusion of vegetable lipid and protein sources in diets for cultivated fish is considered as a sustainable solution for the aquaculture industry to cope with the reduced availability of FO and FM. To fulfil this purpose, industry research must not only determine the effect of replacing feed ingredients on standard farming parameters like growth, survival rates or food conversion ratio (FCR), but also on the nutritional, sensory, processing, and safety characteristics of aquaculture products (Barlow and Pike, 2001). Besides, it is imperative that the impact of potential FM and FO replacements on other factors such as health and welfare will be precisely assessed (Barlow and Pike, 2001).

Nutrition is one of the main factors determining health and welfare status in cultured fish. Dietary supply of amino acids, PUFA, enzyme co-factors and energy is essential to support the fish immune system including lymphocyte proliferation and the synthesis of effectors (e.g. immunoglobulins, lysozyme and complement) and communication molecules (e.g. cytokines and eicosanoids). Moreover, nutritionally adequate diets are recognized to improve the health of convalescent fish, whereas feeding unbalanced diets can lead to gross malnutrition and increase stress and disease susceptibility. Therefore, outbreaks of fish diseases commonly occur when fish are stressed due to a variety of factors including poor nutrition. Thus, substitution of FM and FO by vegetable sources may lead to nutritional imbalances that could affect fish health and welfare.

Some studies have pointed out negative alterations on certain fish health parameters and resistance to stress in fish fed with VO (Bell *et al*., 1993, 1996; Montero *et al*., 2003; Jutfelt *et al*., 2007). For instance, feeding salmon with VOs caused significant reductions in several non-specific immune parameters including haematocrit, total white blood cell and RBC, and macrophage respiratory burst (Good *et al*., 2001). Therefore, the effect of VO inclusion seems to depend on the type of VO, the FO substitution level, the fish species and the culture conditions. In gilthead seabream,

increase in FO substitution levels from 60 to 80% by vegetable oils significantly reduces growth and feed conversion indices (Izquierdo *et al*., 2008) altering normal hepatocyte and enterocyte morphology (Caballero *et al*., 2003), and negatively affecting immune functions and post-stress plasma cortisol response (Montero *et al*., 2003). In addition, inclusion of soybean or rapeseed oil with of 60% VO substitution reduced macrophage phagocytic activity (Montero *et al*., 2003) whereas the reduction in the EPA and DHA acids in diet has been shown to significantly inhibit serum alternative complement pathway activity (Montero *et al*., 1998). However, little is known on the mechanisms involved in the effect of PUFA on stress and disease resistance in cultured fish, which seems to be mediated, at least partly, by the eicosanoid production in target tissues. Indeed, changes in FA composition of phospholipids affect the quantity and the spectrum of eicosanoid synthesis (Bell *et al*., 1992, 1993, 1994, 1996; Ashton *et al*., 1994; Lall, 1998; Balfry and Higgs, 2001) and as in higher vertebrates, altered eicosanoids production may affect immune cell composition and function (Kinsella and Lokesh, 1990; Kelley and Daudu, 1993). Moreover, although these studies about the effects of dietary VO on prostaglandin production in different tissues have been conducted in freshwater and some Nordic marine fish species (Bell *et al*., 1994, 1995, 1996), no studies have been conducted until now on eicosanoid production in seabream, or their alteration by dietary lipids.

Since the different vegetable oils employed as lipid sources to substitute FO markedly differ in their fatty acid profiles, including the relative content of n-3 and n-6 fatty acids, their introduction in fish diets strikingly modify the fatty acid composition of different tissues (Torstensen *et al*., 2000; Bell *et al*., 2001, 2002, Caballero *et al*., 2002; Montero *et al*., 2003; Izquierdo *et al*., 2003, 2005), this in turn can lead to differences in eicosanoid production and immune responses (Balfry & Higgs 2001; Thompson *et al*., 1996; Balfry *et al*., 2006). Thus, Bell and colleagues (1993, 1996) reported that Atlantic salmon fed a diet containing increased levels of sunflower oil (rich in n-6, consequently with decreased n-3/n-6), produced more ARA-derived eicosanoids compared with fish fed diets containing linseed or fish oil (with higher n-3/n-6 ratio) which produced more EPA-derived eicosanoids. In addition, increased activity of head kidney macrophages has been associated with higher levels of dietary n-3/n-6 fatty acids in catfish (Sheldon and Blazer, 1991). Moreover, it is reported that eicosanoids with a more suppressive influence on the immune system are derived from n-3 HUFAs, which are more prevalent in diets containing high concentrations of LNA

and the n-3 HUFA (Higgs *et al*., 1995). Indeed, reports have cautioned that the relative ratios of fatty acids and eicosanoid precursors are the key to understand the effect of dietary lipids on the immune system (Ashton *et al*., 1994).

Furthermore, feeding with decreased n-3/n-6 was associated with enhanced respiratory burst activity of peripheral blood leucocytes (PBLs) (Balfry *et al*., 2006), and increased bactericidal activity of macrophages from channel catfish (*Ictalurus punctatus*). In addition, significantly higher natural-killer-like-cell activity has also been seen in leukocytes isolated from rainbow trout (*Onchorhynchus mykiss*) (Kiron *et al*., 1995a,b), and Atlantic salmon (Balfry *et al*., 2006) that were previously fed an n-3 enriched diet. Other previous reports have suggested that high antibody production is associated with high dietary n-3 HUFA (Erdal *et al*., 1991; Waagbo *et al*., 1993). Indeed, high dietary n-3/n-6 ratios resulted in increased B lymphocyte responses and improved fish survival following a disease challenge (Thompson *et al*., 1996), and higher numbers of thrombocytes in channel catfish, that consequently have reduced (n-6) fatty acid derived eicosanoids (i.e., thromboxane B_2) involved in blood clotting, and compensate for this with elevated numbers of thrombocytes (Klinger *et al*., 1996). Evidence suggests that changing the fatty acid composition of immune cells can influence immune function by changing the physiology of the cell membrane but perhaps more importantly by influencing the production of modulatory prostaglandins and leukotrienes. This is supported by a number of studies showing a reduction in production of PGE_2 and leukotriene B_4 (LTB_4) by stimulated head kidney macrophages from salmon fed a diet containing higher dietary n-3/n-6 ratio (Bell *et al*., 1993, 1996). Montero et al. (2003) found that seabream fed higher n-3/n-6 by including LO in the diet (rich in LNA, increasing n-3/n-6 ratio), decreased the numbers of circulating erythrocytes. It is also demonstrated that dietary lipid determines the fatty acid profile of macrophages and immune cells in cod (Waagbø *et al*., 1995), seabass (Farndale *et al*., 1999) and seabream (Montero *et al*., 2003).

Nevertheless, the impacts that dietary fatty acids have on the immune response are complex and depend on several factors that influence eicosanoid production including competition between n-3 and n-6 fatty acid during metabolism for chain elongation and desaturation. Therefore, there is a clear suggestion that the ratios of n-6 to n-3 fatty acids in the dietary lipids may play an important role in balancing the composition of the fish cell membrane PUFAs (Bell *et al*., 1993, 1996, 2001; Caballero, 2002; Izquierdo *et al*., 2005), that subsequently modulate its immune parameters (Balfry

et al., 2006).

The beneficial effects of fish lipids (rich in n-3 PUFA) in the control of cardiovascular disease and neurological development are well documented in the nutrition literature (Herbaut, 2006; Simopoulos, 2008). Furthermore, high dietary n-6 PUFA and n-6/n-3 PUFA ratios have been suggested to play a role in many Western chronic diseases common in the developed world (Simopoulos, 2008; Yam *et al*., 1996). Therefore, when substituting fish with a blend of VOs, care should be taken not only to establish the correct blends in order to guarantee not only the fish growth, but also to provide them with the adequate n-3/n-6 fatty acids ratios to allow normal function of its physiological processes and to maintain the nutritional benefits of the fish to the human consumer (Bell *et al*., 2001).

1.5- Lipid nutrition and fish resistance to stress

Previous studies have demonstrated the importance of feeding adequate levels of different nutrients in fish to allow adaptation to any chronic or acute environmental stress (Salte *et al*., 1988). Among several nutrients, dietary n-3 HUFA have been found to increase resistance to both chronic and acute stress in seabream (*Sparus aurata*) juveniles (Montero *et al*., 2001). In European eel (*Anguilla anguilla*) and Adriatic sturgeon (*Acipenser naccarii*) dietary n-3 HUFA increased the tolerance to hypoxic conditions (Mackenzie, 2001). Moreover, in adult seabream, feeding diets deficient in n-3 HUFA increased basal plasma cortisol prior to confinment stress denoting the stressor effect of the nutritional deficiency (Montero *et al.*, 1998). Both DHA and EPA have been found to be essential for improving resistance to handling stress in larvae of several fish species such as red seabream (*Pagrus major*) (Izqueirdo *et al*., 1988; Watanabe *et al*., 1989), Japanese flounder (*Paralichthys olivaceous*) (Izquierdo *et al*., 1992; Furuita *et al*., 1999), as well as to temperature change stress in European sea bass (*Dicentrarchus labrax*) (Person-Le Ruyet *et al*., 2004). Generally, the manipulation of dietary fatty acid compositions affects the ability of larval fish to resist several stressors, hence influencing survival following stress (Izquierdo *et al*., 1989; Watanabe *et al*., 1989; Tuncer *et al*., 1993; Kanazawa, 1997; Gapasin *et al*., 1998; Koven *et al*., 2001a,b, 2003; Liu *et al*., 2002). Indeed, feeding n-3 HUFA has been also reported to enhance crustacean's resistance to stress conditions, such as osmotic shock (Palacios *et al*., 2004; Sui *et al*., 2007), temperature fluctuation (Chim *et al*., 2001) and ammonia (Martins *et al*., 2006).

Dietary ARA elevation has also been found to increase overall survival (Bessonart *et al*., 1999) and improve resistance to handling stress in larval gilthead seabream (Koven *et al*., 2001a,b). An optimal concentration of dietary ARA was also found to maximize stress resistance to a hypersaline challenge in larval summer flounder (Willey *et al*., 2003) and striped bass (Harel *et al*., 2001).

Several studies have suggested that ARA is involved in the release of cortisol in fish, although actual mechanisms have not been investigated (Gupta *et al*., 1985; Harel *et al*., 2001; Koven *et al*., 2003). Koven et al. (2003) found that dietary intake of ARA in combination with daily salinity changes were associated with elevated whole body cortisol levels in the seabream larvae at the end of the experimental period. The authors suggested that ARA enhanced the sensitivity of the HPI axis, and in combination with daily stress this resulted in higher levels of cortisol. Thus, ARA has been found to enhance resistance to stress in seabream larvae (Koven *et al*., 2001a,b), and high dietary intake of ARA for the same species lowered the sensitivity of fish to acute stress, as evidenced by decreased plasma cortisol levels and plasma osmolality (Van Anholt *et al*., 2004).

However, the physiological mechanisms by which these fatty acids regulate fish stress resistance or plasma cortisol levels are not clear. In mammals, certain studies suggest that prostaglandins play an important role in mediating the corticosteroidogenic action of ACTH (Kocsis *et al*., 1999), suggesting that the role of fatty acids in stress response seems to be mediated by the production of eicosanoids. Evidence in fish suggested also the mediation of these metabolites in cortisol release (Koven *et al*., 2001a,b; Van Anholt *et al*., 2004)

2- Stress in Fish

2.1- Stress responses

The definition of stress varies among different authors, but it can be described as a series of physiological changes occurring in the organism in order to recover homeostasis threatened by either internal or external factors (stressors). Stress can also be described as an internal hormonal response of a living organism caused by environment or other external factors that move that organism out of its homeostasis (Selye, 1973) by forcing a reallocation of energy within its system. Stress is unavoidable in aquaculture and is one of the main arising problems for the modern intensive aquaculture systems, where fish are frequently exposed to various stressors,

such as grading, transportation and vaccination.

Stressful conditions have negative consequences on fish performance in aquaculture, leading to growth reduction, immune-suppression and increased susceptibility to infectious diseases, resulting in major economic losses to fish farmers. The response of the fish to such stressors involves all levels of organization from the cell (Hightower, 1991) to the individual organism (Barton and Iwama, 1991; Mommsen *et al*., 1999) and to the structure of the population (Adams, 1990).

Under stress situations, fish undergoes a series of physiological reactions in an attempt to recover the homeostasis which could lead to an adaptive response or to a maladaptive response which compromises fish performances. These responses are differentiated in three different types according to the so-called general adaptation syndrome (GAS; Selye, 1975).

a) A primary response, the *alarm reaction*, which includes the organism's perception of the new altered state and the release of stress hormones and neurotransmitters. Therefore, the catecholamines are released from the chromafin tissue situated in the head kidney of teleosts and the endings of adrenergic nerves (Randall and Perry, 1992). Cortisol is released from the interrenal tissue, located in the head kidney, in response to several pituitary hormones, particularly the adrenocorticotrophic hormone (ACTH) that in turn is liberated from the hypothalamus by the corticotropic releasing hormone (CRH) (Donaldson, 1981; Vijayan *et al*., 2005; Alsop and Vijayan 2008).

b) A secondary response or *stage of resistance,* during which the organism adjusts its metabolism to cope with the disturbance. Thus, the released stress hormones activate a number of metabolic pathways including those implying the mobilization and reallocation of energy, osmotic disturbance and increase in cardiac output, oxygen uptake and transfer, resulting in alterations in regular blood chemistry and haematology (Barton and Iwama, 1991, Iwama *et al*., 2006).

c) A tertiary response or *stage of exhaustion* may occur when the organism is unable to adapt or overcome the changes caused by the stressor. Therefore, severe stress can cause massive mortality, and sublethal stress may compromise several behavioural and physiological functions (Campbell *et al*., 1992; Tort *et al*., 1996; Iwama *et al*., 1997; Wendelaar Bonga, 1997; Ortuño *et al*., 2002; Vijayan *et al*., 2005). Thus, fish growth and reproduction performances are reduced in stress situations (Barton *et al*.,

1986; Mesa, 1994; Schreck *et al*., 2001), suppresses cellular immunity by affecting inflammatory signaling pathways (Ortuño *et al*., 2002; Holland *et al*., 2003; MacKenzie *et al*., 2006; Aluru and Vijayan, in press), and also it encourages infectious diseases and can even cause death (Pankhurst and Van der Kraak, 1997).

The neuro-endocrine regulation of the stress response in fish is similar to that of higher vertebrates and involves two regulatory axes (Figure 1.3) (Wendelaar Bonga, 1997; Barton, 2002):

- Hypothalamus-Sympathetic nerves-Chromaffin (HSC) cell axis, equivalent to the Sympathetic-Adrenal-Medullar (SAM) system in vertebrates. As a result of the activation of the sympathetic nerve fibres, which innervate the chromafin cells, stimulating the release of catecholamines (epinephrine and norepinephrine) via cholinergic receptors (Reid *et al*., 1996). Catecholamines, predominantly epinephrine in teleostean fishes, are released rapidly to the circulation system in response to stress stimulus (Randall and Perry, 1992) and are transported to the different organs, altering the normal functions of the different physiological processes (respiration, reproduction, immune system…etc.) (Wendelaar Bonga, 1997).

- Hipothalamus-Pituitary-Interrenal Cell (HPI) Axis: Is equivalent to the Hipothalamus-Pituitary-Adrenal Axis, HPA in high vertebrates. The HPI axis is the regulator of stress response in fish, once activated in response to almost all forms of stress (Wendelaar Bonga, 1997). Briefly, in response to a stress situation, the hypothalamus releases the Corticotropin Releasing Hormone (CRH) that acts on the *Pars distalis* of the adenohypophysis, close to the roof of the mouth, triggering the release of Adrenocorticotropin (ACTH), and, to a lesser extent, α-melanocyte-stimulating hormone (g-MSH) and lipotropic hormone (b-LPH), which are intermediate products of ACTH synthesis. Afterwards, ACTH induces the production and release of cortisol, from the interrenal cells located in the head kidney, to the circulation system (Figure 1.3). Cortisol release is controlled through a negative feedback at different levels of the HPI axis (Donaldson, 1981; Bradford *et al*., 1992; Wendelaar Bonga, 1997).

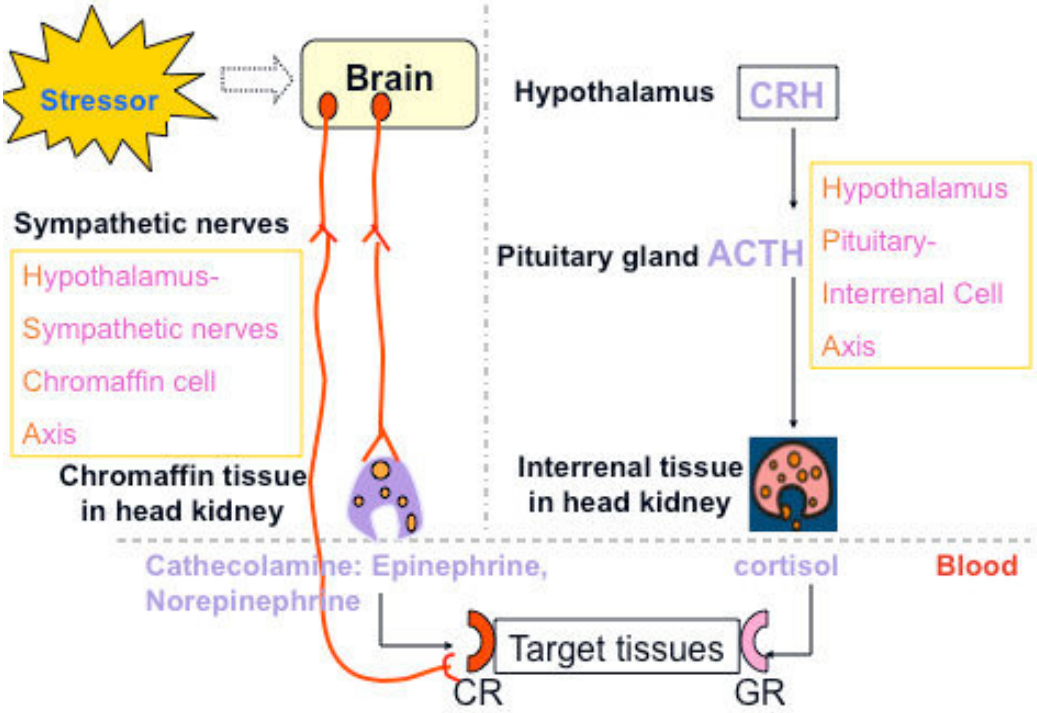

Figure 1.3: A simplified representation of the central and peripheral components of the stress response in fish. Corticotropic releasing hormone (CRH), adrenocorticotrophic hormone (ACTH), catecholamine receptor (CR), glucocorticoid receptor (GR).

According to the intensity and duration of the stress challenge, several types of stress situations are recognized: acute, chronic, repetitive, irregular repetitive, etc. Acute stress is produced by an intense stressor of short duration, whereas a chronic stress situation implies a long-term disturbance that acts as an intense or mild stressor.

2.2- Head kidney as a key organ in stress response

Anatomically, fish don't posses an adrenal gland as in mammals. Teleostean kidney consists of a head and body kidney, deriving from pronephros and mesonephros tissues, respectively. The external form of the fish kidney varies according to species. The head kidney is an organ encased in bone, it must have taken up its position in the head, and have penetrated both the air-bladder and the scapular arch, whereas many nephrons and interstitial lymphoid tissue constitute the body kidney. The head kidney contains the interrenal gland (homologous to the adrenal cortex in mammals) responsible of cortisol release and the chromaffin cells (homologous to the adrenal medulla), surrounding the postcardinal vein and its branches (Milano *et al.*, 1997). Chromaffin cells are located singly or in clusters in the walls of the postcardinal vein

surrounded by the interrenal cells (Imagawa *et al.*, 1996). The location of the interrenal and chromaffin cells near the postcardinal vein facilitates their regulation by the endocrine system via the bloodstream. The interrenal tissue exhibits considerable morphological variation among taxonomic groups (Nandi, 1962), and it is considered as the major endocrine, haematopoietic and lymphatic tissue in fish (Takashima and Hibiya, 1995). Thus, head kidney is considered as a key tissue in stress response.

There is a little available information on the functions of the head kidney tissue in fish, and recent studies are interested in using this organ in different experimental preparations to study its functions. Variability in the size and number of cells of the head kidney between individual fish (Pottinger *et al.,* 1995) and the relatively large numbers of fish required preclude the routine use of whole or fragmented head kidneys in static or perfusion systems (Patiño *et al*., 1986; Benguira and Hontela, 2000, Rotllant *et al*., 2000a,b) for *in vitro* screening tests for adrenotoxicants (example, figure 1.4).

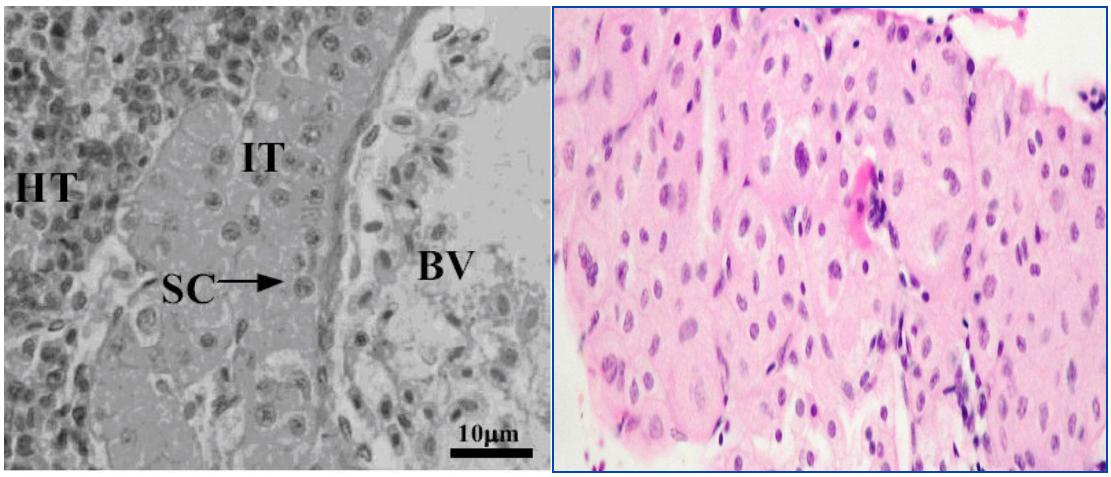

Figure 1.4: Cross sections of paraffin embedded head kidney tissue of teleostean fish consisting of haematopoetic tissue (HT), blood vessels (BV) and interrenal tissue (IT) containing the steroidogenic cells (SC).

2.3- Cortisol: as a stress hormone indicator in fish

The blood circulation level of cortisol is commonly used as an indicator of degree of stress experienced by fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997) since, 1) cortisol is the major glucocorticoid in teleost fish and its plasma levels are known to increase in response to a variety of stressors (Barton and Iwama, 1991), 2) it is easily and reliably measured using radioimmunoassay (RIA) and enzyme-linked immunosorbent assays and 3) it plays a critical regulatory role in many important physiological processes (Mommsen *et al*., 1999). The pathway for cortisol release begins in the HPI axis with the release of CRH by the hypothalamic neurosecretory cells, which in turn stimulates the corticotrophic cells of the adenohypophysis to secrete ACTH. Circulating ACTH, in turn, stimulates the interrenal cells embedded in the head kidney to synthesize and release corticosteroids into the circulation for distribution to target tissues (Figure 1.3).

The production of cortisol is under the control of the HPI (Wendalaar Bonga, 1997; Mommsen *et al*., 1999). For instance, cortisol releasing factor (CRF) secreted from hypothalamic neurons stimulates the release of ACTH from the pituitary. This pituitary peptide binds to melanocortin 2 receptor (MC2R) on the steroidogenic cells in the interrenal tissue leading to corticosteroidogenesis (Aluru and Vijayan, 2009). It is widely believed in mammals that ACTH stimulates cortisol secretion through adenosine 3',5'-cyclic monophosphate (cAMP), which provides substrate cholesterol by activating cholesterol ester hydrolase and facilitating transport of cholesterol to the side-chain cleavage (scc) enzyme. Dempsher et al. (1984) have established a model of cortisol release (Figure 1.5), thus ACTH binds to one or more specific receptors in the adrenocortical cell membrane (MC2R, represented by A and B in the figure below) which has the effect of activating adenylate cyclase (ACase). The cytosolic concentration of cAMP increases, in turn a) activating cholesterol ester (CE) hydrolase, which catalyses the conversion of cholesterol ester into free cholesterol, and b) facilitating the transfer of cholesterol within the mitochondrion to a site accessible to the scc enzyme. Furthermore, low density lipoprotein receptor-mediated uptake of plasma cholesterol together with cytosolic cholesterol synthesis provide substrate for steroid synthesis, and both these processes can be controlled by an intracellular pool of cholesterol.

Furthermore, other studies have pointed out that the main pathway leading to

corticosteroid synthesis by ACTH stimulation involves a signalling cascade integrating G-proteins, adenyl cyclase, cAMP and protein kinase A (PKA) (Miller 1988, Schimmer 1995). Evidence suggests that cortisol exerts a negative feedback effect on the ACTH secretion in the pituitary and also suppresses CRH synthesis in the hypothalamus. In addition, there is also a negative feedback effect of ACTH on the secretion of CRH. Therefore, the entire process can be thought of as a self-regulating system, as summarised in the figure below. In addition, cholesterol modulates its own synthesis by inhibiting beta-hydroxy-beta-methylglutaryl (HMG)-CoA reductase in the adrenocortical cell (Dempsher *et al*., 1984).

Several biotic and abiotic stressors are known to modulate cortisol biosynthesis in fish by altering the expression pattern of genes encoding proteins involved in the functioning of the HPI axis (Alsop and Vijayan, 2009). In teleosts, multiple glucocorticoid receptors (GRs) and one mineralocorticoid receptor (MR) are involved in cortisol signalling (Vijayan *et al*., 2005; Prunet *et al*., 2006; Alsop and Vijayan, 2008). 11-deocycorticosterone was identified as a ligand for trout MR using *in vitro* reporter assays, a physiological role for this ligand is lacking in vivo (Sturm *et al*., 2005; McCormick *et al*., 2008). A distinct physiological role for multiple GR isoforms in cortisol signalling has not been established in vivo (Bury *et al*., 2003).

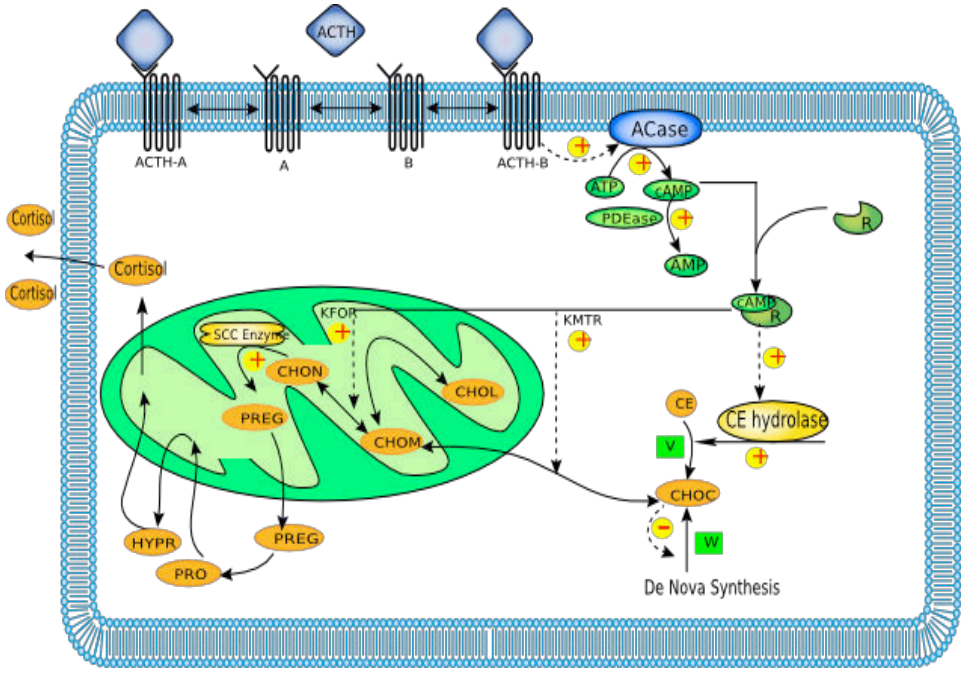

Figure 1.5: A mechanistic model of ACTH-stimulated cortisol secretion in mammals (Dempsher *et al*., 1984). Adenylate cyclase (ACase), cholesterol ester (CE), adenosine 3',5'-cyclic monophosphate (camp), Adenosine triphosphate (ATP).

2.4- Corticosteroids biosynthesis by interrenal cells

Steroid hormones are important regulators of numerous physiological processes, including glucocorticoids, mineralocorticoids and sex steroids, such as oestrogens and androgens (Miller, 1988). However, only low levels of aldosterone have been found in teleosts and the function of mineralocorticoids seems to be carried out by the glucocorticoids (Wendelaar Bonga, 1997).

The steroid hormones are structurally similar and are synthesized from cholesterol in the steroidogenic cells of the head kidney. Thus, cortisol is formed from cholesterol via the intermediates pregnenolone, progesterone, 17-hydroxyprogesterone and 11-deoxycortisol. 17-hydroxyprogesterone can be converted to either corticosteroids by 21-hydroxylase or to sex steroids by 17-hydroxylase which thus is a branch step in the steroid biosynthesis pathway. The 11-deoxycortisol formed by the microsomal 17-hydroxyprogesterone 21-hydroxylase is eventually metabolised to cortisol by the mitochondrial 11-hydroxylase (Figure 1.6). In fish the enzymes responsible for cortisol production have been poorly examined. In mammals the 21-hydroxylation of 17-hydroxyprogesterone is catalysed by microsomal cytochrome P450c21 in cortisol producing adrenocortical cells (Miller *et al*., 1997). Production of cortisol in extra interrenal tissues of fish is poorly examined. In mammals, the enzyme responsible for conversion of 17-hydroxyrogesterone to progesterone is believed to be another form of P450 than the P45021c that is responsible for the conversion of 17*α*-hydroxyprogesterone to 11-deoxycortisol.

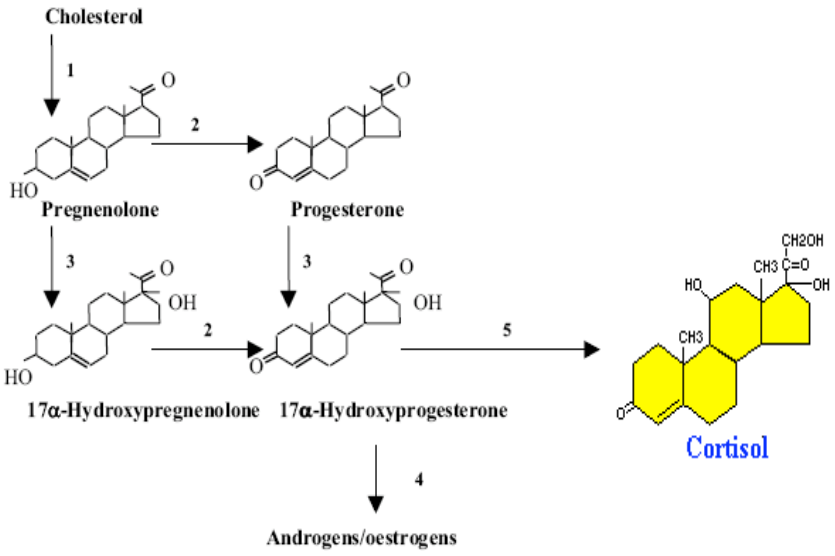

Figure 1.6: Representation of the steroidogenic pathways in teleost fish and the important role of P450c17 in the formation of glucocorticoids and androgens/oestrogens. Enzymes implicated are 1) P450scc, 2) 3β-HSD, 3) P450c17 (hydroxylase), 4) P450c17 (lyase), 5) P450c21 (Ruane, 2002).

2.5- Cortisol roles in fish

While catecholamines induce rapid, short-term elevation in blood glucose primarily through the glycogenolytic pathway (Vijayan and Moon, 1992), cortisol has a key role in regulating many important physiological functions in teleosts. Thus, it is involved in longer-term mobilization of non-carbohydrate energy stores (Wendelaar Bonga, 1997) such as tissues protein and lipids, implying increases in plasma fatty acids (Mazeaud *et al*., 1977). Besides its feedback actions on the corticotropic axis, chronically elevated cortisol levels are responsible for the detrimental effects of stress on vital physiological functions of fish, such as reproduction (Foo and Lam, 1993, Small, 2004), osmoregulation (Redding *et al*., 1991; Mancera *et al*., 1994), growth (Barton *et al*., 1987; Small, 2004) and immune system (Rottlant *et al*., 1997; Weyts *et al*., 1998, MacKenzi *et al*., 2006; Aluru and Vijayan, in press). In freshwater fish, the response to chronic stress may also include: loss of electrolytes, decreases in hematocrit and hemodilution from catecholamine-induced increases in gill permeability (Randall and Perry, 1992) or increased loss of ions through the urine (McDonald and Milligan, 1997).

Cortisol value levels vary and should serve as general guidelines since, individual conditions, including species differences, strain genetic characteristics, prior rearing history, and local environment will modify the plasma values of control and stressed states (Barton *et al*., 2002). For instance, results of confinement studies in rainbow trout, *Oncorhynchus mykiss*, (Pottinger *et al*., 1992) and Atlantic salmon, *Salmo salar* (Fevolden *et al*., 1991) have indicated that the cortisol response to stress in teleost fish is a highly individualized trait. Some individuals display a consistently high cortisol stress response while others have a consistently low cortisol response. The reasons for these differences are presently unclear. Furthermore, selective breeding programs have demonstrated that stress responsiveness is heritable and that individual responsiveness is stable over time (Fevolden *et al*., 1991; Afonso *et al*., 1998).

3- Gilthead Seabream as a model species for this thesis

Gilthead seabream is a marine fish species that has a long history in the Mediterranean region, with evidence of fish capture and fattening dating back more than 2000 years. But, from 1980, the production started to grow rapidly, expanding from 1100 tonnes in 1985 to 8400 MT in 1990. This increase was supported by the

development of new technologies and the availability of national and EU financial aids. Nowadays, gilthead seabream constitutes one of the most important marine fish species cultured in the Mediterranean, and the total European aquaculture production of seabream attained approximately 140.000 tonnes in 2007, about 40.000 tonnes more than 2005 (APROMAR, 2008) (Figure 1.7). In Spain, seabream is a much appreciated fish species and has a high economic importance. Gilthead seabream production techniques are therefore well developed and it constitutes an excellent model for physiological studies in marine warm water species. However, since its culture is relatively new in comparison to salmonids and other cold water species, many physiological and nutritional aspects remain unstudied.

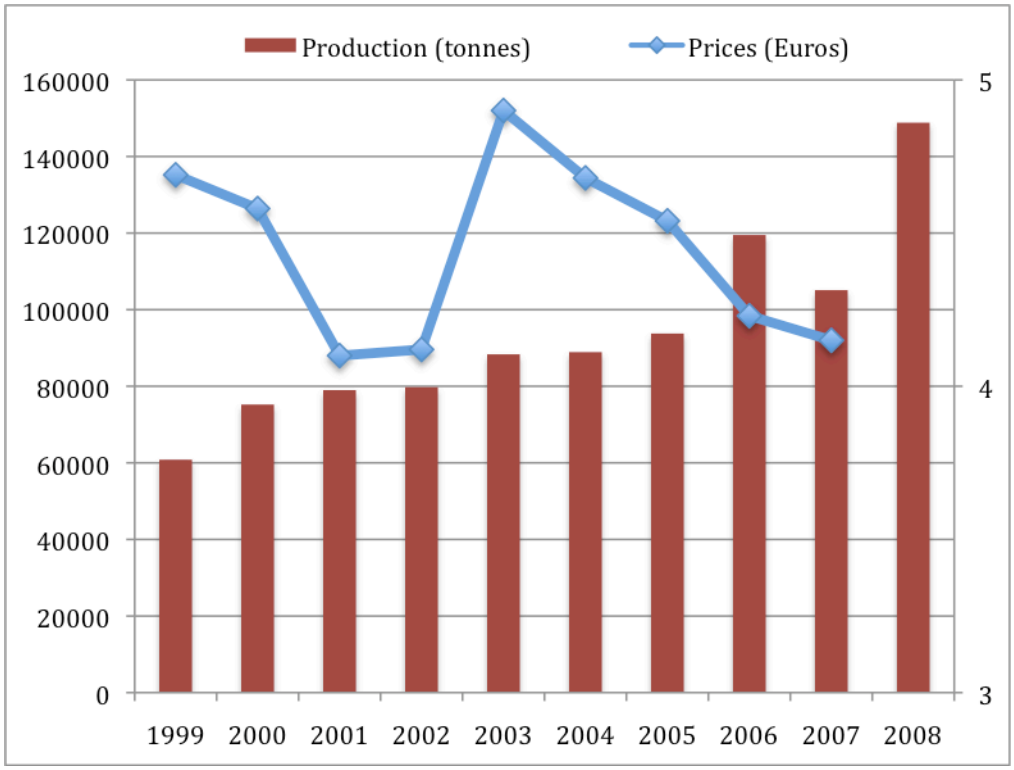

Figure 1.7: Gilthead seabream global production evolution in Europe during the last decade. APROMAR (2008)

4- Objectives

The overall aim of this thesis is to promote the substitution of fish oil by vegetable oils in on-growing diets for gilthead seabream without compromising fish welfare by improving our knowledge on the mechanisms involved in the regulation of stress resistance by dietary lipids.

For that purpose several objectives were addressed:

1- To determine the effect of high levels of fish oil substitution by n-3 or n-6 fatty acids rich vegetable oils on culture performance and fatty acid composition of different tissues in gilthead seabream until commercial size.

2- To study the effect of fish oil substitution levels and n-3/n-6 fatty acid ratios on seabream welfare and their resistance to several types of stress.

3- To investigate the effects of different levels of substitution of fish oil by vegetable oils blends on gilthead seabream health and welfare in terms of plasma fatty acid compositions and prostaglandin and leptin production.

4- To better understand the mechanisms involved in the regulation of stress resistance by polyunsaturated fatty acids by studying the production and release of cortisol in gilthead seabream interrenal cells incubated with adrenocorticotropic hormone.

5- To clarify the effect of feeding vegetable oils along gilthead seabream on-growing on welfare in terms of cortisol production and release by head kidney and the physiological pathways involved.

To achieve these goals, tow feeding experiments along the whole on-growing period and several “in vitro” studies were conducted. The results were organized in five scientific studies that have been already published or submitted for publication. Some of those studies were included in two research projects: **RAFOA** (2001-2005) funded by the EU (**Q5RS-2000-30058**) and **LINOSALUD** (2005-2007) funded by the Spanish Government (**AGL2004-08151-CO302**).

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Chapter 2

General Materials and Methods

CHAPTER 2
GENERAL MATERIALS AND METHODS

Two main feeding experiments are included in this chapter, the first trial aimed to replace fish oil in diets for gilthead seabream with vegetable oils, without compromising the health, welfare and growth performance of the fish, this experiment was a part of the EU project RAFOA (Researching Alternatives to Fish Oils in Aquaculture) **Q5RS-2000-30058**. The second experiment was included in the Spanish Ministry of Education funded project LINOSALUD (Efecto de la Sustitución parcial del aceite de pescado por aceite de Lino en la dieta sobre la salud yla resistencia a estrés de dorada (*Saprus aurata*)) (**AGL2004-08151-CO3 02/ACU**). In both trials fish were grown from juveniles to market size on diets containing different ratios of FO to VO with the latter being either rapeseed (RO), linseed (LO) or palm oils (PO) for a period of 281 days in the first trial, and LO and Soybean oil (SO) for a period of 240 days in the second trial. The blend of oils was formulated to replicate the FO in terms of saturated, monounsaturated and polyunsaturated fatty acid concentrations and ratios except there would be none of the HUFA found in FO.

The two feeding trials were carried out at the Marine Culture facilities of the Grupo de Investigación en Acuicultura (ULPGC & ICCM) (Spain). The prostaglandins and leptins analysis were done in the Fish Nutrition Department facilities, Institute of Aquaculture, University of Stirling (Scotland). Part of the *in vitro* experiments, and all the cortisol analysis were conducted in the facilities of Department of Cell Biology, Physiology and Immunology, Universitat Autonòma de Barcelona, Bellaterra, Spain.

2.1. Experimental conditions

All the fish juveniles used in this thesis were provided by a local fish farm (Alevines y Doradas, S.A.: ADSA, Las Palmas, Spain), maintained for 4 weeks of acclimatising in 1000 l (Figure 2.1) tanks and distributed in 500 l experimental tanks (Figure 2.2).

Figure 2.1: A 1000 l acclimatization tank

Cynlinder conical tanks with a total volume of 500l are settled in pairs and separated by a central drainage channel. Tanks have a diameter of 1.5 m and depth of 1.0 m in the central part (Figure 2.2). Water inlet is located on the tank bottom and water outlet is located near the drop of the tank in the later side. Both inlet and lateral water outlet can be modified with different movable accessories, throughout the culture period according to the feeding sequence.

Figure 2.2: A sample of the intensive 500l rearing tank

2.2. Studied species

Gilthead sea bream (*Sparus aurata* Linnaeus, 1758) (Figure 2.3), is a teleost, belonging to *Sparidae* family, which has a total of 11 dorsal spines, 13-14 of dorsal soft rays, 3 of anal spines; 11-12 anal soft rays. It is characterised by a body tall, with large black spot on the gill cover. This fish has a large distribution (Figure 2.4), as it is present in eastern Atlantic (by British Isles), strait of Gibraltar to Cap Verde and around the Canary Islands, and it is also present in the Mediterranean. Seabream could be found in sea grass beds and sandy bottoms as well as in the surf zone commonly to depths of about 30m, but adults may occur to 150 m depth. It is a sedentary fish, either solitary or in small aggregations. In spring, they often occur in brakfish water coastal lagoons and estuaries. It is a mainly carnivorous species, but accessorily herbivorous, which feeds on shellfish, including mussels and oysters (Bauchot et al., 1990). This is one of the most important fishes in saline and hypersaline aquaculture.

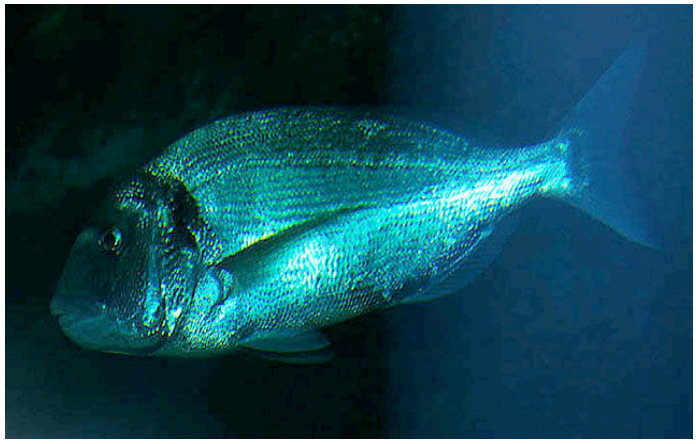

Figure 2.3: Adult gilthead seabream

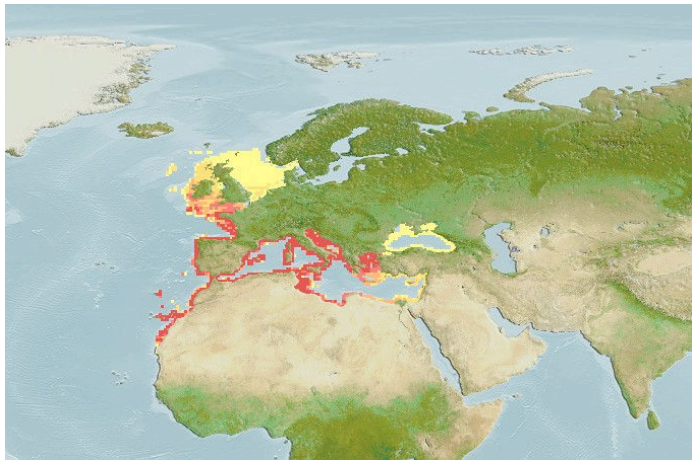

Figure 2.4: Geographical distribution of gilthead seabream (Fish base, 2009)

2.3 - Trial I: part of RAFOA project

2.3.1- Animals and diets

Two thousand four hundred juveniles sea bream of initial weight 24 g were maintained at Instituto Canario de Ciencias Marinas (ICCM). Fish were distributed randomly into 16, 1000 l, polyethylene circular tanks (150 fish / tank, each diet assayed in quadruplicate) supplied with continuous seawater (36 ‰) flow and aeration. Fish were fed under natural photoperiod (approximately 12:12 l/d). Water temperature and dissolved oxygen during the experimental period ranged between 21.88-22.41ºC and 5.5-7.2 ppm, respectively. Baseline samples for lipid composition comprising livers and fillet samples from 10 juveniles of average weight approximately 24 g were collected just before feeding the experimental diets. After two weeks of fish acclimation to the experimental tanks and control diet, the experimental diets were hand-fed until apparent satiation three times a day at 9:00, 12:00 and 15:00h, six days per week. All individual fish in each tank were weighted once per month and the ration adjusted accordingly. Feed intake was determined daily. Fifteen fish were sampled for biochemical parameters were taken at the beginning and at the middle (98 days) of the experimental period.

Four iso-energetic and iso-proteic experimental diets were formulated with a constant lipid content of about 22%. Two different blends included at a 60 % substitution of dietary fish oil in FO diet were used for sea bream (Table 1), 15:60:25 by volume in diet 60LO and 40:40:20 by volume in diet 60RO, of rapeseed, linseed and palm oils, respectively. A 100LO diet with 100% substitution of fish oil by the blend in diet 60LO was also included. The diets were prepared and delivered by Nutreco ARC. The lipid and fatty acid composition of 5 mm grain size diet were analysed and are shown in Tables 2. The control diet contained more saturates, DHA, EPA and ARA than the other diets, with diet 100LO containing the lower levels of these fatty acids. Whereas, diet 60LO and 60RO (with 60 % of substitution) had medium levels of EPA, DHA and ARA, but diet 60RO was higher in monoenoic fatty acids and lower in n-3 (lower in 18:3n-3 and higher in 18:2n-6) to compare the difference in the n-9 & n-3 fatty acids utilization by sea bream, subsequently n-3/n-6 ratio lower than in diet 60LO. Diet 100LO was lowest in EPA, DHA and ARA and highest in 18:2n-6 and monoenoic (oleic acid).

Conversion index (CI), specific growth rate (SGR) and hepatosomatic index (HSI) were calculated according to the following formulas:

CI= Feed Intake/ Weight Gain

SGR= ((Ln Final Weight- Ln Initial Weight/ t))*100

With t= experimental period (days)

HIS= Liver weight/ total weight

Table 2.1: The types and % of oils in the experimental diets

	FO	**60 LO**	**60 RO**	**100 LO**
Fish oil	100	40	40	-
Rapeseed oil	-	10	24	17
Linseed oil	-	35	24	58
Palm oil	-	15	12	25

Table 2.2: Main fatty acids of the different experimental diets (g/100 g fatty acid) (5mm)

	FO	60LO	60RO	100LO
% Lipids (d.w)	20.24	21.36	22.79	25.14
14:0	5.92	3.05	2.93	0.79
15:0	0.50	0.28	0.24	0.08
16:0	19.30	14.99	15.95	15.92
16:1n-7	7.21	3.93	3.23	1.23
16:2n-6	-	-	-	-
17:0	0.26	0.15	0.13	0.02
16:4n-3	0.17	0.04	0.03	0.02
18:0	3.37	3.21	3.36	3.40
18:1n-9	11.71	24.61	29.89	32.98
18:2n-6	5.84	11.87	12.50	13.67
18:3n-6	0.07	0.10	0.08	-
18:4n-6	0.17	0.03	0.03	-
18:3n-3	1.62	14.36	12.25	23.02
18:4n-3	2.18	1.28	0.87	0.17
20:0	0.21	0.24	0.32	0.23
20:1n-9	2.38	2.05	1.98	2.23
20:1n-7	-	-	-	-
20:2n-6	0.15	0.10	0.08	0.03
20:4n-6	0.66	0.34	0.28	0.06
20:4n-3	0.54	0.27	0.21	0.05
20:3n-3	0.08	0.05	0.04	0.02
20:5n-3	11.90	6.10	4.86	1.06
22:0	0.08	0.10	0.24	0.13
22:1n-11	2.98	2.35	2.20	2.41
22:1n-7	-	0.02	-	-
22:4n-6	0.18	-	0.03	0.01
22:5n-6	0.24	0.12	0.11	-
22:4n-3	-	-	0.03	0.02
22:5n-3	1.17	0.56	0.47	0.08
22:6n-3	14.14	7.36	3.21	2.10
Saturated	30.01	22.22	23.33	20.63
Monoenoics	27.70	33.12	37.44	38.85
Σ n-3	32.23	30.27	25.22	26.57
Σ n-6	7.37	12.59	13.13	13.78
Σ n-9	14.56	26.81	31.91	35.22
Σ n-3 HUFA	27.84	14.34	11.82	3.33
n-3/n-6	4.37	2.40	1.92	1.93

2.3.2. Sampling procedure

At the end of the feeding trial, day 281, fish were individually sampled from each tank. Blood was collected from caudal veins in heparinised syringes from 6 fish per each tank (18 fish per diet) and transferred to an eppendorf tube coated with lithium heparin as an anticoagulant. The blood was centrifuged immediately at 3000 rpm for 10 min to sediment the cells. One millilitre of plasma was removed, 50 µl/ml of 2 M formic acid was added and the acidified samples were frozen in liquid nitrogen (-80 °C) before the eicosanoids analysis. 250 µl of plasma were removed and stored at -80 °C for leptin analysis and the remainder plasma was pooled per each tank and stored at -80 °C for fatty acid analysis.

Blood leukocytes separation was conducted on only 3 dietary treatments as sufficient fish fed 100 LO diet were not available. Seven millilitres of blood was collected from the caudal vein in heparinised syringes from 9 fish per diet and transferred to clean glass tubes kept on ice. Blood was then centrifuged at 500 g for 10 min at 4 ºC. After elimination of supernatant cells were resuspended in 10 ml of HBSS Ca–Mg-free and then centrifuged at 500 g for 10 min at 4 ºC. Cells were separated into 2 sub-samples which were resuspended in 6 ml of HBSS Ca–Mg-free, and carefully layered over 6 ml of 46% Percoll and centrifuged at 450 g for 40 min at 4 ºC. The leukocytes (white intermediate layer) were collected and washed with 10 ml of HBSS Ca–Mg free. The leukocytes obtained from 3 fish were pooled and resuspended in 4 ml of HBSS, 2 ml of chloroform were added and the sample was stored at -80 ºC prior to lipid extraction.

2.3.3. Lipids extraction and analysis

Extraction of total lipid from plasma samples, diets and leukocytes was performed by the method of Folch et al (1957) using a mixture of chloroform: methanol (2:1)(v:v) containing 0.01% BHT, as an antioxidant, and KCl (0.88 %); 300 µl of pooled plasma were used. Vigorous vortex mixer followed by centrifugation to assist separation of chloroform and aqueous layers extracted the lipids from plasma, diet and leukocytes samples. The lower layers is filtered through Whatman filter paper and dried under a flow of nitrogen, total lipids were weighted. Neutral and polar fractions were separated by adsorption chromatography on silica cartridges Sep-pak, (Waters, Milford, MA) as described by Juaneda and Rocquelin (1985).

Fatty acid methyl esters were produced from aliquots of total lipids extracted from diet and plasma samples by acid-catalyzed transmethylation performed overnight at 50°C as described by Christie (1982). Fatty acids methyl esters were separated and quantified by Gas-chromatography (Shimadzu C-R5A, 30m*0,32mm Silice column with Supleco-10) according to conditions described by (Izquierdo *et al.,* 1990). Individual methyl esters were identified by comparison with known standards and published data.

2.3.4. Extraction, separation and enzyme immunoassay of PGE isomers

a. Purification of eicosanoids

The frozen plasma for eicosanoids analysis was thawed and centrifuged at 1000 x g for 5min to precipitate the debris. The supernatants were extracted using octadecyl silyl (C18) "Sep-Pak" mini-columns (Millipor (UK), Watford) as described in detail by Bell et al (1994). 200 µl of supernatants was applied to the column, which had been pre-washed with 5 ml methanol and 10 ml distilled water. The column was washed successively with 10 ml distilled water, 5 ml of 15% (v/v) ethanol and 5 ml hexane/chloroform (65:35, v/v) before elution of prostanoids with 10 ml of ethyl acetate. This extract was dried under nitrogen and resuspended in 100 µl methanol and stored in a small glass vial in the deep-freezer (- 4 ºC) before analyzing by immunoassay.

b. Separation of PGE_3 by HPLC

PGE3 was separated by reverse-phase HPLC using a Spherisorb 5 µm C18 (ODS2) column. The chromatographic system was equipped with Waters Model M-45 pumps and Waters 680 monitored at 196 nm using a Pye-Unicam LC-UV detector to determine elution of prostaglandin standards. An isocratic solvent system was used containing 17 mM phosphoric acid/acetonitrile (70:30, v/v) at a flow of 0,75ml/min. The remaining 50 µl of purified eicosanoids from plasma extract was injected to the column and 2.25 ml fractions were collected using an LKB 2112 "Redirac". Fractions corresponding to PGE3 were applied to a C18 "Sep-Pak" which had been-prewashed as described above, and the prostaglandin eluted in 5 ml ethyl acetate. Samples were dried under nitrogen and redissolved in 100 µl of immunoassay buffer. Measurement of PGE3 was performed using enzyme immunoassay (EIA) kits for PGE2 according to the same protocol described above (SPI-bio, Gif sur Yvette, France). The specificity of the kit

antibodies used in this immunoassay with PGE_2 is 100% but it is only 43% with PGE3, which is counted when calculating results.

c. Prostaglandins E_2 and E_3 immunoassay

For prostaglandins E2: 50 µl of the methanol extract were taken, dried under nitrogen and re-dissolved in 500 µl of EIA buffer and stored in the fridge (4°C). This assay is based on the competition between PGE_2 and a PGE_2-acetylcholinesterase (AchE) conjugated PGE_2 (tracer) for a limited amount of PGE_2 monoclonal antibody. Because the concentration of PGE_2 tracer is constant while the concentration of PGE_2 varies depending on the sample, the amount of PGE_2 tracer that is able to bind to PGE_2 monoclonal antibody is inversely-proportional to the concentration of PGE_2 in the well. This antibody PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman's Reagent (which contains the substrate to AchE) is added to the well. The product of this enzymatic reaction has distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrometrically, is proportional to the amount of PGE_2 tracer bound to the well, which is inversely proportional to the amount of free PGE_2 present in well during the incubation. All assay-specific reagents were prepared before starting the assay.

- EIA Buffer: Diluting the contents of the vial of EIA Buffer Concentrate with 90 ml of UltraPure water.
- Wash Buffer: Diluting the content of Wash Buffer Concentrate 1:400 with UltraPure water and adding Tween (0.5ml/liter of Wash Buffer).
- Prostaglandin E2 standard: The concentrate of The PGE2 standard (10 ng) is reconstituted with 1ml of EIA Buffer. Eight clean tubes (#1-8) were prepared; 360 µl EIA Buffer to tube #1 and 200 µl EIA Buffer to tubes # 2-8. 40 µl of the bulk standard (10 ng/ml) is transferred to the tube # 1 and mix thoroughly. Serially, the standard was diluted by removing 200 µl from # 1 and placing in tube # 2, mixing. Next, removing 200 µl from tube 2 to tube # 3, mixing. The process was repeated for tubes # 3-8. These diluted standards should not be stored for more than 24 hours.
- Prostaglandin E2 AchE: The concentrate is diluted with 6 ml EIA Buffer, The tracer dye is added at a final dilution of 1:100.

- Prostaglandin E2 Monoclonal Antibody: The concentrate is diluted with 6 ml EIA, the dye to the reconstituted antiserum is added at a final dilution of 1:100.

Once the immunoassay kits was opened, each plat contained eight Blanks (Blk), one non-specific bindings wells (NSB), one maximum binding wells (B0), and eight point standard curve (S1-S8). A test with different samples dilutions was run to choose the best (80 µl).

Pipetting the reagents: different tips are used to pipet the buffer, standard, sample, tracer, and antibody.

1. EIA Buffer: 100 µl EIA buffer were added to non-specific Binding (NSB) wells and 50 µl to maximum binding (B0) wells.
2. Prostaglandin E2 Standard: 50 µl from tube # 8 to the lowest standar well (S8). 50 µl from tube # 7 were added to the next standard well (S7). The same procedure was used until all standards were aliquoted.
3. Samples: 80 µl of sample were added per well.
4. Prostaglandin E_2 AchE Tracer: 50 µl were added to each well except the Blank (Blk) wells.
5. Prostaglandin E_2 Monoclonal Antibody: 50 µl were added to each well except the Non Specific Binding (NSB), and the Blank (Blk) wells.
6. The plate was covered with plastic film and incubated for 18 hours at 4°C which increase the sensitivity of the assay.
7. Developing the plate: Before developing the plate, Ellman's Reagent is reconstituted with 20 ml of ultrapure water. This should be prepared and used the same day it is prepared, and protected from light when not in use. The wells were emptied and rinsed five times with Wash Buffer. 200 µl of Ellman's Reagent were added to each well and 5 µl of tracer to the Total Activity wells. The plate is covered with plastic film and developed by using an orbital shaker and dark during 75 min.
8. Reading the plate: Is accomplished at a wavelength between 405.
9. Calculating the results: The results were calculated manualy as follows:

- Average the absorbace readings from Blk wells.
- Substract average Blk from all the other wells.
- Substract NSB from all th other wells.
- Calculate %B/B0 (% Sample standard Bound/Maximum Bound) for the remaining wells.

- The standard curve is traced (%B/B0 with standard concentration on PGE_2 en pg/ml).
- The concentration of each sample in PGE_2 is calculated.

2.3.5. Leptin immunoassay

a. Principle of the assay

This assay uses a cuantitative sandwich enzyme immunoassay technique. A monoclonic antibody for leptin is applied to the plates. A monoclonal antibody (Human leptin antibody) specific for leptin had been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any leptin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for leptin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of leptin bound in the initial step. The color development is stopped and the intensity of the color is measured.

b. Reagents

- Leptin microplates: 96- well. Mircoplate of polystyrene (12 columns of 8 well) with monoclínic antibody leptin.
- Leptin conjugate (Part 890574): 21 ml of antibody monoclonic against Leptin of rate with preservatif.
- Leptin estandard (Part 890575): 10 ng of recombinant leptin human in buefferd proteins with preservatif.
- Diluents Analysis RD1-19 (Part 895467): 11ml of buffered protein base with preservatif.
- Calibrator diluente RD5P (5x) Concentrate (Part 895151): 21ml of solution concentrate of buffered protein base with preservatif.
- Concentrate wash buffer (Part 895003): 21 ml of 25-fold concentrate of solution buffered surfactant with preservatif.
- Dye reactivif A (part 895000): 12.5 ml of estabilized hyrdogen preoxidase.
- Dye reactif B (Part 895002): 12.5 ml de estabilized chromogen (tetramethylbenzidine).
- Stop solution: 6 ml of 2 N Sulfiric acid.

c. Reagents preparation

All reagents are brought to the room temperature before the assay.

- Wash Buffer: The concentrate vial is mixed gently and diluted into 1:25 with deionized water. 500 ml of wash buffer were prepared.
- Substrate solution: Color reagent A and B were mixed together in equal volumes and put in dark within 15 minutes before using.
- Calibrator Diluent RD5P (1X): the concentrat is diluted to 1:5. 100 ml were prepared.
- Leptin standard: It is prepared 15 minutes before use. The concentrat is mixed gently and diluted with 1 ml of deionized water. This reconstitution produces a stock solution of 10.000 pg/ml. 8 tubes standard are prepared as expained above (Prostaglandin standard).

d. Assay procedure

The frozen plasma were thawed and centrifuged at 1000 X g for 5 min to precipitate the debris.

- 100 µl of assay diluent RD1-19 were added to each well.
- 100 µl of Standard are added to each stadanrd well.
- 100 µl of control were added to each well followed by 200 µl of Plasma sample. Multi-channel pipette was used.
- The plate is covered and incubated for 2 hours at room temperature.
- All the wells were aspirated and washed with 400 µl of Wash buffer. This process is repeated 4 times. At the last wash, the wells were bolted against clean paper to remove any remaining wash buffer
- 200 µl of Leptin Conjugate were added to each well, the plate was covered with new adhesive strip and incubated for 1 hour at room temperature.
- The wells were aspirated and washed as in step 5.
- 200 µl of Substrate Solution were added to each well and the plate was incubated for 30 minutes at room temperature and protected from light.
- 50 µl of Stop Solution were added to each well.
- The plate is developped within 30 minutes in shaker and the optical density of each well was determined using a microplate reader set to 450 nm.
- Calculation of results: The standard curve is drawn by ploting optical density for the standards versus the concentration of the standards. The data is linearized using a logaritmic transformation. The equation of standard curve is determined and the final Leptin concentration in plasma is determined by simple application of their absorbance in the standard curve equation.

2.4. Trial II: Part of LINOSALUD project

2.4.1. Animals and Diets

Gilthead seabream (*Sparus aurata*) jnuveniles (45 g initial body weight) were distributed in 45 tanks of 500l (50 fish/tank, each diet assayed in triplicate) supplied with seawater at a temperature ranging from 20 ºC at the beginning of the experiment to 24.2 ºC at the end, and aeration. Tow thousand tow hundred fifty animals were used. Eight iso-energetic and isonitrogenous diets with lipids content about 18 % were formulated. Anchovy oil was the only added lipid source in Diet FO (fish oil). All the other diets contained a blend of vegetable oils to substitute FO with different levels of substitution as mentioned in the tables (2.3, 2.4, 2.5). The fatty acids composition of the experimental diets total lipids is showed in Table 2.6. Fish were fed the experimental diets until apparent satiation (3 times/day, 6 days/ week), until they reached the commercial size after 26 weeks.

Feed intake was determined daily and all fish were individually weighed monthly. CI and SGR were calculated following the formulas described above. Mortalities were daily recorded and survival was monthly determined.

Table 2.3: Main ingredients contents of the experimental diets (in %)

	% of dry weight
Oils (Fish oil[a]/linseed/soybean)	16.32
South-american fish meal	47.26
Wheat	7.00
Soybean meal 47% [b]	25.00
Sunflower meal	3.67
Vitamins premix[c]	0.27
Minerals premix[c]	0.48

a South-american, anchovy oil.
b Soybean meal with 47% as a brut protein, “no GMO”
c Vitamin and mineral premixes prepared according to Proaqua A/S commercial standards

Table 2.4: Vitamins and minerals contents of the experimental diets

Vitamins/ Minerals	Units
1-Vitamins	
A-Retinol	11200.0 IU/kg
D3- Cholecalciferol	112.0 IU/kg
E- Tocopherol	280.0 mg/kg
C (Stay C)- Ascorbic acid	336.0 mg/kg
B1- Thiamin	9.0 mg/kg
B2- Riboflavin	15.7 mg/kg
B3- Nicotinic acid/Niacin	179.2 mg/kg
B5- Panthothenic acid	31.4 mg/kg
B6- Pyridoxin	13.4 mg/kg
B8- Biotin	0.5 mg/kg
B9- Folic acid	4.5 mg/kg
B12- Cyanocobalamin	0.036 mg/kg
K- Menadion	6.7 mg/kg
Inositol	44.8 mg/kg
2- Minerals	
I	4.5 mg/kg
Zn	44.8 mg/kg
Fe	67.2 mg/kg
Cu	3.6 mg/kg
Mn	14.6 mg/kg
Mg	136.1 mg/kg
Co	0.2 mg/kg
Se	0.06 mg/kg

Table 2.5: The types and % of oils in the experimental diets

	FO	**70L**	**100L**	**70S**	**100S**	**20L50S**	**50L20S**	**50L50S**
FO	100	30	-	30	-	30	30	
LO	-	70	100	-	-	20	50	50
SO	-	-	-	70	100	50	20	50

Table 2.6: Fatty acids profile of the experimental diets (% total identified fatty acids)

Fatty acids	FO	70L	100L	70S	100S	20L50S	50L20S	50L50S
14:0	9.23	3.11	1.57	3.32	1.59	2.67	2.60	1.37
15:0	0.26	0.10	0.06	0.11	0.06	0.32	0.23	0.06
16:0ISO	0.11	0.04	0.03	0.05	0.03	0.01	0.06	0.03
16:0	22.21	12.19	10.21	15.84	13.69	15.46	12.16	11.75
16:1n-7	11.25	3.91	1.98	4.06	2.01	3.07	3.24	1.73
16:1n-5	0.38	0.14	0.09	0.16	0.09	0.07	0.10	0.08
16:2n-4	1.83	0.61	0.28	0.61	0.29	0.11	0.51	0.24
17:0	0.85	0.38	0.25	0.41	0.28	0.52	0.31	0.25
16:3n-4	2.00	0.66	0.29	0.67	0.31	0.59	0.54	0.24
16:3n-3	0.15	0.07	0.05	0.07	0.05	0.57	0.03	0.05
16:3n-1	0.12	0.05	0.04	0.06	0.04	0.07	0.07	0.03
16:4n-3	0.72	0.26	0.13	0.24	0.11	0.04	0.05	0.09
16:4n-1	-	-	-	-	-	0.25	0.29	-
18:0	3.85	3.96	3.99	3.43	3.20	10.20	3.49	3.74
18:1n-9	9.10	13.75	15.31	18.60	21.48	15.30	18.48	19.04
18:1n-7	3.16	1.57	1.36	2.15	1.77	1.15	1.37	1.36
18:1n-5	0.14	0.08	-	0.12	0.11	0.09	0.10	0.10
18:2n-6	4.06	12.38	16.21	29.93	38.51	22.25	18.50	27.25
18:2n-4	0.38	0.13	0.06	0.12	0.06	0.18	0.12	0.05
18:3n-6	0.36	0.13	-	-	0.07	0.13	0.12	0.09
18:3n-4	0.04	-	0.04	0.18	0.09	0.70	0.12	0.05
18:3n-3	0.48	31.94	37.63	5.63	6.01	11.38	20.67	23.03
18:4n-3	1.94	0.76	0.40	0.73	0.40	0.06	0.75	0.36
18:4n-1	-	-	0.03	-	-	-	0.08	-
20:0	0.28	0.21	0.19	0.28	0.28	0.72	0.26	0.24
20:1n-9	2.59	1.94	1.84	2.08	1.89	1.74	2.35	1.91
20:1n-7	0.26	0.13	0.09	0.13	0.10	0.11	0.15	0.09
20:2n-9	-	0.04	-	0.04	0.01	0.02	0.03	0.01
20:2n-6	0.19	0.11	0.08	0.11	0.09	0.10	0.04	0.08
20:3n-9	-	-	-	-	-	0.08	0.15	-
20:3n-6	0.25	0.09	0.04	0.06	0.03	0.08	0.08	0.03
20:4n-6	1.11	0.43	0.24	0.43	0.24	0.40	0.39	0.21
20:3n-3	-	-	0.07	-	-	0.06	0.08	-
20:4n-3	0.96	0.35	0.16	0.33	0.16	0.32	0.38	0.13
20:5n-3	10.05	4.00	2.07	3.77	2.06	3.85	3.99	1.73
22:1n-11	1.79	1.52	1.48	1.67	1.61	1.45	1.91	1.64
22:1n-9	-	-	0.42	-	-	0.33	0.52	-
22:4n-6	0.34	0.15	0.09	0.14	0.09	0.15	0.14	0.08
22:5n-6	-	-	-	-	-	0.64	0.75	-
22:5n-3	1.74	0.66	0.29	0.60	0.29	0.39	0.11	0.24
22:6n-3	7.82	4.16	2.92	3.87	2.91	4.35	4.69	2.63
Saturates	36.68	19.95	16.28	23.40	19.10	29.88	19.06	17.41
Monoenic	28.79	23.10	22.62	29.03	29.09	23.33	28.57	25.98
n-3	23.42	42.19	43.72	15.23	12.00	21.02	30.74	28.26
n-6	6.27	13.26	16.66	30.67	39.02	23.76	19.99	27.74
n-9	22.99	19.66	19.55	24.78	25.40	17.48	24.79	22.69
n-3 HUFA	20.57	9.17	5.50	8.57	5.42	8.97	9.24	4.73
n-3/n-6	3.74	3.18	2.62	0.50	0.31	0.88	1.54	1.02

2.4.2. Sampling procedure

At the end of the feeding trial (after 26 weeks), 6 fish were individually sampled from each tank. Fish were anaesthetized with 2-phenoxyethanol (1:1000 v/v), and blood was collected from caudal veins in heparinised syringes from 6 fish per each tank (18 fish per diet) and transferred to an eppendorf tube coated with lithium heparin as an anticoagulant. The blood was centrifuged immediately at 3000 rpm for 10 min to sediment the cells, and the plasma was stored at -80ºC for basal cortisol analysis. Samples of muscle, liver, gills and head kidney were stored at -80ºC for biochemical analysis from all the dietary treatments.

2.4.3. Preparation and stimulation of head kidney tissue

At the end of the feeding period, 2 fish were randomly taken from each tank (6 per diet) in less than 1 minute, immediately anaesthetized with 2-phenoxyethanol (1:1000 v/v) and blood collected with a hypodermic syringe from the caudal vein to minimize the haemorrhage during the extraction of the tissue. HK tissue was removed from two fish in each superfusion trial, and cut into very small fragments in Hepes Ringer Medium, which was used as the superfusion medium. Afterwards, HK homogenates were pooled and distributed in 8 superfusion chambers (volume: 0.2 ml) in order to obtain an homogeneous aliquot in each of them. Tissues were superfused with a Hepes (pH 7.4) Ringer's solution containing 171 mM NaCl, 2 mM KCl, 2 mM $CaCl_2H_2O$, 0.25 % (w/v) glucose, and 0.03 % (w/v) bovine serum albumin (Rotllant et al., 2001). The system was temperature-controlled at 18 °C and superfusion medium was pumped through the chamber at a rate of 75 μl/min by a Masterplex L/S^R multichannel peristaltic pump (Cole Parmer Intrument Co., Vernon Hills, Illinois)(Rotllant *et al*., 2001).

Trials were started after 3 h of superfusion when cortisol reached stable baseline levels (Rotllant et al. 2000a, b), in order to avoid deviations due to the different dispersion of interrenal cells in the perfusion preparation and the individual differences or the pre-stress level of each fish. After the stabilisation period of 3 h, tissues were stimulated with ACTH at a concentration of 5 nM $hACTH_{1\text{-}39}$ (Sigma) during 20 min. Subsequently, perfusion was maintained for another 170 min, fraction samples being collected every 20 min during this period. In a second series of experiments to clarify the action mechanisms of HUFA and the implication of eicosanoids in this process, tissues were incubated with a COX inhibitor (Indomethacin, INDO), or lipoxygenase

(LOX) inhibitor (Nordihydroguaiaretic acid, NDGA) for 20 min at a concentration of 25 μM diluted in superfusion medium, and subsequently the tissues were stimulated with ACTH as explained before and maintained for another 170 min collecting samples every 20 min. In all series of experiments each treatment was assayed in quadruplicates. Cortisol stimulation factor (SF) was calculated by comparison of maximum cortisol released after ACTH stimulation with baseline cortisol released (maximum release-baseline release)*100/(baseline release) (Rotllant *et al*., 2001).

2.4.4. Stress Panels

After the feeding period, 20 fish per tank were exposed to confinement stress. Thus, fish were assigned in groups of up to 4 held placed into five floating small cage (50x30x15cm) (Figure 2.5). 4 fish per tank were bled at the beginning of the tests and considered as controls. The five cages, containing 4 fish each, were maintained in each tank for different time intervals, consisting to encompass "early" and "late" response to the stressor, based on primary and secondary stress responses of plasma cortisol. Thus, after 2h one cage per tank was extracted carefully, opened and the fish blood was sampled in less than 2 min of handling time, blood was obtained by caudal sinus puncture with a 1 ml plastic syringe. Aliquots of blood samples were immediately transferred to an Eppendorf tube coated with lithium heparin as anticoagulant. The plasma was obtained by centrifugation at 3000 rpm for 10 min and stored at -80º C prior to cortisol determination and the fish were liberated. The same procedure was repeated after 5h, 24h, 48h and 1 week. This approach was taken to keep any acute disturbance to remaining fish from fish removal as low as possible. Thus, total capture time was less than 8 min per tank to minimize capture stress effects on analyzed parameters (Sumpter, 1997).

Figure 2.5: A sample of cage used for stress panel tests.

2.4.5. Biochemical analysis

Dietary and fish tissues moisture, ash and protein were determined in triplicates according to the Association of Official Analytical Chemists (AOAC) described methods. Moisture was evaporated in an oven at 110 ºC until constant weight and the difference from the initial weight was then calculated as before. The protein content was calculated from the N content according to the Khedjal method. The sample was distilled with NaOH at 40% in Kejeltek System 1003 distiller (Tecator, Höganäs, Switzerland). Finally, the sample was validated with HCl (1 N) to measure N content and protein content was calculated utilizing a nitrogen conversion factor of 6.75.

Extraction of total lipid from diets, fillets, liver, gills and head kidney was performed by the method of Folch et al (1957) as explained in the experiment before.

2.4.6. Cortisol measurements

Cortisol concentration in the perfused fluid was determined by radioimmunoassay (RIA) (Rotllant *et al*., 2001). The antibody, Biolink, S.L. (Costa Mesa, California), was used in a final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11.40% with 21-desoxycorticosterone, 8.90% with 11-desoxycortisol and 1.60% with 17a-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels are given as ng g^{-1} h^{-1}.

2.5. Statistical analysis

Significance of difference ($P<0.05$) between dietary treatments was determined by one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test (Sokal and Rolf, 1995). Analyses were performed using SPSS software (SPSS for windows 13).

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Chapter 3

Effect of feeding seabream (*Sparus aurata*) with a blend of soybean and linseed oil on growth, feed utilization and body fatty acids composition

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To be submitted

Effect of feeding seabream (*Sparus aurata*) with a blend of soybean and linseed oil on growth, feed utilization and body fatty acids composition

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Abstract

Triplicate groups of gilthead seabream were fed eight practical-type diets in which the added lipid was substituted with a blend of linseed oil (LO) and soybean oil (SO) at different levels for a period of 26 weeks. Anchovy oil was the only lipid source in the FO (fish oil) diet, whereas the other diets contained different levels of vegetable oils, 70L (70% LO and 30 FO), 70S (70% SO and 30FO), 100L (100% as LO), 100S (100% as SO), 50S20L (50% as SO, 20% as LO and 30% as FO), 20S50L (20% with SO, 50% with LO and 30% with FO) and 50S50L (50% as SO and 50% with LO). Results showed that substitution up to 70% or more of FO by vegetable oils in diets for gilthead seabream, significantly reduced growth and affected feed utilization. Fatty acid compositions of muscle lipid correlated with SO or LO inclusion in the diet, thus the proportions of 18:2n-6, 18:3n-3 and 18:1n-9 all increased with increasing dietary VO %. The concentrations of eicosapentaenoic acid (20:5n-3), docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) in muscle lipid were significantly reduced ($P<0.05$), along with total saturated fatty acids, with increasing dietary VOs, while the latter 2 fatty acids were less reduced in the muscle than in the diet, indicating their selective retention. The elongase and Δ6 destaurase products from linoleic acid (18:2n-6) and linolenic acid (18:3n-3) were also increased with increasing VOs. Diet-induced changes in liver and gill fatty acid compositions were broadly similar to those in muscle, with some exceptions. Limited supplies of marine fish oils require that substitutes be found without compromising fish welfare. Thus, LO and SO can be used successfully in the culture of gilthead seabream at levels less than 70% of dietary lipid without compromising growth, but substantial reductions occur in muscle 20:5n-3, 22:6n-3 and the n-3/n-6 polyunsaturated fatty acid (PUFA) ratio, which will result in reduced product quality for the consumer.

Key words: Gilthead seabream, Fish Oil, Linseed oil, Soybean oil, growth, EPA, DHA, n-3 HUFA, n-3/n-6.

Introduction

Marine fish oils (FO), naturally rich in omega 3 highly unsaturated fatty acids (HUFA) (>30%) which play very important roles in aquatic organisms, have been traditionally used as a preferred lipid source in aquafeeds, due to its high digestibility and content of essential fatty acids. Since those fatty acids also have beneficial effects on human health (Craig-Schmidt, 2001), global consumer demand for seafood has grown tremendously and aquaculture is playing an increasingly important role in fulfilling the increased demand (Tacon, 2004; FAO, 2007a). Therefore, global demand of FO for aquaculture has been increasing with fish production. Indeed, FO supply is becoming a challenge for the aquafeeds industry, since its production relies on fisheries; an overexploited source of HUFA (FAO, 2007b) and research on alternative lipid sources has become a major goal for sustainable aquaculture development.

Vegetable oils (VO), more sustainable, reliable and, frequently, with more competitive prices than FO, are seen as good alternative sources. VOs assayed in fish feeds include linseed (LO) (Bell *et al*., 2003; Izquierdo *et al*., 2003, 2005; Benedito-Palos *et al*., 2007; Jobling *et al*., 2008), rapeseed (Bell *et al*., 2001, Montero *et al*., 2005), palm (Bell *et al*., 2002), soybean (SO) (Grisdale-Hellan *et al*., 2002) and sunflower oils (Torstensen *et al*., 2000; Bransden *et al*., 2003). In gilthead seabream (*Sparus aurata*), an important fish in Mediterranean aquaculture, it is possible to partially replace FO by VO without compromising growth or feed utilization (Caballero *et al*., 2002; Izquierdo *et al*., 2003, 2005; Montero *et al*., 2005). However, complete substitution might be more difficult as the VO lack the n-3 HUFA essential for this species (Ibeas *et al*., 1994). This means that, as in other marine fish (Watanabe *et al*., 1983; Sargent *et al*., 1989; Castell *et al*., 1994), seabream has a very low Δ5 desaturase and elongase activities to allow synthesis of eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic (22:6n-3; DHA) acids from their precursor linolenic (18:3n-3; LNA) acid (Seiliez *et al*., 2003; Izquierdo *et al*., 2005, 2008). Therefore, seabream minimum requirements for n-3 HUFA, particularly EPA and DHA should be included in diet in order to maintain fish growth, health and welfare (Izquierdo *et al*., 2003, 2005; Montero *et al*., 2003; Caballero *et al*., 2004), as well as filet quality (Kalogeropoulos *et al*., 1992; Izquierdo *et al*., 2003, 2005). Moreover, certain VOs such as soybean oil, have a high content in n-6 fatty acids, particularly linoleic acid (18:2n-6, LA) which are not present in high amounts in marine environments.

Inclusion of those n-6 rich vegetable oils, with a low n-3/n-6 fatty acids ratio (0.3-1) will produce diets with a n-3/n-6 fatty acids profile very different from FO (n-3/n-6 of 9-6:1) (Robaina *et al*., 1998; Bransden *et al*., 2003) and the natural food of marine fish. Indeed, feeding high lipid diets with a 60% FO replacement by certain VOs, particularly soybean oil, increased lipid deposition in hepatocytes (Caballero *et al.*, 2002) and affected some immune parameters (Montero *et al*., 2003, 2008) suggesting undesirable effects on fish health. A well balanced n-3/n-6 fatty acid ratio is determinant for good human health (Simopoulos, 2008), since the functions and efficiency of these fatty acids differ in many aspects of lipid metabolism such as digestion, absorption and transport in fish (Izquierdo *et al*., 2000, 2001; Caballero *et al*., 2006). In addition, energy production, cell membranes structural components and signaling molecules and regulators of gene expression can all be affected by changes in dietary fatty acids (Calder and Burdge, 2004; Yaqoob and Calder, 2007). Elsewhere, n-6 and n-3 PUFA can influence mechanisms concerned with different physiological and inflammatory processes in humans (Sanders, 1993), sometimes in opposing ways, so high dietary n-6 PUFA and n-6/n-3 PUFA ratios have been suggested to play a role in many chronic diseases endemic in Western populations (Simopoulos, 2008), thus altered balance between the n-6 and n-3 series in aquaculture products may reduce the beneficial effects of FO for human health. Producing fish rich in LA with consequently higher n-6/n-3 ratio when feeding soybean oil (Caballero *et al*., 2002; Montero *et al*., 2003, Izquierdo *et al*., 2005), not only reduces the health promoting properties of the fish but worsens an already imbalanced human diet. Therefore, the challenge when substituting FO with different VOs is to maintain high quality fish products by assuring a high n-3/n-6 ratio in the aquaculture products available to the consumer.

The present study was conducted to better understand the consequences of feeding commercial diets containing n-3 and/or n-6 fatty acid rich oils, and blends of these oils giving different n-3/n-6 fatty acids ratios, on culture performance of gilthead seabream, particularly in relation to feed utilization, fish growth, and lipid metabolism in different tissues.

Materials and methods

Fish husbandry

Gilthead seabream (*Sparus aurata*) juveniles (45 g initial body weight) were distributed in 39 tanks of 500 l (50 fish/tank with each diet assayed in triplicate). Seawater was supplied in an open system (10 % renewal/h) at a temperature of 20-24.2 ºC. Fish were fed the experimental diets (Table 3.1) until apparent satiation (3 times/day, 6 days/week) for 26 weeks. Eight iso-energetic and isonitrogenous diets with a lipid content of ~18 % were formulated and provided by Proaqua, S.A. (Dueñas, Spain). Anchovy oil was the only added lipid source in the FO diet, whereas in the other diets the FO was replaced by either LO, SO or blends of both oils (Table 3.2).

Feed intake (FI) was determined daily and all fish were weighed individually every month. Feed conversion ratio (FCR = feed intake/weight gain) and specific growth rate (SGR= ((Ln final weight- Ln initial weight)/t)*100, *t*=experimental period (days)) were calculated monthly. Mortalities were recorded daily and survival was determined monthly.

After 26 weeks of feeding, samples of muscle, liver and gills from 18 fish fed each experimental diet were collected, immediately frozen and stored at -80 ºC until they were analysed. Due to the complexity and the high number of samples produced, and accordingly to the effect on fish growth, we chose only the 6 extreme diets for fatty acid analysis (100FO, 70L, 100L, 70S, 100S, 50S50L). Lipids from diets, liver, gills and muscle were extracted with chloroform: methanol (2:1 v:v) (Folch *et al*., 1957). The fatty acids methyl esters were obtained by transesterification with 1% sulphuric acid in methanol (Christie, 1982), purified by absorption chromatography on NH_2 Sep-pak cartridges (Water, S.A., Milford, Massachusetts) and separated, identified and quantified by gas-liquid chromatography under the conditions previously described (Izquierdo *et al*., 1990). Fatty acid methyl esters were identified by comparison to external standards.

Table 3.1: Main ingredients contents of the experimental diets

	% of dry weight
Oils (Fish oil[a]/linseed/soybean)	16.32
South-American fish meal	47.26
Wheat	7.00
Soybean meal 47%[b]	25.00
Sunflower meal	3.67
Vitamins premix[c]	0.27
Minerals premix[c]	0.48

[a] South-American, anchovy oil.
[b] Soybean meal with 47% gross protein, "no GMO"
[c]Vitamin and mineral premixes prepared according to Proaqua A/S commercial standards.

Table 3.2: Proximate composition (% dry weight) of experimental diets and proportions of the different lipid sources (% of oil inclusion) contained in the experimental diets

Lipid source	**FO**	**70L**	**100L**	**70S**	**100S**	**50S20L**	**20S50L**	**50S50L**
FO (Anchovy oil)	100	30	-	30	-	30	30	-
LO	-	70	100	-	-	50	50	50
SO	-	-	-	70	100	20	20	50
Composition	***Dry Weight (%)***							
Lipid	17.97	17.97	17.51	16.22	17.59	15.51	17	17.67
Protein	44.35	46.97	45.83	47.42	47.51	47.62	44.83	46.35
Ash	4.06	5.22	4.61	5.05	5.42	5.5	4.55	4.83

Table 3.3: Fatty acid compositions of the experimental diets (g/100 g total fatty acids)

Fatty acids	FO	70L	100L	70S	100S	50S20L	20S50L	50S50L
14:0	9.23	3.11	1.57	3.32	1.59	2.67	2.60	1.37
15:0	0.26	0.10	0.06	0.11	0.06	0.32	0.23	0.06
16:0ISO	0.11	0.04	0.03	0.05	0.03	0.01	0.06	0.03
16:0	22.21	12.19	10.21	15.84	13.69	15.46	12.16	11.75
16:1n-7	11.25	3.91	1.98	4.06	2.01	3.07	3.24	1.73
16:1n-5	0.38	0.14	0.09	0.16	0.09	0.07	0.10	0.08
16:2n-4	1.83	0.61	0.28	0.61	0.29	0.11	0.51	0.24
17:0	0.85	0.38	0.25	0.41	0.28	0.52	0.31	0.25
16:3n-4	2.00	0.66	0.29	0.67	0.31	0.59	0.54	0.24
16:3n-3	0.15	0.07	0.05	0.07	0.05	0.57	0.03	0.05
16:3n-1	0.12	0.05	0.04	0.06	0.04	0.07	0.07	0.03
16:4n-3	0.72	0.26	0.13	0.24	0.11	0.04	0.05	0.09
16:4n-1	-	-	-	-	-	0.25	0.29	-
18:0	3.85	3.96	3.99	3.43	3.20	10.20	3.49	3.74
18:1n-9	9.10	13.75	15.31	18.60	21.48	15.30	18.48	19.04
18:1n-7	3.16	1.57	1.36	2.15	1.77	1.15	1.37	1.36
18:1n-5	0.14	0.08	-	0.12	0.11	0.09	0.10	0.10
18:2n-9	0.04	0.02	-	-	-	-	0.03	-
18:2n-6	4.02	12.36	16.21	29.93	38.51	22.25	18.47	27.25
18:2n-4	0.38	0.13	0.06	0.12	0.06	0.18	0.12	0.05
18:3n-6	0.36	0.13	-	-	0.07	0.13	0.12	0.09
18:3n-4	0.04	-	0.04	0.18	0.09	0.70	0.12	0.05
18:3n-3	0.48	31.94	37.63	5.63	6.01	11.38	20.67	23.03
18:4n-3	1.94	0.76	0.40	0.73	0.40	0.06	0.75	0.36
18:4n-1	-	-	0.03	-	-	-	0.08	-
20:0	0.28	0.21	0.19	0.28	0.28	0.72	0.26	0.24
20:1n-9	2.59	1.94	1.84	2.08	1.89	1.74	2.35	1.91
20:1n-7	0.26	0.13	0.09	0.13	0.10	0.11	0.15	0.09
20:2n-9	-	0.04	-	0.04	0.01	0.02	0.03	0.01
20:2n-6	0.19	0.11	0.08	0.11	0.09	0.10	0.04	0.08
20:3n-9	-	-	-	-	-	0.08	0.15	-
20:3n-6	0.25	0.09	0.04	0.06	0.03	0.08	0.08	0.03
20:4n-6	1.11	0.43	0.24	0.43	0.24	0.40	0.39	0.21
20:3n-3	-	-	0.07	-	-	0.06	0.08	-
20:4n-3	0.96	0.35	0.16	0.33	0.16	0.32	0.38	0.13
20:5n-3	10.05	4.00	2.07	3.77	2.06	3.85	3.99	1.73
22:1n-11	1.79	1.52	1.48	1.67	1.61	1.45	1.91	1.64
22:1n-9	-	-	0.42	-	-	0.33	0.52	-
22:4n-6	0.34	0.15	0.09	0.14	0.09	0.15	0.14	0.08
22:5n-6	-	-	-	-	-	0.64	0.75	-
22:5n-3	1.74	0.66	0.29	0.60	0.29	0.39	0.11	0.24
22:6n-3	7.82	4.16	2.92	3.87	2.91	4.35	4.69	2.63
Saturates	36.68	19.95	16.28	23.40	19.10	29.88	19.06	17.41
Monoenoics	28.79	23.10	22.62	29.03	29.09	23.33	28.57	25.98
n-3	23.42	42.19	43.72	15.23	12.00	21.02	30.74	28.26
n-6	6.27	13.26	16.66	30.67	39.02	23.76	-	27.74
n-9	22.99	19.66	19.55	24.78	25.40	17.48	24.79	22.69
n-3 HUFA	20.57	9.17	5.50	8.57	5.42	8.97	9.24	4.73
n-3/n-6	3.74	3.18	2.62	0.50	0.31	0.88	1.54	1.02

Statistical analysis

All the data were statistically treated using ANOVA and Tukey's test at $P<0.05$ was applied as a multiple sample comparison analysis using a SPSS Statistical Software System 10.0 (SPSS Inc., Chicago, Illinois) (Sokal and Rolf. 1995).

Results

Lipid peroxidation products were determined as thiobarbituric acid reactive substances (TBARS) and they showed no significant differences between the diets, ranging between 8.56 and 3.85 μmole of malonaldehyde (MDA)/kg of wet diet. No dietary effect was observed on TBARS concentration ($P<0.05$). The fatty acid profile of the different diets reflected the inclusion level of different VOs (Table 3.3). Saturated fatty acids decreased with higher FO replacement in the diets, ranging from 36.68 in FO diet to 17.41 % in 50SO50LO diet. Total monounsaturates (MUFA; mainly oleic acid (18:1n-9, OA) tend to be reduced with the inclusion of LO and to increase with the inclusion of SO, ranging from 22.62 % in 100LO diet to 29.09 in 100S. LA in diet increased with increasing VO inclusion, particularly SO, ranging from 4.02% in FO diet to 38.51% in 100S diet, while LNA was also increased, particularly by LO inclusion, ranging from 0.48% in FO diet to 37.63% in 100L diet. The percentages of ARA, EPA and DHA were proportionally reduced by the replacement increase, ranging from 0.21, 1.73 and 2.63 %, respectively, in 50S50L diet to 1.11, 10.05 and 7.82%, respectively, in FO diet. Thus, in diets with 70% FO substitution by plant oils the contents of ARA, EPA and DHA were reduced by 56%, 62% and 46% respectively, while when substituting up to 100% of FO, the reduction of these fatty acids was 74%, 83% and 62% compared to their respective values in the control diet.

All fish accepted all the experimental diets and no fish died during the feeding period. All treatments showed good growth but, after 4 months of feeding fish fed FO diet showed the highest final body weight (Figure 3.1)**.** Monthly SGR increased with temperature along the experiment being slower at the beginning and higher at the end of the feeding period. Average SGRs were significantly lower in fish fed 70S20L, 20S50L and 50S50L compared to the control diet (Table 3.4). CI ranged from 1.23 for fish fed FO to 1.64 for fish fed 20L50S at the end of the trial (Table 3.4). Thus, fish fed the control diet showed a significantly higher final weight, with 277.1 g, compared to the other treatments. The weight gain followed a similar pattern with a significantly higher

value for fish fed the control diet and the lowest values for fish fed 100S and 50S50L diets.

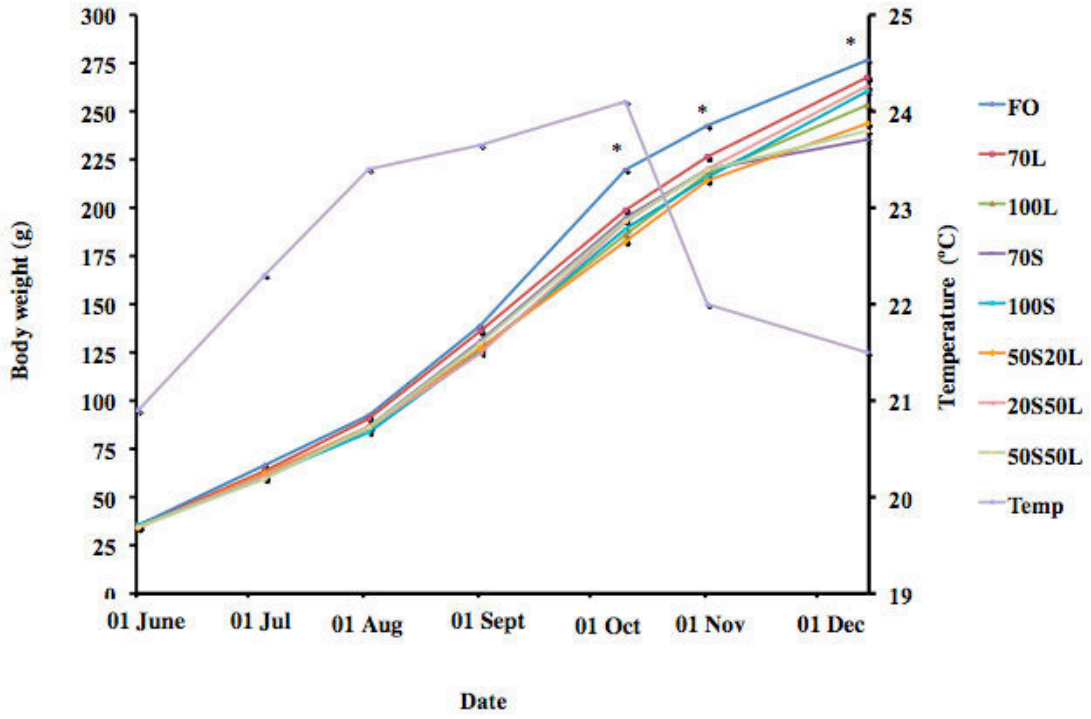

Figure 3.1: Effect of different dietary oils on gilthead seabream body weight (experiment I) (n = 150). * Denotes significant differences ($P < 0.05$) between 100F and the rest of the experimental groups.

Fatty acid compositions of fillet total lipid of sea bream after 26 weeks of feeding the experimental diets are shown in Table 3.5. Total saturates (mainly 16:0) in gilthead sea bream fillets were proportionally reduced by the inclusion of dietary plant oils, but to a lower extent than in the diets. Total MUFA contents were similar in all fillets but oleic acid was significantly higher in fillets from fish fed the VO diets compared to the control treatment. Muscle contents of LA were also highest in fish fed diets with partial substitution of dietary FO, due to the high levels of LA in SO and to a lesser extent in LO diets. The same pattern was true for total n-6 PUFA since LA constituted the main n-6 component. In contrast, ARA was significantly lower in fish fed 70L, 70S, 100S and 50S50L compared to the FO. The content on LNA in flesh total lipids was highest in fish fed 100L followed by fish fed 70L, then those fed 100S and 50S50L, and the lowest content was found in fish fed 70S and FO. EPA, DHA and total n-3 HUFA were significantly highest in fish fed FO due to its highest content of these

fatty acids and decreased with increased substitution by VO. The end products of Δ6-desaturase and elongase from LA, including 18:3n-6, 20:2n-6 and 20:3n-6, were increased in muscle from fish fed SO. In addition the dead end product of C_{18}/C_{20} elongase from LNA, 20:3n-3, was increased in fish fed LO.

The effects of the experimental diets on fatty acid compositions of liver were qualitatively the same as described for flesh. However, a number of notable quantitative differences occurred, particularly with respect to MUFA. Liver total lipid fatty acid composition from sea beam fed the experimental diets is shown in Table 3.6. After the feeding period, total saturates were significantly higher in fish fed FO. Total MUFA, primarily OA, were significantly higher in liver total lipids from fish fed 100L, 70S, 100S and 50S50L compared to the FO diet, due to the higher inclusion of this fatty acid in the blends of VO. LA was significantly higher in all liver total lipids from fish fed VO and the highest content was registered in fish fed 100S. Total n-6 was significantly higher in fish fed SO and 50S50L reflecting its higher content of LA. The liver total lipid content of LNA was also affected by the feeding trial, as fish fed LO showed significantly the highest contents on this fatty acid, followed by 50S50L. Liver total lipid percentage of EPA, DHA and total n-3 HUFA was significantly higher in fish fed FO as a consequence of the dietary abundance of these fatty acids. Liver content of LNA also reflected the dietary input of this fatty acid, such that fish fed the LO diet showed significantly the highest levels, followed by 50S50L and 100S. The Δ6-desaturase and elongase products from LA and LNA, such as 18:3n-6, 20:2n-6, 20:3n-6 and 20:3n-3, were significantly increased in fish fed VO comparing to FO and according to the amount of the precursor fatty acid in the diet.

Fatty acid composition from the gills showed a similar pattern (Table 3.7), thus total saturates were significantly reduced when feeding seabream LO and SO compared to the FO diet. This can be explained mainly by the high content in FO of 16:0. OA was significantly increased in gills from fish fed all the experimental VO diets compared to FO. LA was significantly increased in all fish fed the VO diets with the maximum observed for the 100S diet. LNA was also increased significantly in fish fed LO according to the level of inclusion and consequently total n-3 content was higher. ARA was significantly reduced in gills from fish fed VO compared to FO, and the levels of this FA were higher in this organ compared to the liver or muscle up to almost 2 fold. EPA, DHA and total n-3HUFA were significantly decreased in fish fed LO and SO

compared to the control diet. The amounts of 18:3n-6, 20:2n-6 and 20:3n-6 were increased in SO fed fish, while 20:3n-3 were increased in LO fed fish compared to FO. The n-3/n-6 ratio was clearly affected by both the type and the level of inclusion of each oil and higher n-3/n-6 ratios were observed in all the tissues of fish fed FO. However, increasing dietary VO concentrations led to a progressive decrease in the n-3/n-6 ratio. Diet induced changes on saturated and MUFA concentrations for all the tissues.

Table 3.4: Growth and efficiency in utilization of feed by gilthead seabream fed the experimental diets.

	FO	**70L**	**100L**	**70S**	**100S**	**50S20L**	**2**
Initial Weight (g)	35.6±0.8	35.3±0.4	34.8±0.5	35±0.5	35.4±0.6	34.2±0.3	3
Final Weight (g)	277.1±23.87^{a}	251.12±15.5^{bc}	249.9±14.5^{bc}	260.5±21.7^{ab}	232.8±18.73^{c}	243.1±35.47^{bc}	244.
% Weight Gain	671±14.2^{a}	613.8±18.9^{abc}	610.7±10.5^{abc}	612.8± 8^{b}	553.3±12.3^{c}	612.8±12.8^{abc}	603
SGR	0.73±0.01^{a}	0.71±0.04^{ab}	0.71±0.01^{ab}	0.74±0.13^{a}	0.70±0.02^{ab}	0.71±0.06^{ab}	0.

Table 3.5: Fatty acid profile of muscle from gilthead sea bream fed the experimental diets

		Diets					
Fatty Acids	**Initial**	**FO**	**70L**	**100L**	**70S**	**100S**	**50S50L**
14:0	5.53	5.54±1.14	3.63±0.41	2.12±0.22	2.18±0.5	1.64±0.14	1.63±0.06
14:1n-7	-	0.52±0.03	0.26±0.13	0.22±0.05	0.04	0.03±0.02	0.04
14:1n-5	-	-	-	-	0.07±0.02	0.04±0.03	0.05
15:0	0.56	0.64±0.11	0.37±0.06	-	0.23±0.05	0.2±0.02	0.19±0.02
15:1n-5	0.06	1.19±0.9	0.06±0.03	0.28±0.11	-	0.01±0.01	0.01±0.01
16:0ISO	0.05	0.44±0.32	0.13±0.08	0.11±0.1	0.11±0.04	0.1±0.06	0.07±0.06
16:0	22.97	21.66±2.4^{a}	15.13±1.9^{b}	12.93±1.43^{b}	14.96±0.52^{b}	13.55±0.92^{b}	12.87±0.75^{b}
16:1n-7	6.69	7.86±5.06	5.81±0.23	3.32±0.3	3.62±1.05	2.65±0.24	2.58±0.01
16:1n-5	0.2	0.11±0.02	0.05±0.01	0.07	0.09±0.03	0.07±0.01	0.07
16:2n-6	0.91	0.32±0.39	0.15±0.04	0.15±0.03	0.4±0.16	0.26±0.02	0.25
16:2n-4	0.45	0.86±0.56	-	0.49±0.12	0.24±0.05	0.19	0.19
17:0	-	0.73±1.04	0.93±0.08	0.42±0.11	0.41±0.15	0.28±0.03	0.27
16:3n-4	1.18	1.73±1.07	0.65±0.1	0.3±0.17	0.04±0.01	0.05±0.03	0.07
16:3n-3	0.15	0.34±0.3	0.07±0.04	0.15±0.07	0.08±0.03	0.03±0.03	0.04
16:4n-3	0.05	1.11±0.49	0.3±0.11	0.23±0.25	0.14±0.02	0.11±0.09	0.12±0.03
16:3n-1	0.95	0.28±0.3	-	0.03	0.1±0.14	0.12±0.05	0.12±0.02
18:0	5.2	4.9±0.89^{a}	4.35±0.67^{a}	4.38±0.44^{a}	3.35±0.24^{b}	3.45±0.52^{b}	3.39±0.2^{b}
18:1n-9	13.4	11.43±1.4^{c}	16.14±1.1^{b}	17.33±1.49^{ab}	20.45±1.96^{a}	21.16±1.44^{a}	20.31±0.18^{a}
18:1n-7	2.66	3.06±0.52	2.31±0.14	1.63±1.18	2.22±0.19	1.62±0.36	1.76±0.05
18:1n-5	0.09	0.28	0.16±0.11	0.06±0.02	0.12±0	0.08±0.07	0.05±0.07
18:2n-9	0.13	0.39	0.15±0.09	0.11±0.1	0.07±0	0.06±0.05	0.05±0.07
18:2n-6	10.81	3.68±0.55^{c}	10.92±0.5^{b}	13.45±0.1^{b}	29.58±5.01^{a}	27.86±5.2^{a}	25.05±0.12^{a}
18:2n-4	0.3	0.31±0.23	0.09	0.05± 0.04	0.09±0.05	0.05±0.02	0.06±0.01
18:3n-6	0.21	0.25±0.17	0.15±0.08	0.11±0.08	0.36±0.02	0.32±0.04	0.34±0.02
18:3n-4	0.22	0.3±0.2	0.6±0.09	0.45±0.37	0.16±0.1	0.04±0.04	0.06±0.03
18:3n-3	1.41	1.69±0.55^{d}	21.46±0.6^{b}	31±2.05^{a}	4.26±0.73^{d}	12.89±7.38^{c}	17.5±0.37^{c}
18:3n-1	-	1.25±0.28	-	0.21	0.43±0.14	0.25±0.22	0.47±0.02
18:4n-3	1.21	1.41±0.31	0.2±0.03	0.45±0.21	0.06±0.03	0.03±0.02	0.04
18:4n-1	0.26	0.14±0.09	0.11± 0.07	0.08	0.19±0.01	0.19±0.02	0.19±0.01
20:0	1.05	0.51±0.49	0.17±0.1	-	1.43±0.07	1.64±0.29	0.84±0.78
20:1n-9	0.9	1.41±0.13	0.95±0.06	0.66±0.35	0.1±0.01	0.09±0.01	0.9±1
20:2n-9	0.17	0.19±0.09	0.25±0.02	0.58±0.48	0.12	0.11±0.01	0.29±0.27
20:2n-6	0.04	0.11±0.08	0.17±0.11	0.19	0.6±0.14	0.58±0.13	0.34±0.22
20:3n-6	0.19	0.13±0.05	0.18±0.06	0.20±0.1	0.28±0.03	0.22±0.04	0.26±0.1
20:4n-6	0.76	1±0.24^{a}	0.53±0.1b	1.09±0.61^{a}	0.49±0.24b	0.35±0.1b	0.42±0.22b
20:3n-3	0.07	0.08±0.05	0.82±0.1	0.89±0.35	0.16±0.03	0.44±0.24	0.49±0.16
20:4n-3	0.63	0.92±0.53	0.61±0.13	0.35±0.07	0.4±0.15	0.35±0.05	0.19±0.27
20:5n-3	7.56	7.43±1.44^{a}	3.51±0.96^{b}	1.99±0.39^{b}	2.6±1.28^{b}	1.66±0.21^{b}	1.55±0.05^{b}
22:1n-11	0.82	0.66±0.27	0.31±0.27	0.22±0.19	0.88±0.04	0.45±0.48	0.71±0.44
22:4n-6	-	0.21±0.18	0.3±0.06	0.17±0.09	0.2±0.08	0.16±0.03	0.15±0.01
22:5n-3	3.02	3.68±0.49	1.76±0.54	0.99±0.2	1.46±0.61	0.94±0.09	0.86±0.01
22:6n-3	9.14	11.25±1.4^{a}	6.57±2^{ab}	4.68±1.02^{b}	7.22±2.2^{ab}	5.69±1.13^{b}	5.18±0.39^{b}
Saturates	35.31	33.98±5.6^{a}	24.47±2.8^{b}	19.85±2.13^{b}	22.57±1.38^{b}	20.76±0.83^{b}	19.19±1.81^{b}
Monoenoics	24.82	26.5±4.84	25.84±1.1	23.67±1.95	27.59±0.69	26.2±2.17	26.47±1.85
n-3	23.24	26.8±4.6^{bc}	35.3±4.5^{ab}	38.88±3.69^{a}	16.38±3.5^{c}	22.13±7.69^{bc}	25.97±0.34^{bc}
n-6	12.99	5.7±0.6	12.4±0.41	14.76±0.68	31.92±4.66	29.75±5.24	26.81±0.05
n-3 HUFA	20.42	23.36±4^{a}	13.28±3.7^{b}	7.89±1.35^{b}	11.84±4.21^{b}	9.08±1.35^{b}	8.27^{b}
n-3/n-6	1.79	4.89±0.98^{a}	2.85±0.47^{b}	2.7±0.33^{b}	0.53±0.19^{c}	0.79±0.36^{c}	0.97±0.01^{c}
EPA/DHA	0.83	0.66	0.53	0.43	0.35	0.29	0.3

*Different letters in the same line denote statistically significant differences (P<0.05).

Table 3.6: Fatty acid profile of liver from gilthead sea bream fed the experimental diets

		DIETS					
Fatty Acids	**Initial**	**FO**	**70L**	**100L**	**70S**	**100S**	**50S50L**
14:0	2.91	6.9±0.4	2.54±1.17	1.76±0.09	2.7±0.61	1.61±0.07	1.7±0.34
14:1n-7	-	0.02±0.02	0.03±0.01	0.02	0.03±0.01	0.02	0.02±0.01
14:1n-5	-	0.17±0.05	0.1±0.06	-	0.1±0.03	0.05	0.07±0.01
15:0	0.34	0.9±0.2	0.74±0.4	0.56±0.3	0.85±0.17	0.46±0.05	0.78±0.26
15:1n-5	0.05	0.03±0.01	0.03±0.01	0.02±0.01	0.03±0.01	0.02	0.03±0.01
16:0ISO	0.03	0.19±0.1	0.16±0.14	0.02±0.03	0.03±0.01	0.02	0.17±0.04
16:0	23.16	18.7±5.1	14.53±1.26	13.76±0.82	18.12±3.46	18.9±0.41	15.44±1.89
16:1n-7	4.48	7.5±3.6	4.03±1.53	2.62±0.08	4±1.04	2.49	2.45±0.18
16:1n-5	0.22	0.15±0.08	0.08±0.03	0.09±0.05	0.14±0.03	0.11±0.01	0.11±0.01
16:2n-6	0.55	0.4±0.14	0.44±0.14	0.33	0.42±0.1	0.28	0.28±0.02
16:2n-4	0.6	0.9±0.55	0.41±0.05	0.41±0.09	0.46±0.1	0.37	0.39±0.01
17:0	-	1.8±0.93	0.39±0.13	0.3±0.01	0.4±0.08	0.29	0.3
16:3n-4	0.64	0.1±0.07	0.17±0.03	0.16±0.02	0.04	0.03	0.11±0.01
16:3n-3	0.01	0.15±0.03	0.09±0.02	0.08±0.01	0.08±0.02	0.06±0.01	0.07±0.01
16:4n-3	0.12	0.05±0.04	0.02±0.01	0.01±0.01	0.03±0.04	0.01	0.01±0.01
16:3n-1	0.32	0.2±0.13	0.06±0.01	0.04±0.01	0.05±0.02	0.03	0.03±0
18:0	11.34	5.9±0.17	6.59±1.32	6.88±1.82	5.99±1.25	7.23±0.19	7.27±2.63
18:1n-9	14.89	14.64±5.4^{b}	17.36±0.73^{ab}	18.85±1.56^{ab}	24.58±2.2^{a}	23.78±0.31^{a}	23.43±2.18^{a}
18:1n-7	2.99	2.69±1.35	1.7±1.04	1.22±0.01	2.1±0.52	1.4±0.15	1.43±0.53
18:1n-5	0.09	0.17±0.03	0.11±0.02	0.08±0.01	0.18±0.01	0.16±0.01	0.14±0.02
18:2n-9	0.14	0.14±0.04	0.11±0.03	0.07±0.02	0.18±0.03	0.21±0.08	0.14±0.03
18:2n-6	9.21	5.19±2.82^{d}	11.67±1.86^{c}	13.23±0.56^{c}	23.86±4.71^{a}	26.57±0.14^{a}	20.71±1.18^{b}
18:2n-4	0.34	0.32±0.14	0.11±0.07	0.12±0.02	0.13±0.04	0.08±0.01	0.05±0.02
18:3n-6	0.28	0.29±0.01	0.39±0.11	0.21±0.01	0.74±0.26	1.6±0.22	0.93±0.42
18:3n-4	0.31	0.35±0.35	0.1±0.14	-	0.15±0.05	0.09	0.04±0.06
18:3n-3	1.02	4.09±4.4^{c}	22.98±4.46^{a}	25.47±2.81^{a}	2.85±0.37^{c}	3.02±0.04^{c}	12.76±0.7^{b}
18:3n-1	-	1.46±0.58	0.43±0.6	0.63±0.1	0.33±0.05	0.35±0.03	0.78±0.25
18:4n-3	0.61	0.13±0.08	0.67±0.86	0.05	0.05±0.02	0.03	0.03
18:4n-1	0.29	0.2±0.04	0.16	0.16±0.02	0.18±0.04	0.19±0.01	0.17±0.02
20:0	0.11	1.71±0.57	0.84±0.11	0.83	1.38±0.14	1.32±0.02	1.24±0.02
20:1n-9	0.77	0.18±0.04	0.11±0.03	0.1±0.02	0.13±0.02	0.1	0.11
20:2n-9	0.31	0.01±0.02	0.02±0.03	-	0.01±0.02	-	-
20:2n-6	0.05	0.16±0.02	0.12±0.02	0.12±0.03	0.19±0.03	0.27±0.03	0.18±0.03
20:3n-9	0.4	0.22±0.07	0.3±0.05	0.44±0.05	0.76±0.18	0.92±0.02	0.62±0.09
20:3n-6	0.31	0.21±0.05^{b}	0.16±0.02^{b}	0.17±0.01^{b}	0.34±0.08^{b}	0.56±0.05^{a}	0.3±0.05^{b}
20:4n-6	0.98	1.44±0.18^{a}	0.47±0.07^{b}	0.43±0.08^{b}	0.44±0.11^{b}	0.35±0.02^{b}	0.31±0.02^{b}
20:3n-3	0.11	0.55±0.61^{c}	1.17±0.31^{ab}	1.8±0.14^{a}	0.19±0.02^{c}	0.21±0.01^{c}	0.73±0.08^{c}
20:4n-3	0.77	0.88±0.24	0.64±0.03	0.64±0.02	0.33±0.07	0.28±0	0.2±0.29
20:5n-3	5.91	6.97±0.45^{a}	1.87±0.41^{b}	1.38±0.33^{b}	1.34±0.4^{b}	1±0.05^{b}	1±0.1^{b}
22:1n-11	0.41	0.53±0.05	0.39±0.11	0.3±0.06	0.75±0.05	0.57±0.02	0.67±0.1
22:4n-6	0.18	0.25±0.14	0.13±0.02	0.11±0.01	0.09±0.02	0.09±0	0.07±0.01
22:5n-6	0.21	0.03±0.05	0.03±0.05	0.06±0	0.03±0.01	0.03±0	0.04±0.01
22:5n-3	4.51	3.28±0.71	1.47±0.27	1.22±0.01	1.02±0.26	0.84±0.05	0.74±0.14
22:6n-3	10.04	9.83±3.91^{a}	6.08±0.30^{ab}	5.18±1.03^{ab}	4.19±0.72^{b}	3.99±0.21^{b}	3.94±0.54^{b}
Saturates	37.86	35.9±3.2	25.63±1.75	24.09±3.04	29.44±5.48	29.81±0.36	26.73±3.9
Monoenoics	23.9	26.08±0.1	23.94±3.6	23.36±1.66	31.95±0.96	28.6±0.46	28.39±3
n-3	23.1	25.93±0.5^{b}	34.99±4.57^{a}	35.83±4.08^{a}	10.08±1.37^{c}	9.44±0.37^{c}	19.48±0.29^{b}
n-6	11.77	8.06±3.08^{d}	13.59±1.81^{c}	14.98±0.61^{c}	26.68±5.06^{a}	30.4±0.46^{a}	23.26±0.82^{b}
n-3 HUFA	21.34	21.51±3.8^{a}	11.23±0.71^{b}	10.22±1.25^{b}	7.07±1.40^{c}	6.32±0.32^{c}	6.61±0.4^{c}
n-3/n-6	1.96	3.22±1.25	2.57±0.01	2.39±0.18	0.38±0.09	0.31±0.01	0.84±0.02

Table 3.7: Fatty acid profile of gills from gilthead sea bream fed the experimental diets

	Diets					
Fatty Acids	**FO**	**70L**	**100L**	**70S**	**100S**	**50S50L**
14:0	5.91±0.36	3.5±0.08	2.32±0.01	3.13±0.1	1.77±0.06	1.61±0.07
14:1n-7	0.15±0.02	0.08	0.06	0.09±0.01	0.05	0.06±0.01
14:1n-5	0.18±0.01	0.11	0.07	0.1±0.01	0.06	0.07±0.01
15:0	0.06±0.01	0.04	0.04	0.04±0.01	0.03±0.01	0.03±0.01
15:1n-5	0.66±0.08	0.43±0.03	0.34±0.02	0.34±0.02	0.34±0.02	0.36±0.08
16:0ISO	0.09	0.06	0.04	0.05	0.03	0.03
Me16:0	0.6±0.21	0.25±0.02	0.17	0.2±0.07	0.4±0.09	0.3±0.07
16:0	20.66±1.23^{a}	16.34±0.27^{bc}	15.06±0.45^{c}	17.18±0.99^{b}	17.42±0.08^{b}	14.45±1.08^{c}
16:1n-9	10.86±0.68	6.09±0.06	3.75±0.02	5.68±0.27	3.22±0.18	3.38±0.47
16:1n-7	0.2	0.11	0.07	0.12±0.01	0.08	0.07±0.01
16:1n-5	0.23±0.01	0.14	0.11	0.11±0.02	0.05±0.01	0.06±0.01
16:2n-6	1.56±0.01	0.75	0.44±0.01	0.64±0.02	0.43±0.03	0.39±0.05
16:2n-4	0.55±0.04	0.37	0.31	0.3±0.02	0.16±0.08	0.12±0.03
17:0	1.19±0.14	0.61±0.01	0.39±0.01	0.53±0.02	0.3±0.01	0.32±0.04
16:3n-4	-	0.07	0.06±0.04	0.01±0.01	0.01	0.06±0.01
16:3n-3	0.07±0.05	0.09	0.08	0.07	0.05	0.06±0.01
16:3n-1	0.38±0.44	0.31±0.04	0.2±0.09	0.24±0.09	0.54±0.06	0.43±0.08
16:4n-3	0.45±0.15	0.17±0.01	0.25	0.25±0.04	0.38±0.09	0.33±0.07
16:4n-1	0.17±0.05	0.04±0.03	0.1±0.09	0.04±0.02	0.08±0.02	0.06±0.01
18:0	4.7±0.79	4.59±0.17	4.98±0.09	3.62±0.46	4.73±0.09	4.59±0.62
18:1n-9	11.95±0.18^{c}	17.11±0.17^{b}	20.5±0.1^{a}	22.55±0.64^{a}	22.35±1.24^{a}	20.97±0.45^{a}
18:1n-7	3.48±0.01	2.31±0.02	1.85±0.01	2.68±0.15	2.35±0.04	2.21±0.28
18:1n-5	0.1±0.01	0.07	0.07	0.13	0.11±0.01	0.11±0.01
18:2n-9	0.05±0.02	0.03	0.03	0.05	0.05±0.01	0.11±0.01
18:2n-6	3.79±0.23^{e}	10.12±0.12^{d}	12.74±0.11^{c}	23.92±0.9^{b}	29.46±0.05^{a}	22.08±1.58^{b}
18:2n-4	0.37±0.02	0.2	0.12	0.14	0.06	0.08±0.01
18:3n-6	0.29±0.01^{b}	0.17^{c}	0.15^{c}	0.25±0.02^{bc}	0.29±0.05^{b}	0.4±0.05^{a}
18:3n-4	0.49±0.04	0.24	0.14	0.2±0.02	-	0.04±0.04
18:3n-3	1.51±0.07^{d}	18.88±0.52^{b}	24.03±0.31^{a}	3.17±0.4^{d}	3.28±0.12^{d}	14.56±0.77^{c}
18:3n-1	0.96±0.18	0.48±0.02	0.32±0.01	0.38±0.06	0.18±0.02	0.41±0.08
18:4n-3	0.16±0.03	0.09	0.05	0.06±0.01	0.02±0.02	0.01±0.02
18:4n-1	0.21±0.01	0.19±0.01	0.21	0.21	0.25±0	0.23±0.03
20:0	-	-	-	0.27±0.01	0.14±0.1	0.18±0.13
20:1n-9	0.71±0.15	0.68±0.11	0.82±0.02	1.6±0.1	1.15±0.18	1.31±0.3
20:1n-7	0.18	0.11±0.01	0.1	0.13	0.04±0.05	0.1±0.02
20:2n-9	0.1±0.02	0.07	0.06	0.09±0.01	0.07±0.01	0.1±0.01
20:2n-6	0.15±0.01^{d}	0.24±0.01^{cd}	0.3±0.01^{c}	0.54±0.02^{b}	0.8±0.03^{a}	0.61±0.07^{b}
20:3n-6	0.18±0.01	0.1±0.01	0.08	0.17±0.03	0.22±0.05	0.24±0.03
20:4n-6	2±0.43^{a}	0.95±0.05^{b}	0.49±0.02^{b}	0.56±0.12^{b}	0.78±0.2^{b}	0.6±0.09^{b}
20:3n-3	0.12^{c}	0.72±0.04^{b}	0.94±0.01^{a}	0.13±0.01^{c}	0.15±0.01^{c}	0.62±0.07^{b}
20:4n-3	0.84±0.13	0.48±0.01	0.34	0.33±0.05	0.18±0.01	0.35±0.06
20:5n-3	6.98±0.57^{a}	2.99±0.1^{b}	1.52±0.06^{c}	1.94±0.27^{c}	1.2±0.16^{c}	1.33±0.26^{c}
22:1n-11	0.26±0.04	0.24	0.27±0.01	1.08±0.13	0.69±0.07	0.86±0.1
22:1n-9	0.19	0.22	0.32±0.02	0.48±0.02	0.3±0.03	0.38±0.05
22:5n-6	0.33±0.03	0.16	0.1	0.12±0.01	0.05±0.04	0.06±0.04
22:4n-6	0.43±0.02	0.23±0.01	0.13	0.14±0.02	0.14±0.03	0.13±0.02
22:5n-3	2.83±0.46	1.55±0.06	0.81±0.01	1.1±0.19	0.68±0.07	0.68±0.15
22:6n-3	12.66±0.71^{a}	7.22±0.22^{b}	4.69±0.08^{c}	4.84±0.58^{c}	4.84±0.76^{c}	4.47±0.89^{c}
Saturates	32.53±1.63^{a}	25.08±0.52^{b}	22.78±1.61^{b}	24.76±1.72^{b}	24.41±0.09^{bc}	21.18±1.23^{c}
Monoenoics	29.15±0.96	27.71±0.39	28.31±0.62	35.1±1.37	30.8±1.99	29.93±1
n-3	25.63±1.74^{b}	32.18±0.9^{a}	32.71±1.9^{a}	11.89±1.59^{c}	10.78±1.46^{c}	22.41±2.07^{b}
n-6	8.55±0.19^{d}	12.62±0.06^{c}	14.36±0.37^{c}	26.17±0.93^{b}	31.95±0.39^{a}	24.27±1.66^{b}
n-9	23.85±0.97^{c}	24.2±0.33^{c}	25.47±0.58^{bc}	30.45±1.2^{a}	27.14±1.84^{b}	26.24±0.48b^{c}
n-3 HUFA	23.43±1.66^{a}	12.95±0.5^{b}	8.31±0.56^{c}	8.35±1.29^{c}	7.05±1.24^{c}	7.45±1.71^{c}
n-3/n-6	3±0.23	2.55±0.07	2.28±0.08	0.45±0.05	0.34±0.04	0.92±0.13

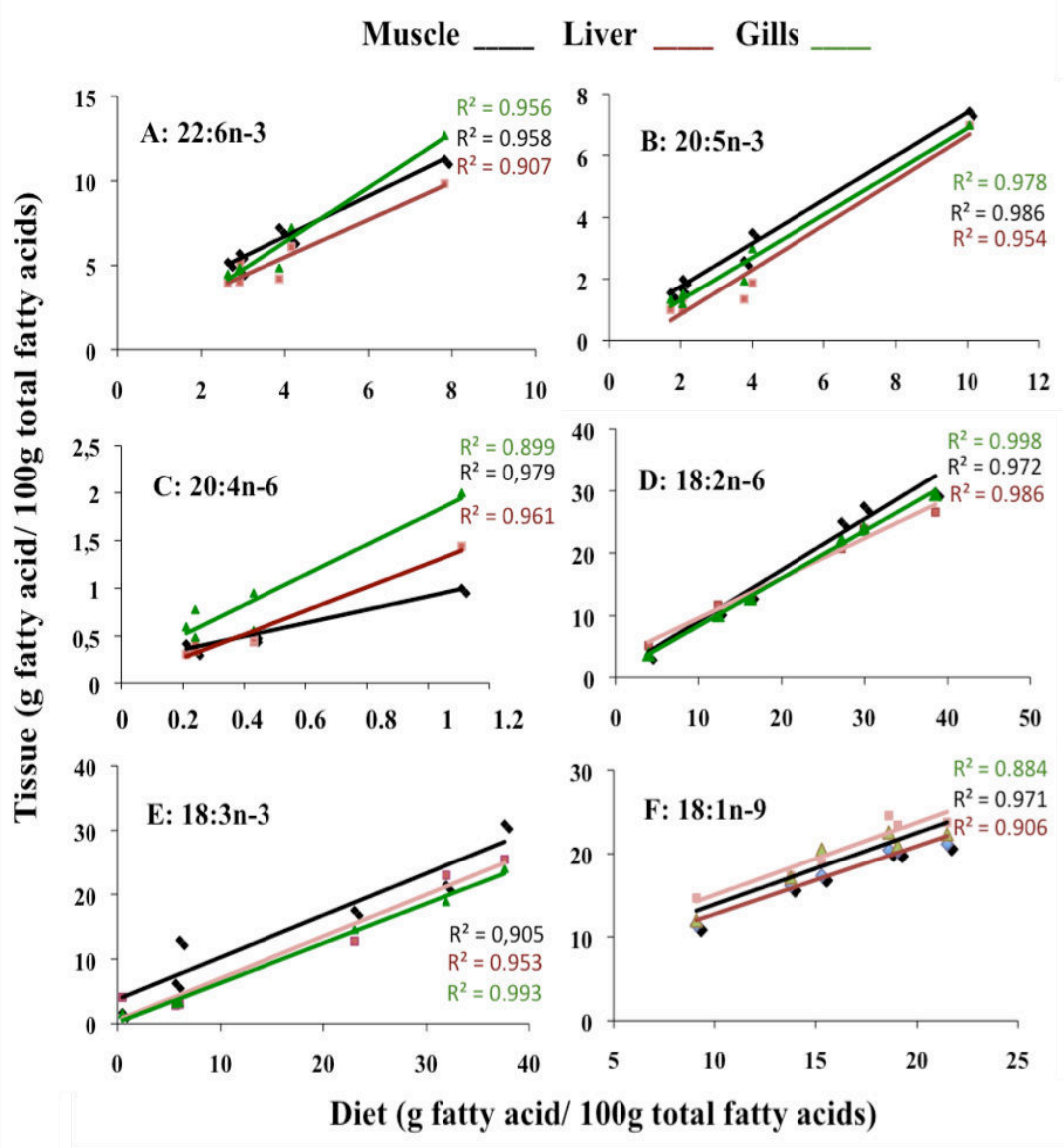

Figure 3.2: Relationship between dietary fatty acid concentrations and liver fatty acid concentrations of 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 in total lipids of gilthead seabream fed either 100% FO, 70L, 100L, 70S, 100S or 50S50L.

Discussion

The present study was focused on the development of cost-effective feed supplies, utilising alternatives to FO that could be used in the sustainable production of aqua feeds without compromising fish growth performances. The results showed that substituting FO with up to 70% or more with LO and SO as the only oil or as a blend of oils significantly decreased fish final weight, except for the 70S treatment that showed no difference to the control. Previous studies with seabream showed that it is possible to substitute up to 60% of FO by rapeseed oil, LO or SO without affecting fish growth and feed utilization, and that 80% substitution decreased fish growth performances (Caballero *et al*., 2002; Montero *et al*., 2003; Izquierdo *et al*., 2003, 2005). The present study, feeding seabream with intermediate substitution levels of 70% or more of its dietary oil as VO significantly decreased growth and CI. These results confirmed again the ability of gilthead sea bream to accept VOs in their diets in comparison with other marine fish species (Regost *et al*., 2003a,b; Izquierdo *et al*., 2005).

The present study was unusual in using a diet with lower lipid content (around 18%) compared to the other previous seabream studies (Caballero *et al*., 2002; Montero *et al*., 2003, 2005; Izquierdo *et al*., 2005) that used up to 22%. Consequently the total n-3 HUFA provided in diets for the present study are supposed to be lower, while the role of these essential fatty acids in different fish species at different stages of fish development is well documented (Watanabe *et al*., 1982; Izquierdo, 1988; 1996, et al., 2000; Sargent *et al*., 1999). Furthermore, FO and FM used in the present study were from south-American origin known to be higher in n-3HUFA, especially EPA, but also DHA, total saturated fatty acids, and lower MUFA content in comparison to those from the northern hemisphere origin (Allen, 1995). For instance, southern hemisphere fish oils can deliver similar levels of n-3HUFA at lower inclusion levels than the northern hemisphere fish oils (Sargent *et al*., 2002). The VOs used in this study are rich in C18 fatty acids, n-3 (mainly LNA) and n-6 (mainly LA) fatty acids, in LO and SO respectively. The inclusion of these oils alone (separately) or as a blend changed the n-3/n-6 input in the diet and consequently changed their incorporation in different fish tissues. Thus, there was a positive correlation between fish final weight and the dietary n-3/n-6 ratio with r^2=0.66, the same positive correlation was observed between final weight and the n-3/n-6 ratio in muscle, liver and gills with r^2= 0.71, 0.63 and 0.62, respectively. Therefore, care should be taken when substituting FO with plant oils, to maintain the correct n-3/n-6 derived from the different oils in order to assure good

growth performances. Although, Montero and colleagues (2008) have demonstrated that fish fed VOs in the present experiment have decreased phagocytic activity, reduced serum alternative complement pathway activity and elevated basal constitutive levels of Mx transcript expression in the liver.

Previous studies in seabream have demonstrated that fatty acid compositions reflect the dietary fatty acid composition (Montero *et al*., 1996; 2003; 2005, 2008; Izquierdo *et al*., 2000; 2003, 2005). This was clearly shown in the present study, in muscle, liver and gills. Specifically, in muscle and liver, positive correlations between dietary and tissue FA concentration were shown for 18:2n-6, 18:3n-3, 18:1n-9, 20:4n-6, 20:5n-3 and 22:6n-3 (Figure 3.2). In agreement with these studies, the linear correlation obtained in the present trial revealed differences between the relationships of dietary and tissue FA for each individual FA. In particular, EPA and DHA were present in higher concentrations in muscle compared to diet in all the treatments. In line with previous studies, these suggest that when these FA were provided to the fish in low concentrations they were selectively retained in the tissues (Bell *et al*., 2004; Montero *et al*., 2005). The differences in retention and utilisation of specific FA in muscle and liver are related to the different functions of FA in the two tissues. Liver is the primary lipid storage organ in marine fish and this is largely FA stored as triacylglycerols while the muscle tissue has most of its FA contained in membrane phospholipids. Since HUFA, especially EPA and DHA, are vital for the function of cell membranes the retention of these HUFA in phospholipids is generally more efficient than in triacylglycerols (Izquierdo et al., 2000; Sargent *et al*., 2002).

HUFA levels significantly decreased in flesh, liver and gills from fish fed VO compared to the control fish. Fatty acids deposited in the flesh reflected those ingested in the diet. However, in particular, DHA and ARA levels in flesh were higher than in diet, indicating a selective deposition and retention. DHA is known to be more abundant in polar lipid of flesh (Izquierdo *et al*., 2005) with the content being double that of EPA. The possible mechanisms underlying this selective deposition include the high specificity of fatty acyl transferases for DHA and the relative resistance of DHA to β-oxidation stemming from the complex catabolic pathway for this HUFA (Frøyland *et al*., 2000). In spite of this selective retention of DHA, the contribution of the desaturase and/or elongase activity upon its precursor (LNA) is very limited and, in consequence, DHA and also EPA are essential and must be provided by the diet (Watanabe *et al*., 1989; Watanabe, 1993; Ibeas *et al.*, 1994; Izquierdo, 1996, *et al*., 2000; Montero *et al*.,

1998). Indeed, fish fed VOs showed a lower EPA in fillet than in the diets provided, indicating that this fatty acid is not subject to specific retention. The same pattern was found in previous studies and the authors suggested a preferential oxidation of EPA over DHA in the muscle (Izquierdo *et al*., 1989a,b, 2005), since the mitochondrial oxidation is predominant in the muscle (Froyland *et al*., 2000) and EPA had a high affinity with this type of oxidation (mitochondrial beta-oxidation) (Madsen *et al*., 1998). In addition, it has been demonstrated that DHA inhibits EPA incorporation into phosphatidylcholine and phosphatidylethanolamine in sea bream larvae (Izquierdo *et al*., 2000). Consequently, the fillet nutritional quality could be affected by high levels of VO inclusion, due to the decrease of n-3 HUFAs, that are essential nutrients for human health (Simopoulos, 1999; Sargent *et al*., 2001).

Humans are not very efficient at synthesizing very-long-chain PUFAs and it has been postulated that this is because they have evolved with diets abundant in fish and game meats, which are quite rich in these types of fats (Cordain *et al*., 2000, 2005). It is thought that a deficiency of n-3 HUFA, especially EPA and DHA found in FO, can be linked to many inflammatory diseases of the developed world, such as cardiovascular disease and inflammatory disorders such as arthritis (Hibbeln *et al*., 2006). Furthermore, DHA has been recognized as being vitally important for neural function, and a deficiency of this HUFA has been linked to depression, cognitive disorders, and mental illness (Conklin *et al*., 2007).

In turn, C18 fatty acids are selectively deposited in flesh, when present at high concentrations in the diet (Bell *et al*., 2001, 2002, 2003; Caballero *et al*., 2002; Izquierdo *et al*., 2003, 2005, 2008). This suggests that the monoenes, mainly 18:1n-9, as well as LA and LNA, are readily oxidized when present at high concentrations (Mourente and Bell, 2006). In muscle, liver and gills, 18:2n-6 and 18:3n-3 were found in much lower concentrations than in the diet when the fish were fed the VO diets, indicating that when these FA were abundant in the diet they were selectively utilized for metabolism, probably for energy production (Hendersen and Sargent, 1985; Stubhaug *et al*., 2006). Interestingly, the concentration of 18:1n-9 in liver and gills was higher than in the diet, suggesting a preferential retention, whereas in muscle presented a higher content than in the diet only for fish fed 70S and 100S. The selective retention of this FA in seabream, rather than mobilization for catabolism, may reflect its structural function in membrane phospholipids where it is often found in the *sn*-1

position of phospholipids with HUFA being favoured in the *sn*-2 position (Bell and Dick, 1991).

LO is rich in 18:3n-3 and previous studies have shown an increased tissue content of this fatty acid and total n-3 when it is included in the diet (Izquierdo *et al*., 2003, 2005; Menoyo *et al*., 2007). In the present study, feeding seabream with diets containing increasing levels of LO significantly increased their content of 18:3n-3 in muscle, liver and gills in a parallel manner. This fatty acid has been demonstrated to be beneficial for human nutrition by improving many physiological functions (Ferretti and Flanagan, 1996; Newcomer *et al*. 2001; Maillard *et al*., 2002; Brouwer *et al*., 2004), including cardioprotective effects, modulation of inflammatory response, and a positive impact on both central nervous system function and behaviour (Stark *et al*., 2008). In addition, it is shown that this fatty acid reduced the number of cardiac deaths by 70% in Mediterranean consumers (De Lorgeril *et al*., 1994, 1999) and it is also used in livestock feeding (Sanderson *et al*., 2002). While SO contains high levels of LA, and is readily available at a favourable price, several authors claim that higher levels of n-6 fatty acids present in some VOs can unbalance the dietary n-3/n-6 ratio and induce arrhythmias which subsequently can lead to cardiac dysfunctions (Jouven *et al*., 2001; Leaf, 2001); Thus, its inclusion in diets for fish should be limited due to the greater reduction of the n-3/n-6 ratio that arises when using the product. In the present study, different tissues from seabream fed SO showed a linear deposition of n-6, mainly LA, with increasing SO inclusion level with a high positive correlation (Figure 3.2). Thus, tailoring the fatty acid composition of farmed fish to suit consumer preference, particularly from the point of view of supplying healthy food, was considered when substituting FO with VOs in diet for marine fish. Previous studies have shown that feeding seabream with diets containing a higher content of LA or LNA rich oils, significantly increased the contents of these fatty acids in the flesh and consequently reduced its quality for the consumer (Bell *et al*., 2001, 2002, 2003; Caballero *et al*., 2002; Montero *et al*., 2003, 2005; Izquierdo *et al*., 2003, 2005).

In fish fed SO and LO, the muscle, liver and gills presented higher levels of products of Δ6 desaturation and elongation from their C_{18} precursors, including 20:2n-6 and 20:3n-6, in SO fed fish and 20:3n-3 in LO fed fish, suggesting a more efficient elongase than desaturase activity, specially for n-3 series. This was also found in another study (not published data) showing higher delta 6 desaturase activity in fish fed LO comparing to rapeseed oil or FO diet in several tissues such as intestine, liver but

with lower levels in the muscle, denoting the important roles of the first 2 organs in lipid metabolism. 20:3n-3 was 9 fold higher in fish fed LO compared to the control, while 20:2n-6 was only increased 5 fold, while 20:3n-6 was only increased 2 fold, which is in accordance with previous studies (Tocher *et al*., 2000; Menoyo *et al*., 2005). Numerous mammalian studies have established that a fatty acid can compete and influence the metabolism of another fatty acid (Garcia and Holman, 1965). In addition, the n-3 PUFA are known to be usually more effective in inhibiting the metabolism of n-6 PUFA than *vice versa* (Horrobin, 1991). The same effects were found in previous studies analysing different tissues from the same species (Montero *et al*., 2005; Ganga *et al*., 2005; Izquierdo *et al*., 2003, 2005) denoting the stimulation effect of VOs in increasing the fatty acyl desaturation/elongation pathway (Bell *et al*., 2002; Tocher *et al*., 2002, 2003). Therefore, in terms of product nutritional quality, there is a marked effect of feeding VOs on seabream flesh quality expressed by its decreased content of n-3 HUFAs.

Liver PUFA composition was affected by VO diets following a similar but more extreme pattern than in the fish fillet (Montero *et al*., 2003; Izquierdo *et al*., 2003). There was a selective retention of 16:0 and 18:1n-9 in VO treatments, which could be considered as a lipid storage depot associated with reserves of metabolic energy rather than an immediate synthesis of cell membrane phospholipids (Sargent *et al*., 2002). Indeed, 16:0 and 18:1n-9 are the preferred substrates for β-oxidation in fish (Henderson and Sargent, 1985, Torstensen *et al*., 2000). In addition, 18:1n-9, LA and LNA have always shown lower concentrations in liver than in the diet (Bell *et al*., 1994, 2001, 2003; Caballero *et al*., 2002; Izquierdo *et al*., 2003, 2005) denoting their ready oxidation when present at higher concentrations. As in flesh there is an increasing amount of 20:2n-6, 20:3n-6 and 20:3n-3 in livers from fish fed VOs, denoting again the presence of the desaturase and elongase activities in this metabolic tissue. The decreased content of EPA and ARA when feeding VO, even when the levels of 20:3n-3 and 20:3n-3 are increased, may indicate that the Δ5 desaturase activity is very low if not absent in marine fish (Ghioni *et al*., 1999; Tocher and Ghioni, 1999; Seiliez *et al*., 2003; Izquierdo *et al*., 2008).

Gill fatty acids profiles showed the same parallel effects caused by the dietary inputs with some exceptions, e.g. ARA content was 2 fold higher compared to the other tissues. This organ has many vital physiological functions such as respiration and osmoregulation. Thus, the fatty acid composition of this organ is determinant for its

optimal function. ARA is known to be the main eicosanoid precursor of highly active hormones with many physiological functions (Horrobin *et al*., 1991; Bell *et al*., 1994). Saturates were decreased in gills from fish fed different VOs compared to FO, while MUFA were not significantly affected. Fish gill is a multifunctional organ that, in addition to providing aquatic gas exchange, plays dominant roles in osmotic and ionic regulation, acid-base regulation and excretion of nitrogenous wastes (Evans *et al*., 2005) Thus all these functions need energy, largely from fatty acids, and especially saturates and MUFA are considered as preferable for oxidation and energy production, as 16:0, 16:1 and 18:1n-9 have been demonstrated to be preferred substrates for β-oxidation (Keissling, 1993; Henderson, 1996; Frøyland *et al*., 2000). In this experiment, MUFA and saturates were more deposited in gills in comparison to the other tissues, suggesting the role of these fatty acids might be to cope with the high energetic demands of this organ. It is fairly well established that the ability of salmon to osmoregulate is directly related to their diet and this may be mediated through changes in gill polar lipid compositions, in particular ARA and EPA levels and the resultant effects on prostaglandin production (Tocher *et al*., 2000).

Some scientists suggested that the retention efficiencies for the n-6 EFAs are lower than n-3 fatty acids, and it is directly related to the quantities of n-6 EFAs present in the diet. This suggests that the catabolic degradation of n-6 EFAs was relatively lower when they were supplied in larger amounts via vegetable oils (Bendiksen and Jobling, 2003) and this is in accordance with our results where we observed that the incorporation of LA in muscle was slightly lower in 100SO compared to 70SO diets (Table 3.5). The C18 fatty acids were incorporated more in the muscle compared to the other tissues. Feeding seabream with VOs in previous studies has demonstrated that the flesh content of n-3HUFA could be re-established by a finishing diet with FO for a period of 3 months before harvesting (Izquierdo *et al*., 2005). Nevertheless, the lower retention of n-6 fatty acids in comparison with n-3 EFAs may be indicative of higher rates of oxidation of the n-6 fatty acids relative to n-3 fatty acids. The oxidation of DHA in fish tissues is low, whereas LA is oxidized more readily (Henderson, 1996). In addition, lipids that contain HUFAs are generally considered to be more easily digested and absorbed than those containing less-unsaturated fatty acids (Henderson and Tocher, 1987; Higgs and Dong, 2000; Johnsen *et al*., 2000). As such, it is also possible that the lower retention efficiency of the n-6 EFAs could also result from reduced digestion and absorption in comparison with n-3 EFAs (Bendiksen and Jobling, 2003).

Tissue lipids of seabream are readily influenced by the fatty acid composition of the diet. The present study clearly establishes linear correlations between the fatty acid compositions of diets, and their incorporation in flesh and liver (Figure 3.2). These correlations are of practical use for predicting outcomes of feeding different blends of a given substituting oil in place of fish oil. They also reveal how different fatty acids in dietary lipid are selected for or against retention relative to tissue lipids. Thus, when substituting FO with VOs in diets for gilthead seabream, care should be taken to choose the correct blend of VOs in order to maintain the adequate n-3/n-6 levels and guarantee the final nutritional quality of the flesh. Given present concerns about imbalance of n-6 and n-3 PUFA in the diets of developed nations and the encouragement to consume oily fish, such as mackerel, sardines, salmon and trout, it is important that cultured seabream maintain a high level of essential (n-3) HUFA in the edible flesh.

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Chapter 4

Stress response in seabream (*Sparus aurata*) held under crowded conditions and fed diets with different levels of inclusion of linseed and/or soybean oil

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Stress response in seabream (*Sparus aurata*) held under crowded conditions and fed diets with different levels of inclusion of linseed and/or soybean oil

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Abstract

The physiological response to stressors in fish, including hormonal profiles and associated tissue responsiveness, are less documented. The aim of this study was to evaluate the effect of feeding gilthead seabream (*Sparus aurata*) with diets containing linseed oil (LO) and soybean oil (SO) as a substitute for fish oil (FO) and their effect on fatty acid profile of head kidney and the consequent effect on stress response to a crowding test. Fish were fed with different diets with different levels of substitution 0% (FO), 70% (70LO, 70SO, 20LO50SO and 50LO20SO) and 100% (100LO, 100SO and 50LO50SO) over a period of 8 months. At the end of the feeding trial, samples for head kidney biochemical analysis were collected and the fish were challenged by a crowding test. Samples of plasma for cortisol analysis were collected at different times during the test, 0h, 2h, 5h, 24h, 48 and 1 week. Basal cortisol levels were significantly increased in fish fed 70LO, 100LO and 50LO50SO. The physiological response to crowding was significantly affected by the diet. After 2 h of crowding, all the treatments showed higher cortisol, with fish fed 100LO significantly different with the maximum response of 131.38 pg/ml compared to 50LO20SO which had the lowest plasma cortisol response with only 18.73 pg/ml. After 5h and 24h, plasma cortisol was reduced in all treatments except for 50LO20SO. After 48 h of crowding, the plasma cortisol was increased in all treatments with the maximum value seen in fish fed 100LO (72.12 pg/ml). These values were decreased after 1 week in fish fed FO, 70LO, 100LO and 50L050SO after 1 week of crowding, but remained higher in fish fed 70SO, 100SO, 20LO50SO and 50LO20SO. In general, fish fed with LO were characterized by a faster and stronger stress response and rapid recuperation, while fish fed SO had a slow response and a longer recuperation.

Keywords: Seabream, stress, cortisol, fatty acids, head kidney, linseed oil, soybean oil.

Introduction

Finfish aquaculture has traditionally used diets containing large amounts of fish meal (FM) and fish oil (FO), primarily as a cost-effective source of highly digestible animal protein and lipids. Moreover, FO is an important dietary source of omega-3 fatty acids, and in particular the long chain polyunstaurated fatty acids (PUFA), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Ackman, 1989; Henderson and Tocher, 1987). In particular, FO is important in fish nutrition as it is used as the main source of these essential fatty acids (EFA) required for fish to ensure optimal growth, development and reproduction (Izquierdo *et al*., 1989; Sargent *et al*., 1999). The steady increase in global production volume in aquaculture of 8-10% a year (Tacon, 2004, 2006) has resulted in increasing prices and limited availability of these ingredients in the market. Consequently, it is expected that the demand for FO by aquaculture will probably exceed available resources over the next decade (Pike and Barlow, 2003; Tacon, 2004, 2005). Therefore, recently much interest has focused on researching alternatives to FO and FM in aquafeeds, and many studies have reported the successful use of vegetable oils (VO) and vegetable proteins as good substitutes without compromising fish growth or feed utilization (Bell *et al*., 2001, 2002; Torstensen *et al*., 2000; Caballero *et al*., 2002; Montero *et al*., 2003, 2005; Izquierdo *et al*., 2003, 2005).

VOs are devoid of C20 n-3 PUFA, including EPA and DHA while the levels of C18 PUFA, linoleic acid (LA; 18:2n-6), linolenic acid (LNA; 18:3n-3) and monounsaturated fatty acids (MUFA; mainly oleic acid OA, 18:1n-9) are high in VO. Thus, including VOs in diets for marine fish have been found to lead to increased C18 PUFAs and reduced n-3 HUFA in the flesh of the fish, potentially compromising the nutritional quality to consumers (Bell *et al*., 2001, 2002; Torstensen *et al*., 2000; Montero *et al*., 2003, 2005; Izquierdo *et al*., 2003, 2005). In addition, modifying the fatty acid profiles of fish tissue by reducing its HUFA content could have many physiological and metabolic impacts in fish; since these components have important roles in metabolism, cannot be synthesized de *novo* and, therefore, must be obtained from the diet (Ghioni *et al*., 1999). Alteration of the phospholipid composition may induce structural modifications that in turn modulate the functional properties of the membranes (Wills, 1985; Christon *et al*., 1992), indicating the fundamental relationship between nutrition and animal's responses to its environment.

PUFA/HUFA are membrane components, sources of energy and cell mediators

that are essential for cell structure and function. It is well documented that dietary fat can influence the fatty acid compositions of different fish tissues, including those of cell membranes (Bell *et al*., 2001, 2002; Izquierdo *et al*., 2003). They may alter various properties of the membranes such as integrity, fluidity, permeability, distribution and numbers of receptor sites (Yaqoob, 1998). HUFAs are also precursors of a range of highly active C20 compounds called eicosanoids, that are formed in small or even trace amounts by virtually every tissue of the body and involved in a wide variety of physiological functions (Bell *et al*., 1991). In broad terms they are produced in response to stressful situations, both at cellular and whole body level. In fish, HUFA are reported to modulate stress response (Montero *et al*., 1998, 2003; Tago *et al*., 1999). Whereas, the mechanisms by which HUFA could modulate fish resistance to stress are still unknown, some recent studies have suggested that these effects could be mediated partly by eicosanoids (Koven *et al*., 2001a,b; Van Anholt *et al*., 2004a,b; Ganga *et al*., 2006). Thus, incubating head kidney tissue with different HUFA in an in vitro superfusion system affected significantly their ACTH-stimulated cortisol release, and this effect was partly mediated by cycloxygenase (COX) and lipoxygenase (LOX) metabolites (Ganga *et al*., 2006).

There is a trend towards increased concern in animal welfare and, recently, this concern has expanded to include the welfare of fish. High densities are generally used in intensive aquaculture compared to the wild fisheries (Turnbull *et al*., 2005) to improve business profitability. Generally this practice is considered as a potential source of stress, with a negative effect on fish growth rate and welfare (Lefrançois *et al*., 2001; Ellis *et al*., 2002; Van de Nieuwegiessen *et al*., 2008, 2009), survival and feeding rate (Rowland *et al*., 2006). In teleosts, the stress response comprises of a number of physiological processes, which are largely regulated by the hypothalamus-pituitary-interrenal (HPI) axis. Following exposure to a stressor, the hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates the pituitary to secrete adrenocorticotropin hormone (ACTH). Binding of ACTH to membrane receptors of the steroidogenic cells activates a series of enzymatic reactions using cholesterol as a substrate for the synthesis of cortisol (Patiño *et al*., 1986; Lacroix and Hontela, 2001). Cortisol production is under the control of the HPI axis activation (Wendelaar Bonga, 1997; Mommsen *et al*., 1999) and is considered a key response to stress, in order to cope with the physiological changes and to recover homeostasis. Several studies have reported a relationship between the stress response in fish under

crowded conditions and increased plasma-cortisol levels (Montero *et al*., 1999; Ruane and Komen, 2003; Trenzado *et al*., 2006; Van de Nieuwegiessen *et al*., 2008, 2009).

Although plasma cortisol response to stressors is well established in teleosts, the magnitude and duration of this steroid response is dependent upon the type, intensity and duration of the stressor as well as the history of the animal (Wendelaar Bonga, 1997; Iwama *et al*., 2006) as well as the feeding history being crucial in determining the stress response. Under conditions of acute stress, cortisol elevation usually lasts hours, but with chronic stress, such as prolonged confinement, values may remain high for days or weeks (Pickering and Pottinger, 1989; Iwama *et al*., 2006), only returning to basal values over considerable time (Pickering and Stewart, 1984).

The aim of the present study was to contribute to our understanding of how feeding lipid from vegetable sources (LO and SO), rich in C18 fatty acids (mainly LA, LNA and OA), could affect fatty acid metabolism and their incorporation in head kidney tissue and their consequent effect on stress resistance caused by crowding. This was studied by measuring the plasma basal and post-stress cortisol levels.

Materials and Methods

Fish and Diets

Two thousand two hundred and fifty juveniles of gilthead seabream (*Sparus aurata*) (45 g initial body weight) were distributed in 45 tanks of 500 l (50 fish/tank, each diet assayed in triplicate) supplied with seawater at a temperature ranging from 20-24.2 ºC and constant aeration. Five iso-energetic and isonitrogenous diets with lipid content ~22 % were formulated. Anchovy oil was the only added lipid source in the FO diet (fish oil). In the other diets, fish oil was replaced by linseed (LO) or soybean oil (SO) oils at 70% (70LO and 70SO, 20LO50SO, 50LO20SO) or 100% (100LO, 100SO and 50LO50SO). Fish were fed the experimental diets containing different ingredients (Table 4.1) until apparent satiation (3 times/day, 6 days/ week) for 240 days, when they reached commercial market size.

Table 4.1: Ingredients of the experimental diets used

	% of dry weight
Oils (Fish oil[a]/linseed/soybean)	16.32
South-American fish meal	47.26
Wheat	7.00
Soybean meal 47%[b]	25.00
Sunflower meal	3.67
Vitamins premix[c]	0.27
Minerals premix[c]	0.48

a South-american, anchovy oil.
b Soybean meal with 47% as a brut protein, "no GMO"
c Vitamin and mineral premixes prepared according to Proaqua A/S commercial standards.

Biochemical analysis

Extraction of total lipid from diets and fish head kidney was performed by the method of Folch *et al*. (1957) using a mixture of chloroform: methanol (2:1)(v:v) containing 0.01% BHT, as an antioxidant, followed by phase partition with KCl (0.88 %). Vigorous vortex mixing followed by centrifugation to assist separation of chloroform and aqueous layers extracted the lipids from diet and head kidney samples. The lower layer was filtered through Whatman filter paper and dried under nitrogen, total lipids were weighed following desiccation.

Fatty acid methyl esters were produced from aliquots of total lipids extracted from different samples by acid-catalyzed transmethylation performed overnight at 50°C as described by Christie (1982). Fatty acids methyl esters were separated and quantified by gas-chromatography (GC; Thermo Finnigan) with He as a carrier gas using a fused silica, carbowax 20M, 30m * 0.32 mm i.d. (df=0.27 m) column (Supleco, Bellefonte, USA). The initial temperature of the column was set to 170ºC for 10min, and then it was raised to 220ºC at 2.5 ºC/min and finally maintained at 215ºC for a further 5min. The temperature of the injection port was 250ºC. The peaks in the chromatogram were identified by comparison to a well-characterised external standard (Sigma). Individual methyl esters were identified by comparison with known standards and published data.

Stress Panels

After the feeding period, 20 fish per tank were exposed to confinement stress. Thus, fish were distributed in small floating cages (50x30x15cm) in 5 groups of 4 fish,

another 4 fish per tank were used as control fish for basal plasma cortisol determination just before confinement. Subsequently, all fish from different cages were sampled at 5 different time intervals (2h, 5h, 24h, 48h and 1 week) after the beginning of confinment to encompass "early" and "late" response to the stressor. At each sampling time one cage was carefully removed from each tank, opened and fish blood was sampled in less than 2 min of handling time, blood was obtained by caudal sinus puncture with a 1 ml plastic syringe. Aliquots of blood samples were immediately transferred to an Eppendorf tube coated with lithium heparin as anticoagulant. The plasma was obtained by centrifugation at 3000 rpm for 10 min and stored at -80º C prior to cortisol determination and then the fish were liberated. The same procedure was repeated after 5h, 24h, 48h and 1 week. This approach was taken to keep any acute disturbance to the remaining fish, from fish removal, as low as possible; both density groups were treated in the same manner. Thus, total capture time was less than 8 min per tank to minimize capture stress effects on analyzed parameters (Sumpter, 1997).

Cortisol measurements

Cortisol concentration in the perfused fluid was determined by radioimmunoassay (RIA) (Rotllant *et al*., 2001). The antibody, Biolink, S.L. (Costa Mesa, California), was used at a final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11.40% with 21-desoxycorticosterone, 8.90% with 11-desoxycortisol and 1.60% with 17a-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels are given as ng/ml of plasma.

Statistical analysis

Significance of difference ($P<0.05$) between dietary treatments was determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparison tests (Sokal and Rolf 1995). Analyses were performed using SPSS software (SPSS for windows 13).

Results

Lipid peroxidation products were determined as thiobarbituric acid reactive substances (TBARS) and they showed no significant difference between the diets, ranging between 8.56 and 3.85 mmole of malonaldehyde (MDA)/kg of wet diet. No dietary effect was observed on TBARS concentration ($P<0.05$).

The control diet (FO), formulated with 100% FO, contained approximately 37% total saturates, mainly 16:0, almost 29% total monounsaturated fatty acids with approximately one-third as 18:1n-9, 6 % n-6 fatty acids, predominantly 18:2n-6, and 29% n-3 fatty acids predominantly n-3HUFA, mainly EPA (Table 4.2). Inclusion of different VO levels resulted in increased percentages of 18:1n-9, 18:2n-6 and 18:3n-3 with concomitant decreased proportions of n-3 HUFA, total PUFA and long chain monoenes. Thus, the levels of 18:1n-9, 18:2n-6 and 18:3n-3 increased to 15, 16 and 38% of total fatty acids, respectively, in the diet formulated with 100% LO, and to 21, 38 and 6% of total fatty acids, respectively, in the diet with 100% SO with an average value of 5.5%. n-3HUFA in both diets. The 20LO50SO and particularly 50LO20SO diet showed higher contents of n-3, n-6 and n-9 fatty acids. The diet 50LO50SO had 18.97%, 27.15% and 22.95% of 18:1n-9, 18:2n-6 and 18:3n-3 respectively, and registered the lowest content on n-3 HUFA, with only 4.71%.

Fish HK fatty acid profile of total lipids reflected the dietary lipid composition (Table 4.3). N-3 fatty acids were significantly increased in fish fed the LO diet, while n-6 content was significantly increased in SO fed diet. N-9 fatty acids were also increased in fish fed the LO and VO diets compared to control diet, due mainly to an increase in OA. However, the content of n-3 HUFA was significantly ($P<0.05$) reduced in fish fed the two vegetable oils separately, or as a blend, compared to control diet fed fish oil. DHA was reduced by 45 % in HK of fish fed 70LO, 55 % in fish fed 100LO, 58 % in fish fed 70SO, 61 % in fish fed 100SO, 35,5% in fish fed 20LO50SO, 19% in fish fed 50LO20SO and 64% in fish fed 50LO50SO. Similarly EPA was reduced by 64 % in HK of fish fed 70LO, 76 % in fish fed 100LO, 73 % in fish fed 70SO, 80 % in fish fed 100SO, 57% in fish fed 20LO50SO, 42% in fish fed 50LO20SO and 82% in fish fed 50LO50SO. Regarding ARA, a 47 % reduction was found in fish fed 70LO, 16% in fish fed 100LO, 88% in fish fed 70SO, 86% in fish fed 100SO, 45% in fish fed 20Lo50SO, 40% in fish fed 50LO20SO and 52% in fish fed 50LO50SO. Dietary inclusion of vegetable oils reduced DHA/EPA ratios in fish HK, regardless of the type of oil used. In fish fed LO and/or SO the end products of Δ6 desaturase and elongase from 18:2n-6 and 18:3n-3 (mainly 20:2n-6, 20:3n-6 and 20:3n-3) were increased in fish fed SO and LO respectively compared to the control fish. Figure 1 shows a highpositive correlation between dietary LA, LNA, EPA and their content in the head kidney with a r^2=0.99, 0.93 and 0.91, respectively, and to a lesser extent dietary DHA was also positively correlated with dietary DHA content in the head kidney with r^2=0.71.

Table 4.2: Fatty acid composition of the experimental diets (g fatty acid/ 100g of total fatty acids)

Fatty acids	FO	70L	100L	70S	100S	20L50S	50L20S	50L50S
14:0	9.23	3.11	1.57	3.32	1.59	2.99	2.6	1.36
15:0	0.26	0.10	0.06	0.11	0.06	0.45	0.27	0.09
16:0ISO	0.11	0.04	0.3	0.05	0.03	0.04	0.08	0.09
16:00	22.21	12.19	10.21	15.84	13.69	27.42	12.21	11.87
16:1n-7	11.25	3.91	1.98	4.06	2.01	3.44	3.23	1.79
16:1n-5	0.38	0.14	0.09	0.16	0.09	0.21	0.10	0.08
16:2n-4	1.83	0.61	0.28	0.61	0.29	0.57	0.64	0.29
17:00	0.85	0.38	0.25	0.41	0.28	0.56	0.31	0.25
16:3n-4	2.00	0.66	0.29	0.67	0.31	0.62	0.53	0.24
16:3n-3	0.15	0.07	0.05	0.07	0.05	0.03	0.03	0.05
16:3n-1	0.12	0.05	0.04	0.06	0.04	0.07	0.07	0.03
16:4n-3	0.72	0.26	0.13	0.24	0.11	0.04	0.05	0.09
16:4n-1	-	-	-	-	-	0.26	0.28	-
18:00	3.85	3.96	3.99	3.43	3.20	7.69	3.48	3.73
18:1n-9	9.10	13.75	15.31	18.60	21.48	12.49	18.42	18.97
18:1n-7	3.16	1.57	1.36	2.15	1.77	1.23	1.37	1.35
18:1n-5	0.14	0.08	-	0.12	0.11	0.09	0.10	0.10
18:2n-9	0.04	0.02	-	-	-	-	0.03	-
18:2n-6	4.02	12.36	16.21	29.93	38.51	16.40	18.42	27.15
18:2n-4	0.38	0.13	0.06	0.12	0.06	0.12	0.12	0.05
18:3n-6	0.36	0.13	-	-	0.07	0.14	0.12	0.09
18:3n-4	0.04	-	0.04	0.18	0.09	0.18	0.12	0.05
18:3n-3	0.48	31.94	37.63	5.63	6.01	11.36	20.61	22.95
18:4n-3	1.94	0.76	0.40	0.73	0.40	0.75	0.75	0.36
18:4n-1	-	-	0.03	-	-	0.07	0.08	-
20:00	0.28	0.21	0.19	0.28	0.28	0.51	0.26	0.24
20:1n-9	2.59	1.94	1.84	2.08	1.89	1.65	2.34	1.90
20:1n-7	0.26	0.13	0.09	0.13	0.10	0.11	0.15	0.09
20:2n-9	-	0.04	-	0.04	0.01	0.02	0.03	0.01
20:2n-6	0.19	0.11	0.08	0.11	0.09	0.04	0.04	0.08
20:3n-9	-	-	-	-	-	0.10	0.15	-
20:3n-6	0.25	0.09	0.04	0.06	0.03	0.09	0.08	0.03
20:4n-6	1.11	0.43	0.24	0.43	0.24	0.43	0.39	0.20
20:3n-3	-	-	0.07	-	-	0.06	0.08	-
20:4n-3	0.96	0.35	0.16	0.33	0.16	0.34	0.38	0.13
20:5n-3	10.05	4.00	2.07	3.77	2.06	4.15	3.98	1.72
22:1n-11	1.79	1.52	1.48	1.67	1.61	1.33	1.91	1.63
22:1n-9	-	-	0.42	-	-	0.31	0.52	-
22:4n-6	0.34	0.15	0.09	0.14	0.09	0.15	0.14	0.08
22:5n-6	-	-	-	-	-	0.70	0.74	-
22:5n-3	1.74	0.66	0.29	0.60	0.29	0.24	0.11	0.23
22:6n-3	7.82	4.16	2.92	3.87	2.91	2.59	4.68	2.62
Saturates	36.68	19.95	16.28	23.40	19.10	39.17	18.86	17.45
Monoenoics	28.79	23.10	22.62	29.03	29.09	20.86	28.13	25.92
n-3	23.42	42.19	43.72	15.23	12.00	19.55	30.65	28.15
n-6	6.27	13.26	16.66	30.67	39.02	17.98	19.95	27.60
n-9	22.99	19.66	19.55	24.78	25.40	14.57	21.49	20.88
n-3 HUFA	20.57	9.17	5.50	8.57	5.42	7.38	9.22	4.71
n-3/n-6	3.74	3.18	2.62	0.50	0.31	1.09	1.54	1.02

Table 4.3: Effect of feeding vegetable oils on HK fatty acids profile (g fatty acid/100g total fatty acids)

	FO	70LO	100LO	70SO	100SO	20LO50SO	50LO20SO	50LO50SO
14:00	7.85±0.17	3.98±0.16	2.79±0.13	3.86±0.12	2.34±0.03	3.11±0.04	4.58±0.53	2.34±0.07
15:00	0.25	0.15±0.01	0.12±0.02	0.14	0.08±0.01	0.52±0.09	0.69±0.36	0.09±0.01
15:1n-5	0.49±0.33	0.45±0.03	0.33±0.03	0.54±0.06	0.33±0.05	0.07±0.05	0.03±0.02	0.35±0.06
16:0 ISO	0.13	0.23±0.03	0.29±0.09	0.10±0.03	0.04±0.03	0.11±0.03	0.11±0.03	0.08±0.01
Me16:0	0.16±0.03	0.14±0.01	0.10±0.01	0.12±0.01	0.09±0.01	-	-	0.1
16:00	22.72±0.12	18.90±0.51	17.13±0.52	20.65±0.58	17.95±0.47	16.75±0.4	18.16±1.27	17.7±0.48
16:1n-9	0.07	0.06	0.28±0.01	0.05±0.03	0.07	-	-	0.15±0.08
16:1n-7	12.33±0.2	5.79±0.15	3.69±0.07	5.76±0.12	3.35±0.01	5.18±0.1	7.11±0.44	3.26±0.06
16:1n-5	0.28	0.17±0.01	0.13±0.01	0.13±0.02	0.09±0.02	0.13±0.01	0.16±0.01	0.1±0.01
16:2n-6	0.34	0.19	0.15	0.20±0.01	0.18	0.21	0.21±0.02	0.16
16:2n-4	1.31±0.02	0.52±0.01	0.35	0.50	0.30	0.52	0.68±0.03	0.28
17:00	0.60	0.41±0.01	0.33±0.01	0.39	0.28	0.36±0.01	0.42±0.08	0.3±0.01
16:3n-4	1.21±0.02	0.49	0.35	0.49±0.01	0.34	0.08±0.01	0.11±0.02	0.32
16:3n-3	0.16	0.37±0.05	0.09±0.01	0.09	0.07	0.51±0.02	0.67±0.04	0.07
16:3n-1	0.35±0.09	-	0.39±0.06	0.28±0.03	0.25	0.03±0.03	0.04	0.33±0.04
16:4n-3	0.30±0.09	0.13±0.06	0.20±0.02	0.12±0.08	0.19	0.16±0.14	0.21±0.12	0.17±0.02
16:4n-1	0.02±0.02	0.05±0.01	0.02	0.02±0.02	0.02±0.02	0.31±0.1	0.26±0.09	0.02±0.02
18:00	4.57±0.12	5.94±0.1	5.91±0.23	4.94±0.07	4.60±0.17	5.77±1.35	4.31±0.76	5.5±0.13
18:1n-9	14.06±0.1^{e}	19.94±0.4^{d}	21.57±0.18^{c}	23.50±0.31^{b}	25.04±0.4^{a}	20.39±0.9^{bc}	20.72±1.2^{bc}	24.64±0.35^{a}
18:1n-7	3.98±0.01	2.52±0.05	2.05±0.06	2.94±0.07	2.51±0.03	1.62±0.45	2.38±0.28	2.33±0.05
18:1n-5	0.19±0.02	0.05±0.07	0.12	0.10±0.07	0.14	0.13±0.02	0.16±0.01	0.13
18:2n-9	0.06	0.04±0.01	0.03±0.01	0.02±0.02	0.05	0.04±0.04	0.02±0.01	0.03±0.02
18:2n-6	3.47±0.04^{f}	9.50±0.17^{e}	11.69^{d}	20.62±0.46^{b}	27.94±0.3^{a}	20.52±0.4^{b}	12.85±1.02^{c}	19.63±0.27^{b}
18:2n-4	0.35	0.17	0.11±0.03	0.12±0.01	0.07	0.14±0	0.14±0.08	0.07±0.01
18:3n-6	0.28	0.18	0.15±0.78	0.22±0.01	0.30±0.01	0.25±0.13	0.18±0.05	0.23±0.02
18:3n-4	0.55±0.02	0.14±0.1	0.07±0.02	0.13±0.09	-	0.17±0.15	0.53±0.12	-
18:3n-3	1.16±0.02^{f}	16.39±0.55^{b}	20.42±0.02^{a}	2.79±0.14^{ef}	3.19±0.13^{e}	7.71±0.1^{d}	6.3±1.13^{d}	11.53±0.31^{c}
18:3n-1	0.95±0.02	0.39±0.03	0.03	-	-	0.01±0.01	-	-
18:4n-3	0.15	0.06	0.31±0.01	0.28±0.02	0.22±0.02	0.44±0.02	0.14±0.25	0.26±0.02
18:4n-1	-	0.09±0.13	0.04±0.03	0.03±0.02	0.02±0.02	0.06	0.1±0.02	0.01±0.01
20:00	0.23	0.15±0.11	0.24	0.24	0.23	0.23±0.01	0.23±0.04	0.24
20:1n-9	1.80±0.04	1.54±0.02	1.47±0.04	1.84±0.02	1.66±0.01	2.03±0.01	3.05±0.28	1.74±0.03
20:1n-7	0.08±0.12	-	0.13±0.01	0.16	0.12	0.15±0	0.2±0.03	0.13
20:2n-9	0.10±0.01	0.09±0.01	0.05	0.08±0.07	0.05±0.04	0.1±0.05	0.03±0.05	0.04±0.03
20:2n-6	0.17	0.21±0.03	0.28±0.01	0.44±0.01	0.65±0.01	0.1±0.02	0.15±0.12	0.49±0.03
20:3n-9	-	-	0.08±0.04	0.14±0.02	0.23±0.02	0.46±0.02	0.31±0.01	0.14±0.02
20:3n-6	0.18±0.01^{d}	0.08±0.01^{e}	0.34±0.01^{b}	0.39±0.01^{a}	0.29±0.01^{c}	0.2±0.01^{b}	0.13±0.04^{d}	0.26±0.02^{c}
20:4n-6	0.96±0.08^{a}	0.81±0.03^{c}	0.51±0.04^{b}	0.11±0.01^{c}	0.13±0.01^{c}	0.53±0.06^{b}	0.58±0.11^{b}	0.46±0.03^{b}
20:3n-3	0.11b	0.31±0.02b	0.51±0.13a	0.26±0.01b	0.19±0.01b	0.31±0.01	0.22^{b}	0.23±0.02
20:4n-3	0.79±0.01	0.37±0.02	0.12±0.09	-	0.07±0.05	0.41±0.01	0.53±0.12	0.04±0.03
20:5n-3	5.07±0.08^{a}	1.83±0.18^{cd}	1.22±0.06^{de}	1.38±0.09^{cde}	1.03±0.1^{de}	2.18±0.16^{bc}	2.93±0.78^{b}	0.92±0.07^{e}
22:1n-11	1.12±0.03	0.99±0.02	0.93±0.02	1.19±0.02	1.06±0.02	1.23±0.06	1.96±0.19	1.14±0.01
22:1n-9	0.53	0.67±0.04	0.65±0.04	0.57±0.02	0.62±0.07	0.59±0.07	0.68±0.05	0.71±0.05
22:4n-6	0.25	0.12±0.01	0.09	0.07±0.05	0.03±0.04	0.14±0.01	0.16±0.04	0.05±0.03
22:5n-6	0.27±0.01	0.12±0.01	0.28±0.27	0.09±0.01	0.07±0.01	1.19±0.02	1.52±0.4	0.07±0.01
22:5n-3	2.40±0.04	0.94±0.07	0.41±0.29	0.75±0.05	0.59±0.06	0.08±0.01	0.07±0.03	0.5±0.04
22:6n-3	7.40±0.19^{a}	4.07±0.28^{cd}	3.35±0.09^{cd}	3.08±0.23^{d}	2.89±0.23^{d}	4.77±0.33^{bc}	5.98±1.34^{ab}	2.69±0.19^{d}
Saturates	36.6±0.18^{a}	29.8±0.76^{bc}	26..7±0.9^{bcd}	30.49±0.74^{b}	25.6±0.66^{d}	26.7±1.05^{cd}	28.4±2.99^{bcd}	26.16±0.7^{cd}
Monoenes	34.8±0.51^{a}	32.03±0.5^{bc}	31.2±0.14^{c}	36.54±0.34^{a}	34.8±0.37^{a}	31.58±1.1^{c}	36.55±2.07^{a}	34.68±0.5^{ab}
n-3	17.7±0.3^{b}	24.73±1.15^{a}	26.48±0.72^{a}	8.82±0.57^{c}	8.48±0.59^{c}	16.56±0.3^{b}	17.05±3.12^{b}	16.41±0.69^{b}
n-6	5.97±0.05^{f}	11.02±0.23^{e}	13.87±0.51^{d}	22.33±0.46^{b}	29.75±0.3^{a}	23.06±0.4^{b}	15.77±1.36^{c}	21.33±0.38^{b}
n-9	16.79±0.2^{e}	22.55±0.43^{d}	24.28±0.1cd	26.42±0.2ab	27.85±0.3^{a}	23.62±0.9^{cd}	24.81±1.47^{bc}	27.38±0.26^{a}
n-3 HUFA	15.91±0.3^{a}	7.89±0.57bc	5.34±0.28cd	5.51±0.37cd	4.79±0.45c	7.74±0.51^{bc}	9.72±2.21^{b}	4.38±0.36^{d}
n-3/n-6	2.96±0.02	2.24±0.06	1.91±0.04	0.4±0.02	0.28±0.02	0.72±0.02	1.07±0.11	0.77±0.02
DHA/EPA	1.46±0.02	2.23±0.07	2.76±0.05	2.24±0.09	2.80±0.07	2.18±0.02	2.06±0.16	2.94±0.05

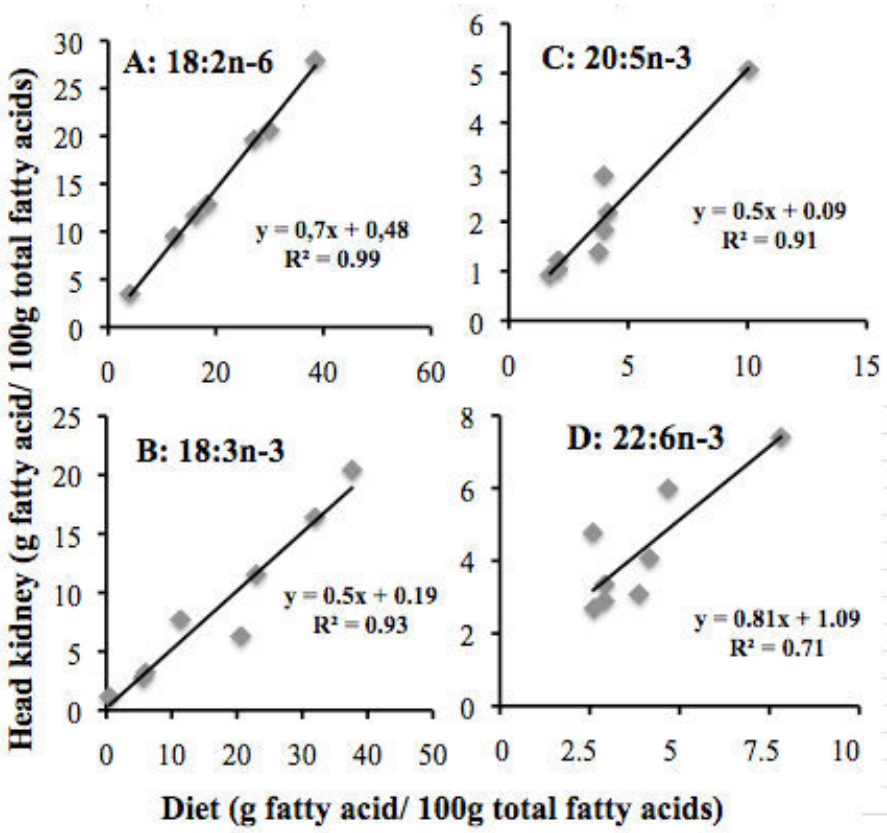

Figure 4.1: Relationship between dietary fatty acid concentrations and head kidney fatty acid concentrations of 18: 2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 in total lipids gilthead seabream fed either 100% FO, 70LO, 100LO, 70S, 100S 20LO50SO, 50LO20SO or 50S50L.

Table 4.4: Cortisol in plasma of fish from the crowding trial (ng/ml of plasma)

	FO	70 LO	100LO	70SO	100SO	20LO50SO	50LO20SO	50L50SO
0 H	2.4±0.8^{c}**D**	6.9±5.8^{bc}**C**	21.1±12^{a}**C**	1.4±0.5^{c}**C**	2.5±1.1^{c}**C**	6.7±5.5^{c}**B**	14±8^{abc}**B**	19.2±5.8^{ab}**B**
2 H	87.5±13.6^{b}^{c}**A**	99.8±40.9^{ab}**A**	131.4±57.8^{a}**A**	43.1±13.4^{cd}**AB**	65.4±29.1^{bcd}**A**	26.5±13.9^{d}**A**	18.7±6.9^{d}**B**	58.3±17^{bcd}**A**
5 H	10.2±7.1^{d}**C**	31.4±2.2^{bc}**BC**	44.8±11.5^{ab}**BC**	27.4±4.4^{bcd}**BC**	50.7±6.7^{a}**AB**	24.3±5^{cd}**AB**	23.2±6.6^{cd}**AB**	32.4±11.4^{abc}**A**
24 H	11.8±2.3^{c}**C**	15.5±2.9^{bc}**C**	27.9±13^{ab}**C**	13.9±7.1^{bc}**C**	20.9±11.6^{abc}B C	25.5±3.6^{ab}**AB**	21±8.1^{abc}**AB**	33.8±4.8^{a}**AB**
48 H	48.7±21^{ab}**B**	64.8±18^{a}**AB**	72.1±30^{a}**B**	46.5±12.3^{ab}**AB**	58.1±24.6^{ab}**AB**	34.9±17.1^{b}**A**	48.5±15.7^{ab}**A**	30.3±18.4^{b}**AB**
1 week	17.6±4.3^{c}**C**	20.5±6.3^{c}**C**	31.6±11.6^{bc}**C**	48.8±17.4^{ab}**A**	52.4±9.9^{a}**AB**	31.9±18.8^{bc}**A**	48.4±21.8^{ab}**A**	24.7±9.2^{c}**B**

Different capital letters mean significant differences between values from the same column

Different small letters mean significant differences between values from the same line

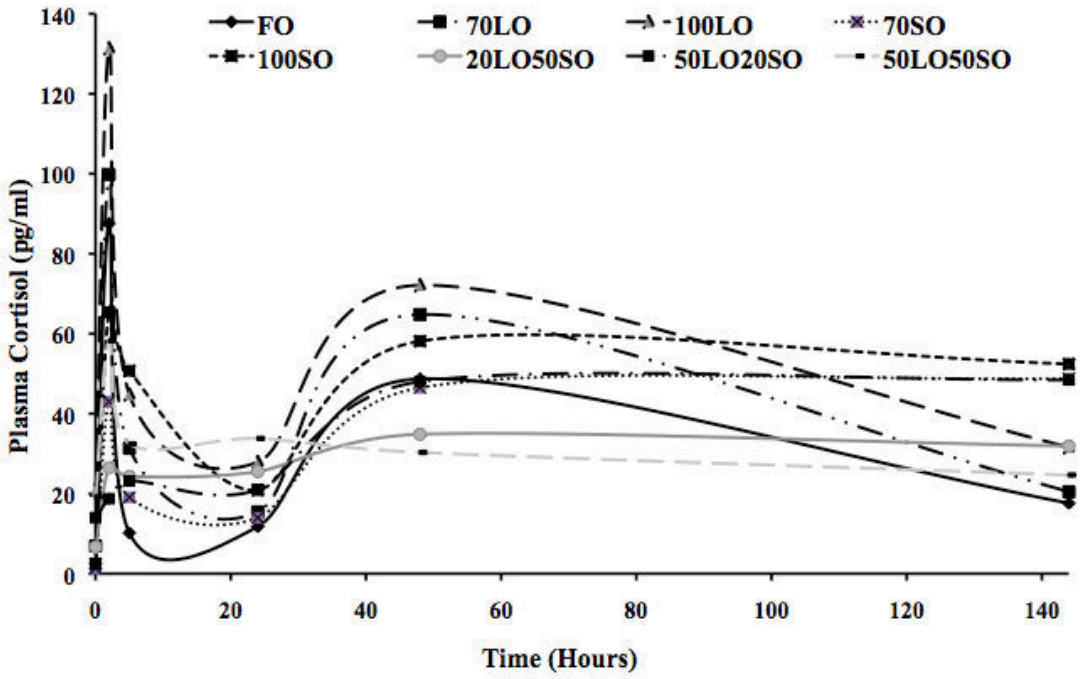

Figure 4.2: Plasma cortisol evolution during the crowding period (pg/ml)

Plasma cortisol

Cortisol production was increased significantly after confinement and manipulation in all the treatments. Fish fed FO, 70LO, 100LO and 50LO50SO showed significantly the highest cortisol levels after 2H of crowding and it decreased after 5H or 24H of crowding but increased significantly after 48H of crowding followed by a drop at 1 week. Fish fed 70SO, 20LO50SO and 50LO20SO showed a significantly increased cortisol level after 2H of crowding followed by a decrease after 5H and 24H of crowding, but it increased again to its maximum level 48H after crowding and maintained these higher levels even after 1 week of crowding.

The plasma cortisol levels from fish within the same time of sampling, showed differences between the dietary treatments for each time point. Fish fed 70LO, 100LO, and 50LO50SO showed significantly higher basal plasma cortisol levels in comparison to FO fed fish (Table 4.4), with fish fed diet 100LO registering the maximum levels, 9 fold higher than those of the FO fed fish. Two hours after handling and crowding confinement, plasma cortisol was increased in all the treatments and in comparison with control fish, those fed 100LO showed significantly higher values, while fish fed

20LO50SO and 50LO20S also showed significantly higher plasma cortisol levels. Accordingly, fish fed 70SO and 100SO also showed low cortisol values but not significantly different from control fish. Five hours after handling and crowding confinement, plasma cortisol levels were decreased in all the treatments, particularly in control fish, whereas fish fed diets 70LO, 100LO, 100SO and 50LO50SO registered significantly higher values than control fish. After 24 hours of crowding, plasma cortisol levels in control fish were similar to those registered after 5 h, whereas there was a reduction trend in all the other fish with values being still significantly higher than controls in fish fed 100SO, 20LO50SO and 50LO50SO. After 48 hours of crowding plasma cortisol levels increased in all treatments without significant differences to controls. Finally, after 1 week of crowding plasma cortisol levels were again similar to those obtained after 24 h, except in fish fed 70SO, 100SO and 50LO20SO which were higher than after 24 h and significantly higher than in control fish.

Table 4.5: Correlation coefficients between cortisol levels and different head kidney fatty acids

	0H	**2h**	**5H**	**24H**	**48H**	**1 week**
18:2n-6	0.06	-0.45	0.60	0.38	-0.30	0.67
18:3n-3	0.17	0.61	0.43	0.50	0.46	-0.39
20:5n-3	-0.14	-0.05	-0.82	-0.57	-0.09	-0.38
22:6n-3	-0.06	-0.13	-0.80	-0.50	-0.09	-0.32
20:4n-6	0.02	0.44	-0.46	-0.46	0.16	-0.69
n-3	0.09	0.64	-0.03	0.19	0.46	-0.66
n-9	0.30	-0.31	0.71	0.54	-0.12	0.66
N-3 HUFA	-0.16	0.01	-0.81	-0.60	-0.04	-0.43

Discussion

In common with all marine fish species studied to date, the cellular lipids for seabream are enriched in n-3 HUFA reflecting an absolute dietary requirement for DHA and EPA (Izquierdo, 1996; Izquierdo *et al*., 1990; Sargent *et al*., 1995, 1997, 1999). The reason for this requirement is the low activity of the Δ5 desaturase and/ or C18 to C20 elongase enzymes in all marine fish that prevents synthesis from 18:3n-3 (Tocher and Ghioni, 1999; Bell and Sargent, 2003), and particularly in gilthead seabream (Izquierdo *et al*., 2008). In addition, it is generally accepted that dietary fatty acid profile is closely reflected in fatty acid composition of fish tissues (Rosenlund *et al*., 2001; Bell *et al*., 2002; Caballero *et al*., 2002; Izquierdo *et al*., 2003, 2005). In the present study, feeding LO and SO increased C18 fatty acids in the total lipids of seabream head kidney and their Δ6 desaturase and elongase end products 20:2n-6, 20:3n-6 and 20:3n-3, whereas the content on n-3 HUFA was significantly reduced, in agreement with previous studies from the same species and in different tissues (Caballero *et al*., 2002, Montero *et al*., 2003, 2005; Izquierdo *et al*., 2003, 2005). The fatty acid compositions of total lipid from head kidney suggested that while EPA is incorporated at the same level as is present in the diet, DHA and ARA were selectively incorporated into cellular lipids and retained (Linares and Henderson, 1991; Bell *et al*., 2001) denoting the importance of these fatty acids in many physiological processes (Lands, 1991; Bell and Sargent, 2003). 18:1n-9, 18:2n-6 and 18:3n-3 were selectively utilized in flesh, when present at high concentrations in the diets, whereby dietary concentrations were always higher in head kidney than in other tissues such as flesh (Bell *et al*., 2001, Montero *et al*., 2005, Izquierdo *et al*., 2003, 2005), suggesting that these C18 fatty acids are readily oxidised when present at high concentrations (Bell *et al*., 2001). The relationship between the dietary LA, LNA and EPA and head kidney content of these fatty acids, showed higher correlation with r^2= 0.99, 0.93 and 0.91, respectively, denoting the direct incorporation of these fatty acids in the head kidney and that their content is determined by the dietary input. However, head kidney content of DHA was relatively correlated with diet but even when its content is lower in the diet, the levels in head kidney are still higher, suggesting selective uptake and retention

The basal plasma cortisol in the control fish in this study were similar to those found in the same species in previous studies (Montero *et al*., 1999, 2001, 2005). The basal cortisol levels were significantly increased in fish fed 70LO, 100LO and 50LO50SO in accordance with previous studies in the same species in vivo (Montero *et al*., 2003) confirming again the role of this oil rich in LA in increasing cortisol secretion. The physiological changes in cortisol, are typical of changes found in fish exposed to a variety of stressors and have been extensively documented (Maule *et al*., 1989, Van de Nieuwegiessen et al., 2008, 2009). The response to stress is often characterized as being composed of a primary and a secondary phase (Wendelaar Bonga, 1997). These responses were different according to the dietary treatment. Therefore, in fish fed FO, 70LO, 100LO and 50LO50SO, there was a rapid response to the stress test, as plasma cortisol increase was most dramatic after 2 h of crowding but had decreased markedly after 5 h, which is in accordance with the results found in catfish (Davis *et al*., 2002) and this continued to decrease after 24 h. This may be due to the inability of fish to continue secreting the hormone or an adjustment of the fish to the somewhat mild, non-life threatening intensity of the stressor. Thereby, at 48 h the plasma cortisol was significantly increased, followed by a decrease to lower levels after 1 week.

The same pattern was observed by Maule and colleagues (1989) who found that plasma cortisol levels were increased significantly and reached its maximum 4 hours after stress handling. These levels were decreased 24 hours after the handling stress, but 48 hours after stress plasma cortisol levels were increased again, and finally dropped to levels similar to the control 4 days after the crowding challenge. However fish fed 70SO, 100SO, 20LO50SO and 50LO20SO, showed a later response to the crowding stress, as 2 h after the challenge, the cortisol was increased significantly, but it continued to increase slightly after 5 h and 24 h and reached its maximum after 48 h and/or 1 week of crowding. This is in accordance with the in vitro results found with the head kidney from the fish of the same trial and also with other previous studies denoting the role of n-3 PUFA in increasing cortisol release by head kidney and the role of n-6 (20:3n-6) in decreasing this response (Ganga *et al*., 2006). After crowding

challenge as a acute stress, the fish fed SO generally registered a lower (but not significant) response compared to FO which is in accordance with previous studies (Ganga *et al*., 2006) where the 20:3n-6 was increased in head kidney from fish fed SO. This fatty acid was found previously to decrease cortisol release in vitro (Ganga *et al*., 2006).

However, numerous studies have elucidated the effects of stressors on physiological responses in fish (Veiseth *et al*., 2006; Miller *et al*., 2007), and our results demonstrated significant findings on how seabream respond to overcrowding in relation to cortisol levels. 2 hours of crowding were sufficient such that seabream responded to this challenge in order to cope with the new metabolic needs, and thus the plasma cortisol concentrations were increased to different degrees in all the treatments, and with fish fed LO showing the highest cortisol response in accordance with previous studies (Montero *et al*., 2005). These cortisol concentrations were decreased 5 hours post crowding and continued to decrease until 24 hours. This could be attributed to physiological acclimation of fish after the initial handling stress, together with a potential increase of cortisol degradation or decreased stressor influence (Pickering and Pottinger, 1987; Barton *et al*., 2005; North *et al*., 2006; Trenzado *et al*., 2006). The same response was reported in previous studies using Atlantic cod signaling a significant increase in plasma cortisol levels 2 hours post crowding but returned to pre-crowding levels 24 h after exposure (Caipang *et al*., 2008). Therefore, it is clear that overcrowding as a stressor activated the HPI-axis (Pottinger *et al*., 1994), and consequently elevated plasma cortisol as it was shown in earlier studies done using same species (Ortuño *et al*., 2001, Barton *et al*., 2005). A similar response was seen in salmonids (Pickering and Pottinger, 1989; Mazur and Iwama, 1993; Wiseman *et al*., 2007; Fast *et al*., 2008), ayu (*Plecoglossus altivelis*) (Iguchi *et al*., 2003) and African catfish (*Clarias gariepinus Burchell*) (Van de Nieuwegiessen *et al*., 2008, 2009) showing that cortisol is an immediate and transitory response to overcrowding (Caipang *et al*., 2008).

Moreover, after 48 hours of crowding the cortisol concentrations increased again for all the treatments with the LO fed fish registering the highest concentrations as

expected (Montero *et al*., 2005). Nevertheless after 1 week of crowding, the plasma cortisol levels decreased in FO, 70LO and 100LO fed fish compared to their values at 48 hours, but remained at the same levels in the other treatments with 100SO registering the highest concentration. This dietary effect on stress response could be explained by the type and the quantity of different dietary fatty acids (Ganga *et al*., 2006). While the mechanism of the stress response is still controversial in fish and it depends on many factors. Crowding of brown trout caused increased cortisol levels for the first 2 days, however by 6 days, plasma cortisol levels returned to values seen prior to crowding (Pickering and Pottinger, 1987). In addition, recent observations indicated that GR mRNA levels were lowered in sea bass (*Dicentrarchus labrax*) liver in response to chronic crowding stress (Terova *et al*., 2005) suggesting that the stress effect on the GR message may be either species-specific and/or dependent on the type, intensity and duration of the stressor (Wiseman *et al*., 2007) It may be that there was a specific response due to the specific fatty acids present in each VO (LO or SO) in the present study. . However, it is thought that the adaptive significance of GR turnover is autoregulated by cortisol (Sathiya and Vijayan, 2003) suggesting that GR response may be an important part of the adaptive stress response that is regulated by plasma cortisol level. Increased clearance rate of corticosteroids from the blood has been suggested as one of the possible mechanisms to explain acclimation to chronic stress in salmonids (Redding *et al*., 1984). Alternatively, the lack of increase in cortisol in long-term stressed individuals may be due to a negative feedback of this daily increase in cortisol on the HPI (Pickering, 1992; Pickering and Pottinger, 1987).

In the present study, the cortisol response to crowding was different according to the dietary treatment with fish fed the LO diet registering the highest cortisol value. This was supported by its highest correlation with the head kidney LNA content (r^2= 0.61), and this in accordance with the results from a previous study showing increased plasma cortisol levels when feeding seabream with diets rich in this fatty acid (Montero *et al*., 2005).

The conserved stress response has adaptive value and is thought to allow animals to regain homeostasis after a stressor insult (Mommsen *et al*., 1999; Iwama *et*

al., 2006). Some reports have suggested that prolonged exposure to a stressor can lead to allostasis, which is the ability of the body to return to physiological levels seen prior to stress challenge (McEwen, 1998, Schreck, 2000). However, even though animals chronically stressed seem to compensate physiologically to the stressor, their ability to perform important functions at the whole organism level may be affected (Schreck, 2000). Therefore, many studies have identified effects of cortisol on different physiologic processes, including osmoregulation, immune function, neuroendocrine function and behaviour. Thus, cortisol supresses cellular immunity by affecting inflammatory signaling pathways (Holland *et al*., 2003; MacKenzie *et al*., 2006; Aluru and Vijayan, 2009). Moreover, 1h post-handling disturbance was found to increase the activities of glycolytic enzymes that may be critical to cope with the increased liver energy demand (Iwama *et al*., 2006; Wiseman *et al*., 2007). It is also demonstrated that a 24 h restraint stress was shown to modulate Il-1β and its receptor expression in the head kidney and brain of common carp, leading to the hypothesis that IL- 1β plays a key role in the stress-mediated peripheral immune response as well as centrally in the activation of the HPI axis (Metz *et al*., 2006).

It was also demonstrated that when subjecting seabream to confinement, the fatty acid composition of the head kidney was affected and PUFA were decreased (Van Anholt *et al*., 2004a). In the present study, we demonstrated that feeding fish with LO and/or SO seriously affected the head kidney fatty acid composition by increasing C18 and decreasing n-3 HUFA, indicating the effect of feeding higher levels of LO and/or SO in increasing plasma cortisol concentration over a long period (Montero *et al*., 2005). Consequently the cortisol release after crowding stress was significantly different between treatments. n-3 PUFA, in addition to affecting general properties of cells as membrane components, play a role in modulating the production of both lipid (eicosanoids) and protein (cytokines) mediators. Feeding fish with decreasing levels of n-3 HUFA, by including LO in the diets, decreased significantly the plasma levels of PGE3 (Ganga *et al*., 2005) known as an anti-inflammatory local hormone. Moreover, a number of authors have attributed a DHA effect on stress resistance (Kanazawa, 1997; Tago *et al*., 1999; Harel *et al*., 2001).

These results indicate clearly that cortisol release is directly correlated to the head kidney composition of different fatty acids, particularly n-3 HUFA (EPA and DHA) with r^2 =0.82 and 0.8, respectively after 5 h of crowding. This is in accordance with previous results showing the stimulatory effect of EPA and DHA on cortisol release in head kidney (Ganga *et al*., 2006). In another study, Welker and Congleton (2004) found that feeding salmon juveniles with LO and SO in different proportions showed that the highest cortisol levels were registered in fish fed SO. They suggested that the cortisol response may have been influenced by the ratio of prostaglandin 1 and 2 series to prostaglandin 3-series precursor fatty acids provided by different diets (Welker and Congleton, 2004) concluding that there is a relationship between dietary lipid source and stress in chinook salmon (*Oncorhynchus tshawytscha*).

Many other studies have reported that dietary ARA improves fish survival and resitance to stress (Castell *et al*., 1994, Bessonart *et al*., 1999; Koven *et al*., 2001a,b, Van Anholt *et al*., 2004a,b). Neverthless, the mechanisms by which these HUFAs could modulate the stress response are still unknown, but many recent studies have suggested the implication of COX and LOX metabolites in this response (Koven *et al*., 2001a,b, 2003; Van Anholt *et al*., 2004a). Prostaglandin and PGE2, in particular, have been shown to modulate the sensitivity of the mammalian HPA axis and consequently change the stress response (Di Luigi *et al*., 2001). In a previous study, we demonstrated that feeding seabream with higher levels of LO, increased significantly plasma PGE3 concentration (Ganga *et al*., 2005). Although less studied in fish, the prostaglandins likely modulate the release of hypothalamic CRH and/or pituitary ACTH, as shown in mammals (Abou-Samra *et al*., 1986). Furthermore, Koven *et al*., (2001a, b, 2003) have demonstrated the importance of feeding diets rich in ARA to seabream larvae in improving its response to stress and increasing their survival, and they *suggested* that this response is mediated by COX metabolites derived from ARA.

In conclusion, high stocking densities could be considered as a chronic stress factor that affects fish welfare in aquaculture (Wedemeyer, 1997, Van de Nieuwegiessen *et al*., 2008, 2009), and good practices in aquaculture business are of great importance to improve fish welfare and can minimize stress on farmed fish and

maintain high standards of fish welfare (Terova *et al*., 2005). This study showed clearly that feeding seabream with LO and SO as substitutes to FO resulted in a profound alteration of head kidney fatty acids profiles. N-3 HUFA were decreased and OA, LA and LNA were decreased. Short-term overcrowding (2 h) in seabream resulted in short term increases in plasma cortisol. These alterations, affected the stress response to crowding for all the dietary treatments with fish fed with higher contents of LO and/or SO showing significantly higher plasma basal and post-stress cortisol levels. Thus, when substituting fish oil with vegetable oils in diets for seabream, care should be taken to guarantee that sufficient content of EFAs necessary for normal stress response are provided to satisfy fish physiological requirements.

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Chapter 5

Effect of dietary lipids on plasma fatty acid profiles and prostaglandina and leptin production in gilthead seabream (*Sparus aurata*)

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Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*)

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Abstract

The aim of this study was to investigate the effects of different levels of substitution of fish oil by vegetable oils rich in oleic, linoleic and linolenic acids on gilthead seabream plasma and leukocyte fatty acid compositions and prostaglandin (PG) and leptin production. Juvenile seabream of 24 g initial body mass were fed four iso-energetic and iso-proteic experimental diets for 281 days. Fatty acid composition of plasma lipids was markedly affected by the inclusion of vegetable oils (VO). ARA (arachidonate), EPA (eicosapentaenoate) and DHA (docosahexaenoate) were preferentially incorporated into polar lipids of plasma, and DHGLA (di-homogammalinoleate) accumulated with increased vegetable oil inclusion. Dietary treatments resulted in alterations of DHGLA/ARA ratios, but not ARA/EPA. ARA-derived PGE_2 production in plasma was not affected by vegetable oils, in agreement with similar eicosanoid precursor ratio (ARA/EPA) in leukocytes total lipids and plasma phospholipids among fish fed with the different dietary treatments. Feeding vegetable oils leads to a decrease in plasma EPA which in turn reduced plasma PGE_3 concentration. Moreover, PGE_3 was the major prostaglandin produced in plasma of fish fed fish oil based diet. Such findings point out the importance of EPA as a precursor of prostaglandins in marine fish, at least for the correct function of the blood cells, and correlates well with the predominant role of this fatty acid in immune regulation in this species. A negative correlation was found between plasma PGE_2 and leptin plasma concentration, suggesting that circulating levels of leptin may act as a metabolic signal modulating PGE_2 release. The present study has shown that increased inclusion of vegetable oils in diet for gilthead seabream may profoundly affect the fatty acid composition of plasma and leukocytes, specially HUFA (highly unsaturated fatty acids), and consequently the production of PGE_3, which can be a major PG in plasma. Alteration in the amount and type of PG produced can be at least partially responsible for the changes in the immune system and health parameters of fish fed diets with high inclusion of VO.

Keywords: Leptin; Leukocytes; Seabream; Plasma; Prostaglandins; Polyunsaturated fatty acids

1. Introduction

Supply of fish oil (FO) and fish meal for aquaculture feeds has shown a reduction due to stagnation in capture fisheries as a consequence of over fishing and natural events such as El Niño. FO production has suffered a decrease and fluctuations which results in higher prices and uncertainty of its availability. Some scientists expect that supplies of FO for aquaculture production will become critical between 2005 and 2010 (Bell and Sargent, 2003). Thus, there is a strong need for diversification of feed ingredients used in aquaculture (Kaushik, 2000), and considerable research efforts have been directed towards the evaluation of other non-marine ingredients as a potential substitutes in fish diets (Hardy et al., 2001).

Previous experience has shown that it is possible to replace up to 60% of the FO by vegetable oils (VO) in diets for seabream without compromising growth, survival, fish feed utilisation or fillet organoleptic properties, when fish are fed either for a medium (3 months, Izquierdo et al., 2000, 2003) or long period (8 months) (Menoyo et al., 2004; Izquierdo et al.,

Abbreviations: ARA, arachidonic acid; DHGLA, di-homogammalinoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; HUFA, highly unsaturated fatty acid; LA, linoleic acid; LNA, linolenic acid; LO, linseed oil; NL, neutral lipid; OA, oleic acid; PL, polar lipid; PG, prostaglandin; RO, rapeseed oil; VO, vegetable oil.

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in press). However, a substitution increased up to 80%, significantly reduces growth and conversion indices (Izquierdo et al., in press) altering normal hepatocyte and enterocyte morphology (Caballero et al., 2003), and negatively affecting immune functions and post-stress plasma cortisol response (Montero et al., 2003). In addition, inclusion of soybean or rapeseed oil with of 60% substitution reduced macrophage phagocytic activity (Montero et al., 2003) whereas the reduction in the eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids in diet has been shown to significantly inhibit serum alternative complement pathway activity (Montero et al., 1998). The role of dietary highly unsaturated fatty acids (HUFAs) on immune regulation seems to be mediated, at least partly, by the eicosanoid production in target tissues. Eicosanoids are oxygenated derivatives of polyunsaturated fatty acids produced from membrane phospholipids by the action of phospholipases, cyclooxygenases and lipoxygenases (Rowley et al., 1995). In broad terms, eicosanoids are produced in response to stressful situations, both at a cellular and whole body level (Sargent et al., 1999). The principal substrate is arachidonic acid (ARA; 20:4n-6), generating 2-series prostanoids and 4-series leukotrienes, that have potent proinflammatory effects (Secombes, 1996). In addition, EPA, DHA and di-homogammalinolenic (DHGLA; 20:3n-6) are also important eicosanoid substrates in fish due to their high concentration in membrane phospholipids of aquatic organisms (Henderson and Sargent, 1985). Non-esterified ARA, through the action of cyclooxygenase enzymes, yields 2-series prostanoids (prostaglandins (PG) and thromboxanes) and, through the action of lipoxygenase enzymes produce 4-series leukotrienes and lipoxines (Lall, 2000). Alternatively, the metabolic derivatives produced from non-esterified EPA are 3-series prostanoids and 5-series leukotreines and lipoxines whereas the 1-series prostanoids derived from DHGLA. These compounds are known to play essential roles in the regulation of many physiological and immunological processes in the body (Balfry and Higgs, 2001). The effect of dietary VO on prostaglandins production (in different fish organs) has been previously documented in freshwater and some Nordic marine fish species (Bell et al., 1991, 1992, 1993, 1994b; Henderson et al., 1996), but no studies have been conducted until now on eicosanoid production in seabream, or their alteration by dietary lipids.

Leptin is known as a multifunctional hormone that plays numerous important roles in homeostasis, immune function and reproduction. Its major role pertains to the regulation of energy balance by decreasing food intake and increasing energy expenditure (Van Dijk, 2001). The level of secreted leptin is proportional to body fat level and, through its action on hypothalamic centers, leptin suppresses food intake and increases energy expenditure (Frederich et al., 1995). The reduction in energy availability leads to impairments in humoral immunity and hence leptin has been proposed as a neuroendocrine signal between body fat and immunity regulating humoral immune responses (Demas and Sakaria, 2005). Direct actions of leptin on immune cells seem to affect disease resistance through on hematopoiesis, proinflammatory response and other immune cell functions (Gainsford and Alexander, 1999; Fantuzzi and Faggioni, 2000). Leptin stimulates macrophages and neutrophils, its production is increased during inflammation and delays apoptosis of human mature neutrophils "in vitro" (Bruno et al., 2005). Leptins could also affect human Natural Killer Cell lines function (Zhao et al., 2003). Moreover, recently, Zerani et al. (2005) demonstrated the role of leptin as a metabolic signal of PG release in rabbit. However few studies have been conduct with leptin production in fish (Volkoff et al., 2003), and no one of them deal with their relation with dietary fat or prostaglandin production.

The aim of the present study was to investigate the effect of different levels of substitution of fish oil by vegetable oils (rich in oleic (OA), linoleic (LA) and linolenic (LNA) acids) on seabream plasma and leukocyte fatty acid compositions and effects on prostanoids and leptin production.

2. Materials and methods

2.1. Fish and husbandry

Two thousand four hundred juvenile seabream (*Sparus aurata*), obtained from a local fish farm (ADSA, Las Palmas, Spain) of 24g initial weight were maintained at the Instituto Canario de Ciencias Marinas (ICCM) (Canary Islands, Spain). Fish were distributed randomly into 16×1000 L polyethylene circular tanks (150 fish/tank, each diet assayed in quadruplicate) supplied with continuous seawater (36‰) flow and aeration. Fish were fed under natural photoperiod (approximately 12:12 L/D). Water temperature and dissolved oxygen during the experimental period ranged between 21.9–22.4 °C and 5.5–7.2 ppm, respectively. After 2 weeks of acclimation, the experimental diets were hand-fed until apparent satiation three times a day at 9:00, 12:00 and 15:00 h, 6 days per week. Fifteen fish were sampled for biochemical parameters at the beginning and after 98 days of the experiment period.

2.2. Diets

Four iso-energetic and iso-proteic experimental diets were formulated with a constant lipid content of ~22%. Two different VO blends, included at a 60% substitution of dietary FO, were used for seabream. The diets contained rapeseed, linseed and palm oils in ratio 15:60:25 in diet 60 LO and 40:40:20 in diet 60 RO. A 100 LO diet with 100% substitution of fish oil by the blend in diet 60 LO was also included. The diets were prepared by Nutreco ARC, Stavanger, Norway. The fatty acid compositions of the 5 mm diets were analyzed and were shown in Table 1.

2.3. Sampling procedure

After 281days, fish were individually sampled from each tank for final sample collection. Blood was collected from the caudal vein in heparinised syringes from 6 fish from 3 tanks per diet (18 fish per diet) and transferred to an eppendorf tube

Table 1
Fatty acid compositions of the 5 mm experimental diets (g/100 g fatty acid)

	FO	60 LO	60 RO	100 LO
%Lipids (dw)	20.24	21.36	22.79	25.14
14:0	5.92	3.05	2.93	0.79
16:0	19.30	14.99	15.95	15.92
16:1n-7	7.21	3.93	3.23	1.23
16:4n-3	0.17	0.04	0.03	0.02
18:0	3.37	3.21	3.36	3.40
18:1n-9	11.71	24.61	29.89	32.98
18:2n-6	5.84	11.87	12.50	13.67
18:3n-3	1.62	14.36	12.25	23.02
18:4n-3	2.18	1.28	0.87	0.17
20:1n-9	2.38	2.05	1.98	2.23
20:2n-6	0.15	0.10	0.08	0.03
20:3n-6	n.d.	n.d.	n.d.	n.d.
20:4n-6	0.66	0.34	0.28	0.06
20:3n-3	n.d.	n.d.	n.d.	n.d.
20:4n-3	0.54	0.27	0.21	0.05
20:5n-3	11.90	6.10	4.86	1.06
22:1n-11	2.98	2.35	2.20	2.41
22:5n-6	0.24	0.12	0.11	–
22:5n-3	1.17	0.56	0.47	0.08
22:6n-3	14.14	7.36	6.21	2.10
Saturated[a]	30.01	22.22	23.33	20.63
Monounsaturates[b]	27.70	33.12	37.44	38.85
∑n-3[c]	32.23	30.27	25.22	26.57
∑n-6[d]	7.37	12.59	13.13	13.78
∑n-9	14.56	26.81	31.91	35.22
∑n-3 HUFA	27.84	14.34	11.82	3.33
n-3/n-6	4.37	2.40	1.92	1.93

dw—dry weight.
$n=3$. Values in the same row with different superscript letters are significantly different ($P<0.05$).
[a] Includes 15:0, 17:0, 20:0 and 22:0.
[b] Includes 22:1n-7.
[c] Includes 20:3n-3 and 22:4n-3.
[d] Includes 18:3n-6 and 22:4n-6.

coated with lithium heparin as an anticoagulant. The blood was centrifuged immediately at 600×*g* for 10 min 4 °C to sediment the cells. One milliliter of plasma was removed, 50 μL/mL of 2M formic acid was added and the acidified samples were frozen in liquid nitrogen (−80 °C) prior to eicosanoid analysis. 250 μL of plasma was removed and stored at −80 °C for leptin analysis and the remaining plasma was pooled per each tank and stored at −80 °C for fatty acid analysis.

2.4. Blood leukocyte separation

This assay was conducted on only 3 dietary treatments as sufficient fish fed 100 LO diet were not available. Seven milliliters of blood was collected from the caudal vein in heparinised syringes from 9 fish per diet and transferred to clean glass tubes kept on ice. Blood was then centrifuged at 500 *g* for 10 min at 4 °C. After elimination of supernatant cells were resuspended in 10 mL of HBSS Ca–Mg-free and then centrifuged at 500 *g* for 10 min at 4 °C. Cells were separated into 2 sub-samples which were resuspended in 6 mL of HBSS Ca–Mg-free, and carefully layered over 6 mL of 46% Percoll and centrifuged at 450 *g* for 40 min at 4 °C. Cells collected from the white intermediate layer were resuspended in 10 mL of HBSS Ca–Mg-free and centrifuged at 500 *g* for 10 min at 4 °C. Collected cells were resuspended in 6 mL HBSS, Ca–Mg- free, carefully layered over 6 mL of 46% Percoll and centrifuged at 450 *g* for 40 min at 4 °C. The leukocytes (white intermediate layer) were collected and washed with 10 mL of HBSS Ca–Mg-free. The leukocytes obtained from 3 fish were pooled and re-suspended in 4 mL of HBSS, 2 mL of chloroform were added and the sample was stored at −80 °C prior to lipid extraction.

2.5. Lipid analysis

Extraction of total lipid from diets, plasma samples and blood leukocytes was performed by the method of Folch et al. (1957). Neutral and polar fractions were separated by adsorption chromatography on silica Sep-Pak cartridges (Waters, Milford, MA) as described by Juaneda and Rocquelin (1985). Fatty acid methyl esters were produced from aliquots of total lipids extracted from diet, plasma samples and blood leukocytes by acid-catalyzed transmethylation performed overnight at 50 °C as described by Christie (1982).

Fatty acid methyl esters were separated and quantified by gas–liquid chromatography (Shimadzu C-R5A gas chromatograph, 30 m × 0.32 mm ID capillary column (Suplecowax-10, Sigma-Aldrich, Madrid, Spain) according to conditions described by Izquierdo et al. (1990). Individual methyl esters were identified by comparison with known standards and published data.

2.6. Extraction, separation and enzyme immunoassay of eicosanoids

The frozen acidified plasma samples were thawed and centrifuged (1000 *g*, 5 min) to precipitate cell debris. The supernatants were extracted using octadecyl silyl (ODS, C18) 'Sep-Pak' mini-columns (Millipore (UK), Watford) by the method of Powell (1982) and as described in detail by Bell et al. (1994b). The final extract was redissolved in 100 μL of methanol prior to HPLC. PGE_3 was separated by reverse phase HPLC using the methodology similar to that described in Bell et al. (1994a). An isocratic solvent system was employed containing 17 mM phosphoric acid/acetonitrile (70/30, v/v) at a flow rate of 0.75 mL/min. Fifty microliters of the eicosanoid plasma extracts was injected onto the column and 2.25 mL fractions was collected using an Waters Fraction Collector (Waters Ltd., Watford, UK). Fractions corresponding to the PGE_3 elution time were pooled and extracted as follows. The pooled fractions were applied to a C18 'Sep-Pak,' which had been pre-washed with 5 mL methanol and 10 mL distilled water. The column was then washed with further 10 mL distilled water and the PGE_3 eluted in 5 mL of ethyl acetate. Samples were dried under nitrogen and redissolved in immunoassay buffer. Measurement of PGE_3 was performed using an enzyme immunoassay (EIA) kit for PGE_2 according to the manufacturers protocol (SPI-Bio, Gif sur Yvette, France). The cross-reactivity of the PGE_2 antibody with PGE_3 was 43%. The remaining 50 μl of the eicosanoid plasma extracts was used to measure prostaglandin PGE_2 concentration.

2.7. Leptin immunoassay

Plasma leptin concentration was measured with an enzyme immunoassay kit using a monoclonal antibody to human leptin according to the manufacturers protocol (SPI-bio, Gif sur Yvette, France).

2.8. Statistical analysis

Significance of difference ($P<0.05$) between dietary treatments was determined by one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test. Analyses were performed using SPSS software (SPSS for windows 11.0).

3. Results

3.1. Fatty acid composition of plasma

The fatty acid compositions of plasma polar and neutral lipids at the end of the trial are summarized in Tables 2 and 3. In polar lipids, DHA was the most abundant fatty acid in fish fed 100 FO, 60 RO and 60 LO, followed by 16:0 and 18:1n-9. However in fish fed 100 LO, 18:1n-9 and 16:0 were the most abundant fatty acids followed by DHA. 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 fatty acids were all increased with increasing dietary 18:2n-6. 18:3n-3 was also significantly increased in fish fed vegetable oils. The elongase product of 18:3n-3, 20:3n-3, was also higher in fish fed 60 RO, 60 LO and 100 LO diets than in fish fed FO diet, despite the low dietary content. 20:4n-3 was also high, but not significantly, in fish fed VO. DHGLA was significantly higher and ARA lower ($P<0.05$) in fish fed the diet with the highest level of 18:2n-6 (100 LO) when compared with FO fed fish. Besides, EPA content showed a significant reduction ($P<0.05$) in fish fed either 60 RO or 100 LO, compared to fish fed FO. The ARA/EPA ratio was relatively similar in the four treatments. However, the DHGLA/ARA ratio was significantly lower in fish fed FO (0.09) and increased with increasing dietary LA and gammalinolenic acid (18: 3n-6) (GLA), being highest in fish fed 100 LO (0.49). DHA and total n-3 PUFA contents decreased, thought not significantly, according to their respective dietary levels, leading to a lower n-3/n-6PUFA ratio.

Fatty acid composition of neutral lipids was generally higher in LA and GLA content, than that of polar lipids, LA being greater in fish fed VO. Fish fed VO also exhibited higher amounts of GLA, while those fed FO were significantly higher in EPA and DHA. DHA/EPA was increased with feeding VO, and ARA/EPA was also slightly but not significantly increased. Table 4 shows the relationship between the plasma content of some fatty acids and its level in the diet. Oleic acid was particularly incorporated into plasma neutral lipids compared with polar lipids. The ratio of incorporation decreased with increasing dietary content of this fatty acid, the highest ratio being observed in fish fed FO (1.41) and the lowest in fish fed 100 LO (0.75). The ratio of incorporation of palmitic acid was not different among the treatments, being slightly higher in polar lipids. By contrast, ARA EPA and DHA were preferentially incorporated into polar lipid, increasing with VO inclusion.

Table 2
Plasma polar lipid fatty acid compositions (g/100 g of total fatty acids) (mean±SD)

Fatty acids	FO	60 LO	60 RO	100 LO
12:0	1.59±0.36	1.61±0.72	1.44±0.51	1.38±0.85
14:0	2.15±0.16	1.32±0.74	1.01±0.19	0.90±0.34
16:0	22.91±0.93	18.76±4.33	23.30±1.29	21.97±1.78
16:1n-7	2.99±0.88	1.27±0.64	1.43±0.66	1.05±0.69
18:0	6.34±0.13	6.12±2.42	7.16±1.34	7.24±0.49
18:1n-9+18:1n-7	10.47±0.96	16.15±4.81	14.28±0.30	15.73±1.12
18:2n-6	6.04±2.64^{b}	8.47±2.78^{a,b}	8.07±1.89^{a,b}	11.75±1.63^{a}
18:3n-6	0.09±0.00	0.07±0.02	0.05±0.02	0.10±0.03
18:3n-3	0.59±0.23^{c}	3.05±0.95^{a,b}	2.19±0.06^{b,c}	4.98±1.54^{a}
18:4n-3	0.10±0.00	0.11±0.08	0.04±0.05	0.06±0.06
20:0	0.21±0.09	0.20±0.13	0.19±0.14	0.12±0.01
20:1n-9	0.53±0.04	0.70±0.13	0.67±0.01	0.81±0.25
20:2n-6	0.16±0.01	0.32±0.01	0.36±0.06	0.46±0.09
20:3n-6	0.12±0.02^{b}	0.26±0.07^{a,b}	0.31±0.12^{a}	0.31±0.06^{a}
20:4n-6	1.28±0.15^{a}	1.02±0.15^{a,b}	1.05±0.32^{a,b}	0.65±0.12^{b}
20:3n-3	0.09±0.00^{b}	0.34±0.02^{a}	0.38±0.16^{a}	0.32±0.2^{a}
20:4n-3	0.26±0.04	0.39±0.06	0.38±0.13	0.39±0.08
20:5n-3	9.02±0.90^{a}	7.53±1.16^{a,b}	6.87±0.82^{b}	4.52±1.04^{b}
22:1n-11	0.32±0.04	0.29±0.14	0.31±0.01	0.39±0.20
22:5n-6	0.47±0.02	0.64±0.28	0.51±0.30	0.29±0.04
22:5n-3	1.89±0.22	2.04±0.16	1.96±0.34	1.47±0.07
22:6n-3	28.63±3.56	26.28±3.20	26.03±4.86	22.95±1.77
Total saturates[1]	34.11±0.06	28.56±5.07	33.62±3.37	31.92±2.06
Total monoenes[2]	15.24±2.09	19.22±5.93	17.12±1.19	18.58±0.76
n-3[3]	41.01±4.47	40.09±3.47	37.97±6.08	34.87±1.21
n-6[4]	8.44±2.41	10.98±2.23	10.42±1.26	13.67±1.75
n-9[5]	11.43±1.01	17.24±5.08	15.12±0.52	16.97±1.03
n-3 HUFA	39.99±4.71	36.68±4.53	35.64±6.28	29.69±2.65
n-3/n-6	4.86±1.37^{a}	3.65±1.08^{a,b}	3.64±1.03^{a,b}	2.58±0.40^{b}
DHA/EPA	3.22±0.01	3.45±0.01	3.85±0.02	5.01±0.03
ARA/EPA	0.14±0.00	0.14±0.00	0.15±0.03	0.14±0.01
DHGLA/ARA	0.09±0.004^{c}	0.26±0.09^{b}	0.28±0.02^{b}	0.49±0.15^{a}

[1]Includes 15:0, 17:0, and 22:0. [2]Includes 16:1n-5, 18:1n-5, 20:1n-5, 20:1n-7, 22:1n-9, and 22:1n-7. [3]Includes 22:4n-3. [4]Includes 22:4n-6. [5]Includes 18:2n-9, 18:3n-9, 20:2n-9, 20:3n-9 and 22:1n-9. Includes 16:2n-3, 16:2n-4, 16:4n-3 and 16:4n-1.

$n=3\times6$. Values in the same row with different superscript letters are significantly different ($P<0.05$).

The ARA, EPA and EPA/ARA composition of total lipids from blood leukocytes is shown in Fig. 1. Both palmitic and oleic acids, followed by DHA and EPA were the most abundant fatty acids in these cells (data not shown). Fish oil substitution with VO markedly affected fatty acid composition of blood leukocytes by significantly reducing the EPA contents, and slightly ARA ones. Incorporation of linolenic acid was always lower than for linoleic acid. ARA/EPA values were only slightly lower than those found in plasma polar lipids and slightly higher than those found in plasma neutral lipids.

3.2. Plasma prostaglandin analysis.

Plasma PGE_2 and PGE_3 concentrations are shown in Table 5. The concentration of PGE_2 was not significantly different among the four treatments with the lowest value in fish fed FO (33.72 pg/mL) and the highest in fish fed 60 RO (44.74 pg/mL). However, the concentration of PGE_3 decreased accord-

Table 3
Plasma neutral lipid fatty acid compositions (g/100g of total fatty acids) (mean±SD)

Fatty acids	FO	60 LO	60 RO	100 LO
12:0	4.70±2.09	3.14±1.09	3.34±0.69	2.97±0.90
14:0	5.52±3.28	2.42±0.55	2.31±0.46	2.43±0.40
16:0	22.36±0.53	16.96±0.36	18.15±1.11	18.77±1.07
16:1n-7	4.13±1.11	2.55±0.40	1.99±0.15	2.09±1.13
18:0	4.10±0.97	5.70±0.23	4.89±0.55	5.07±0.64
18:1n-9 + 18:1n-7	16.46±2.34	22.78±0.72	27.31±0.60	24.65±3.37
18:2n-6	6.34±2.35[b]	13.37±3.18[a]	11.09±3.26[a,b]	10.61±0.52[a,b]
18:3n-6	0.14±0.06	0.07±0.00	0.16±0.03	0.23±0.5
18:3n-3	1.06±0.09[c]	7.09±0.44[a,b]	6.39±0.33[b]	9.11±3.71[a]
18:4n-3	0.40±0.03	0.50±0.05	0.36±0.13	0.46±0.09
20:0	0.18±0.08	0.40±0.11	0.28±0.10	0.28±0.16
20:1n-9	1.51±0.18	1.46±0.04	1.54±0.13	1.33±0.06
20:2n-9	0.09±0.01	0.18±0.05	0.18±0.01	0.37±0.04
20:2n-6	0.18±0.02	0.32±0.16	0.27±0.02	0.28±0.03
20:3n-6	0.09±0.03	0.10±0.03	0.17±0.11	0.16±0.05
20:4n-6	0.52±0.14	0.30±0.01	0.34±0.10	0.49±0.23
20:3n-3	0.38±0.02	0.38±0.11	0.35±0.07	0.36±0.10
20:4n-3	0.42±0.22	0.33±0.05	0.30±0.08	0.25±0.03
20:5n-3	6.19±1.73[a]	3.27±0.17[b]	2.85±0.59[b]	2.68±1.64[b]
22:1n-11	1.12±0.04	0.89±0.13	0.83±0.04	0.70±0.05
22:5n-6	0.37±0.08	0.21±0.01	0.22±0.01	0.22±0.01
22:5n-3	2.37±0.65	1.36±0.05	1.32±0.20	1.02±0.44
22:6n-3	14.85±4.02[a]	10.67±1.00[b]	10.54±2.07[b]	10.15±2.09[b]
Total saturates[1]	38.08±6.91	29.45±0.70	29.68±1.68	29.84±1.42
Total monoenes[2]	25.28±3.47	29.36±0.69	33.22±0.62	29.81±1.96
n-3[3]	26.32±6.13	24.05±1.40	22.52±3.01	24.92±1.54
n-6[4]	8.19±1.90[b]	14.68±3.05[a]	12.43±3.14[a,b]	12.93±1.30[a,b]
n-9[5]	18.88±2.71	25.35±1.02	29.84±0.77	26.99±2.99
n-3 HUFA	24.42±6.54	16.13±1.04	15.49±2.96	14.58±4.26
n-3/n-6	3.39±1.53[a]	1.68±0.44[b]	1.81±0.73[b]	1.93±0.08[b]
DHA/EPA	2.38±0.00	3.23±0.04	3.70±0.02	4±0.11
ARA/EPA	0.08	0.09	0.12	0.18

[1]Includes 15:0, 17:0, and 22:0. [2]Includes 16:1n-5, 18:1n-5, 20:1n-5, 20:1n-7, 22:1n-9, and 22:1n-7. [3]Includes 22:4n-3. [4]Includes 22:4n-6. [5]Includes 18:2n-9, 18:3n-9, 20:3n-9 and 22:1n-9. Includes 16:2n-3, 16:2n-4, 16:4n-3 and 16:4n-1. $n=3\times6$. Values in the same row with different superscript letters are significantly different ($P<0.05$).

ingly to the EPA content in the diet, being significantly lower ($P<0.05$) in fish fed 100 LO (Fig. 2).

The correlation between PGE_3 and its precursor (EPA) in plasma, is shown in Fig. 3. A high correlation ($r^2=0.97$) was found between plasma PGE_3 and EPA concentration with a positive relationship ($Y=3.26X+18.61$).

Table 4
The ratio between fatty acid percent in plasma lipid classes and dietary percent of the same fatty acid (% Fatty acid in plasma/%fatty acid in diet)

Fatty acid in plasma lipid classes		FO	60 LO	60 RO	100 LO
Oleic acid (OA)	Polar	0.89	0.66	0.48	0.48
	Neutral	1.41	0.93	0.91	0.75
Palmitic acid	Polar	1.19	1.25	1.46	1.38
	Neutral	1.16	1.13	1.14	1.18
Arachidonic acid (ARA)	Polar	1.94	3.00	3.75	10.83
	Neutral	0.79	0.88	1.21	8.17
EPA	Polar	0.76	1.23	1.41	4.26
	Neutral	0.52	0.54	0.59	2.53
DHA	Polar	2.02	3.57	4.19	10.93
	Neutral	1.05	1.45	1.7	4.83

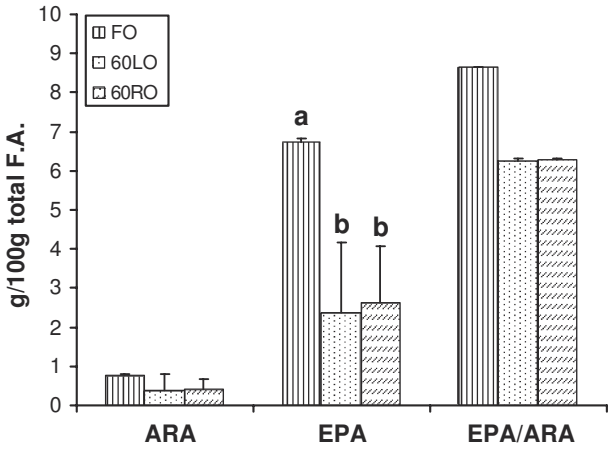

Fig. 1. Proportion (g/100 g total fatty acids) of blood leukocytes EPA, ARA and EPA/ARA. Columns assigned a different letter are significantly different ($P<0.05$).

3.3. Plasma leptin analysis

The production of the hormone leptin in plasma is shown in Fig. 4. No significant differences were seen among the four diets.

However, high negative correlation was also found between plasma leptin and PGE_2 ($r^2=0.85$; Fig. 5).

4. Discussion

Fatty acid composition of plasma lipids was markedly affected by the inclusion of VO. LA and LNA, main components of vegetable oils, significantly increased in plasma of fish fed VO, being preferentially incorporated into NL, as it has been found in other cells and tissues of this specie (Izquierdo et al., 2003, in press) and other species (Waagbo et al., 1995). Interestingly, DHGLA also increased in fish fed VO, with maximum values for the 100 LO group. DHGLA is the predominant product of desaturation and elongation in salmonids, feeding fish with diets containing high level of LA and LNA (Bell et al., 1991, 2002; Tocher et al., 2000).

Plasma DHA content was even higher than in the diets, being particularly incorporated in PL. Similar results were found in cod (*Gadus morhua*) (Waagbo et al., 1995), European sea bass (*Dicentrarchus labrax*) (Farndale et al., 1999) and seabream (Caballero, 2002). Other previous studies have also demonstrated that DHA was preferentially retained under dietary essential fatty acids deficiency (Izquierdo, 1996; Izquierdo et al., 2001; Montero et al., 2001), showing the

Table 5
Concentration of PGE_2 and PGE_3 in plasma from seabream fed experimental diets (mean±SD)

Prostaglandins	FO	60 LO	60 RO	100 LO
PGE_2 (pg/mL)	33.72±6.52	41.87±7.18	44.74±16.47	35.15±4.58
PGE_3/PGE_2	1.45±0.33	1.00±0.22	0.91±0.46	0.96±0.12

$N=3\times6$. Values in the same row with different superscript letters are significantly different ($P<0.05$). The specificity of the antibody was 100% for PGE_2 and 43% for PGE_3.

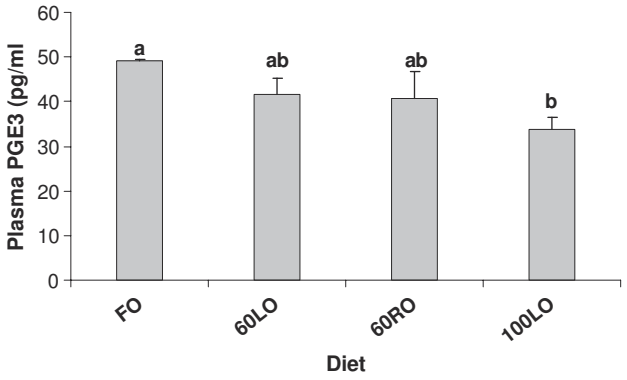

Fig. 2. Plasma concentration of PGE_3 (pg/mL) from seabream fed the experimental diets. Columns assigned a different letter are significantly different ($P<0.05$).

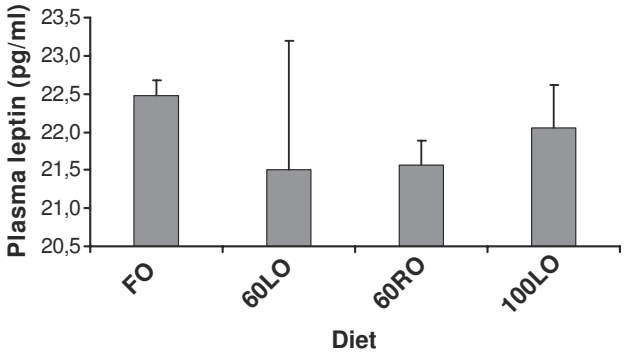

Fig. 4. Leptin concentration in plasma from seabream fed the four experimental diets.

importance of this fatty acid as a major structural component of fish cell membranes (Watanabe, 1993; Sargent et al., 1995). Selective incorporation of DHA into fish lipids is directly related with the specificity of some PL synthesis enzyme complexes such as 1-lysophosphatidylacylCoA transferase (Gurr and Harwood, 1991; Caballero, 2002) and the affinity for fatty acid binding proteins (FABP) (Sire and Vernier, 1981).

ARA, EPA and DHA were preferentially incorporated into PL lipids of plasma, and DHGLA accumulated with the increased VO inclusion. The higher content of EPA in plasma PL suggest the selective incorporation of this fatty acid into membrane lipids in agreement with the results found in sea bass leukocytes (Farndale et al., 1999) and seabream anterior kidney macrophages (Montero et al., 2003). A selective deposition of ARA in PL was found at low dietary concentrations in agreement with previous results found in other cellular types of this specie (Montero et al., 2003; Fountoulaki et al., 2003) or other species such as cod (Waagbo et al., 1995) or sea bass (Farndale et al., 1999), and denoting the importance of this fatty acid for optimal cell function. Besides, this fatty acid is retained in essential fatty acid deficient fish (Izquierdo, 1996) and is incorporated and retained in PI of seabream (Mourente and Tocher, 1993) and turbot (*Scophtalmus maximus*) (Linares and Henderson, 1991).

The dietary treatments employed in the present study have resulted in profound alterations of DHGLA/ARA ratios (ratio in table), but not in ARA/EPA (Table 2). ARA, EPA and DHGLA are all precursors for eicosanoid production (Bell et al., 1994a) and changes in the ratios of these HUFA had important consequences for the quantity and spectrum of eicosanoids produced by turbot (Bell et al., 1998). ARA-derived PGE_2 production in plasma was not affected by VO, in agreement with similar eicosanoid precursor ratio (ARA/EPA) in leukocytes total lipid and plasma phospholipids among fish fed the different dietary treatments. However, reduction in dietary ARA/EPA lead to a significant reduction in PGE_2 concentration in heart, brain and kidney in turbot (Bell et al., 1995). This controversy may be related to dietary, tissue or species differences between both studies.

Interestingly, the production of PGE_3 was significantly different among fish fed the four experimental diets and strongly correlated with plasma polar lipid concentrations of its precursor, EPA. Moreover, PGE_3 was the major prostaglandin produced in plasma of fish fed FO, in contrast with previous studies conducted "in vitro" in brain astroglial turbot cells and pineal organ of Atlantic salmon (*Salmo salar*) (Bell et al.,

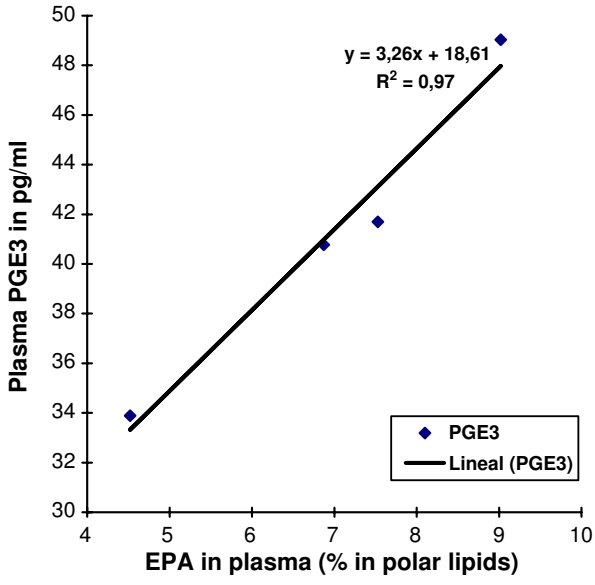

Fig. 3. The relationship between PGE_3 (pg/mL) and EPA (percent in polar lipids) concentration in plasma from seabream fed the experimental diets with pink dots $r^2=0.97$ and line $Y=3.26x+18.61$.

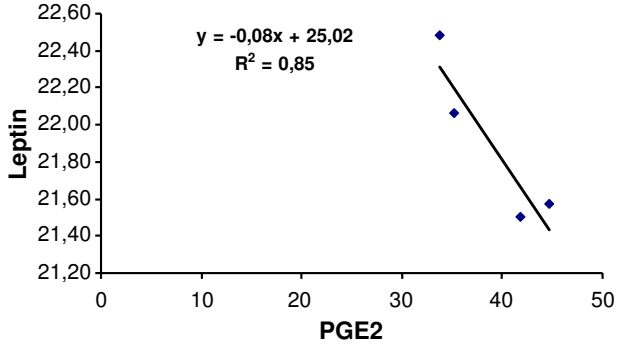

Fig. 5. Relation between plasma leptin and PGE_2 in seabream fed the four experimental diets with pink dots $r^2=0.85$.

1994a; Henderson et al., 1996). Such findings remarks the importance of EPA as a precursor of PG in marine fish, at least for the correct function of their blood cells, and correlates well with the predominant role of this fatty acid in immune regulation in this specie (Montero et al., 1998). Nevertheless, PG production markedly differs among tissues of the same species. For instance, PGE_2 production was higher in turbot kidney macrophages than in blood leukocytes (Tafalla et al., 1999). In the present study, feeding VO lead to a decrease in plasma EPA which in turn reduced plasma PGE_3 concentration. PL fatty acid composition determines the physical properties of cell membranes, influencing the activities of membrane-associated proteins and enzymes (Spector and Yorek, 1985) and the catalytic activity of the phospholipase A_2 enzymes (Bell et al., 1996). Since a previous study conducted in rainbow trout (*Salmo gairdneri*) has demonstrated that blood erythrocytes are not capable of eicosanoid synthesis (Pettit et al., 1989), plasma eicosanoids reflect principally leukocyte or vascular products. Despite that the plasma PGE_2 levels were not being significantly different, the lower production in fish fed FO may be explained by the higher content of EPA in the PL of these fish, since this fatty acid is a potent competitor of ARA. Hence, these results show that substitution of FO with VO in diets for seabream modifies plasma PUFA composition profoundly, affecting the 3-series prostaglandin production.

Increased in DHGLA in polar lipid fatty acid composition could be related with the results obtained in PG production. For instance, supplementation with both DHGLA and EPA significantly reduced ARA-derived prostaglandin production in salmon (Bell et al., 1993) suggesting a competitive inhibition by those fatty acids at the cyclooxygenase active site (Bell et al., 1996). Also, phospholipase A_2 activity, which is key to release of eicosanoid precursor fatty acids from cell membranes, is affected by dietary fatty acids. In salmon fed a diet with reduced n-3/n-6 PUFA ratio, the phospholipase A activity was increased in cardiac tissue compared to those fed fish oil (Bell et al., 1993). The ability of DHGLA and EPA to attenuate the production of ARA-derived eicosanoids is fundamental in the control of pathophysiological processes in numerous inflammatory conditions occurring in humans (Horrobin, 1992).

Recently, the interaction between leptin and prostaglandin release has been described by Zerani et al. (2005) who suggested that circulating levels of leptin may act as a metabolic signal modulating PG release through mediation of the nitric oxide synthase/nitric oxide system. In the present study a negative correlation was found between plasma PGE2 and leptin plasma concentration in agreement with the results found by Zerani et al. (2005). Moreover, Campbell et al. (1998) demonstrated that leptin electrophoretic mobility depends markedly on the free fatty acids in plasma, increases of oleic acid inducing a decrease of leptin mobility. This may result in a change of conformation of leptin and consequently may affect the transport/binding of this protein to target tissues. In agreement with these findings, oleic acid contents in plasma neutral lipids were negatively correlated to leptin levels, which in turn were negatively related with PGE_2. Besides, an effect of arachidonic acid increasing the release of leptin has been also described (Fain et al., 2001), in agreement with the results obtained in the present study, when fish with higher levels of ARA in plasma neutral lipids showed the lower levels of PGE2. However, further experiments are required to clarify the interaction between plasma fatty acids and leptin levels.

In summary, the present study shown that increased inclusion of VO in diets for seabream may profoundly affect the fatty acid composition of plasma and leukocytes, especially HUFA, and consequently the production of PGE_3, which can be a major PG in plasma. Alteration in the amount and type of PG produced can be at least partially responsible for the changes in the immune system and health parameters of fish fed diets with high inclusion of VO (Blazer, 1992; Thompson et al., 1996; Montero et al., 2003). Further research is needed to clarify the effects of dietary fatty acids on plasma leptin levels and its relation with prostaglandins production.

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Chapter 6

Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*

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Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream, *Sparus aurata*

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Abstract

Highly unsaturated fatty acids are essential components of cellular membranes of vertebrates and can modulate physiological processes, including membrane transport, receptor function and enzymatic activities. In gilthead sea bream, dietary deficiencies of essential fatty acids of marine fish raise the basal cortisol levels and alter the pattern of cortisol release after stress. The aim of the present study was to clarify the effect of different essential fatty acids on adrenocorticotropic hormone (ACTH)-induced cortisol production and release in fish, through *in vitro* studies of sea bream interrenal cells maintained in superfusion and incubated with different types of fatty acids and eicosanoid production inhibitors. Results showed the first evidence of the effect of certain fatty acids on cortisol production by ACTH-stimulated interrenal cells in fish. Both arachidonic acid (ARA) and particularly eicosapentaenoic acid (EPA) promoted cortisol production in sea bream interrenal cells. Moreover, incubation with indometacin (INDO) reduced the increased cortisol production induced by EPA and ARA, suggesting mediation by their cyclooxygenase-derived products. Docosahexaenoic acid stimulated cortisol production to a lesser extent than that caused by EPA or ARA, but the inhibitory effect of INDO was not as marked as it was for the other fatty acids. In contrast, supplementation with dihomogammalinoleic acid reduced cortisol production, denoting the inhibitor effect of this fatty acid in cortisol secretion.

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Introduction

Highly unsaturated fatty acids with 20 or more carbon atoms and three or more double bonds (HUFA) are essential components of cellular membranes and can modulate physiological processes, including membrane transport, receptor function and enzymatic activities. Hence, dietary fatty acids have been shown to have marked effects on a variety of immunological and haemostatic parameters (Balfry *et al.* 2001, Montero *et al.* 2001). HUFA possess a wide range of cellular functions. One of the most important functions is to supply precursors for the synthesis of eicosanoids, which are produced in response to various extracellular stimuli by two main types of dioxygenase enzymes: cyclooxygenases (COX) and lipoxygenases (Horrobin 1983). Following cell stimulation, both arachidonic acid (ARA; 20:4n-6) and eicosapentaenoic acid (EPA; 20:5n-3) are released from the membrane by the action of phospholipase A_2. Later these fatty acids are transformed by a range of lipoxygenases and cyclooxygenases to yield prostaglandins (PG), leukotrienes, lipoxins and other compounds, which can modulate several immune functions (Uhing *et al.* 1990).

Eicosanoids have been found in a large range of freshwater and marine fish (Matsumoto *et al.* 1989, Mustafa & Srivastava 1989) and in many tissues (Henderson & Tocher 1987, Bell *et al.* 1994*a*, Tocher 1995). In fish, a preferred eicosanoid precursor for cyclooxygenase seems to be ARA (20:4n-6) (Tocher & Sargent 1987, Bell *et al.* 1994*a*, 1994*b*, 1998), but EPA (20:5n-3) and dihomo-γ-linolenic acid (DHGLA; 20:3n-6) are also important eicosanoid precursors which can modulate production and biological efficacy of ARA-derived eicosanoids (Horrobin 1983, Bell *et al.* 1994*a*, Ganga *et al.* 2005). In addition, the high content of docosahexaenoic acid (DHA; 22:6n-3) in cellular membranes affects eicosanoid production (Nablone *et al.* 1990). This fatty acid is also recognised as a precursor of certain biologically active trioxilated derivatives (German *et al.* 1983, Hong *et al.* 2005). Therefore, the supply of precursor polyunsaturated fatty acids with 18 or more carbon atoms and two or more double bonds (PUFA) for eicosanoid synthesis is directly related to the fatty acid composition of membrane phospholipids, which in turn is influenced by dietary PUFA intake and metabolism (Lands 1989).

In gilthead sea bream, dietary deficiencies on n-3 HUFA, essential fatty acids for marine fish (Izquierdo 1996), raised the basal plasma cortisol levels and altered the pattern of cortisol release after stress (Montero *et al.* 1998). Cortisol is a key corticosteroid hormone for homeostatic response to stress

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in all vertebrates, through its effects on metabolism and immune function (Hontela 1997, Wendelaar Bonga 1997) as well as the osmoregulation process (Wendelaar Bonga 1997). Thus, the increase in plasma cortisol levels is regarded as the most reliable method for differentiating between stressed and non-stressed fish (Thompson *et al.* 1993, Yin *et al.* 1995, Rotllant & Tort 1997). Moreover, feeding relatively low levels of n-3 HUFA, although not affecting growth and feed efficiency, significantly raised plasma cortisol levels (Montero *et al.* 2003).

However, the physiological mechanisms by which these HUFA regulate the hormone-induced plasma cortisol levels are not clear. In fish, several studies have suggested that ARA is involved in the release of cortisol, although the actual mechanisms have not been investigated (Gupta *et al.* 1985, Bessonart *et al.* 1999, Harel *et al.* 2001, Koven *et al.* 2003, Van Anholt *et al.* 2004). In mammals, certain studies suggest that PG play an important role in mediating the corticosteroidogenic action of adrenocorticotropic hormone (ACTH) (Kocsis *et al.* 1999), and thus the role of fatty acids in stress response seems to be mediated by the production of eicosanoids.

The present study aims to clarify the effect of different HUFA on ACTH-induced cortisol production and release by gilthead sea bream interrenal cells.

Material and Methods

Animals

Sexually immature gilthead sea bream (*Sparus aurata*) of body weight $54{\cdot}7 \pm 11{\cdot}2$ g supplied by a Spanish fish farm (Masnou, Barcelona, Spain) were kept for 2 weeks in two fibreglass tanks of 1000 l held in a semi-closed seawater circulation system equipped with physical and biological filters. Water temperature was maintained at 16–18 °C, the salinity at 35–40% and photoperiod at 12 h light:12 h darkness. Fish were fed once a day with a commercial feed until 24 h before the *in vitro* trials to avoid feed interference. A total number of 30 fish were employed in the experiments.

Superfusion trials

After 2 weeks of acclimatisation, fish were randomly taken from the tanks in less than 1 min, immediately anaesthetised with 2-phenoxyethanol (1:1000 v/v) and blood collected with a hypodermic syringe from the caudal vein to minimise the haemorrhage. Head kidney tissue was removed from two fish in each superfusion trial and cut into very small fragments in Hepes Ringer medium, which was used as the superfusion medium. Afterwards, head kidney homogenates were pooled and distributed in eight superfusion chambers (volume: 0·2 ml) in order to obtain a homogeneous aliquot from each of them. Tissues were superfused with a Hepes (pH 7·4) Ringer's solution containing 171 mM NaCl, 2 mM KCl, 2 mM $CaCl_2H_2O$, 0·25% (w/v) glucose and 0·03% (w/v) bovine serum albumin (Rotllant *et al.* 2001). The system was temperature-controlled at 15 °C and superfusion medium was pumped through the chamber at a rate of 75 µl/min by a Masterplex L/S^R multichannel peristaltic pump (Cole Parmer Instrument Co. Vernon Hills, IL, USA).

Trials were started after 3 h of superfusion when cortisol reached a stable baseline level (Rotllant *et al.* 2000*a*, 2000*b*) due to several factors such as the different dispersion of interrenal cells in the perfusion preparation, individual differences and the pre-stress level of each fish. After the stabilisation period of 3 h, tissues were subsequently incubated with different fatty acids. A series of preliminary tests were performed in quadruplicate, to determine the adequate fatty acid concentration (50, 150 or 300 µM) and incubation time (1 or 3 h) for any of the three fatty acids assayed (ARA, EPA and DHA). Best cortisol stimulation was found with fatty acid concentrations of 50 µM and an incubation time of 1 h (Table 1) and these conditions were used afterwards in all the research experiments. Both in these preliminary tests and in the research experiments, perfusion medium was supplemented with the corresponding concentration of different fatty acids ARA, EPA, DHA and DHGLA (diluted in less than 0·5% of ethanol/medium v/v) prior to tissue incubation. In a second series of experiments to clarify the action mechanisms of these fatty acids, tissues were incubated with a COX inhibitor indometacin (INDO) for 20 min at a concentration of 25 µM diluted in superfusion medium. After incubation with the fatty acids, the perfused tissues were stimulated with ACTH at a concentration of 5 nM $hACTH_{1–39}$ (Sigma) for 20 min. Subsequently, perfusion was maintained for another 170 min, fraction samples being collected every 20 min during this period. Cortisol stimulation factor was calculated by the comparison of maximum cortisol released after ACTH stimulation with baseline cortisol released (maximum release − baseline release)/(baseline release) (Rotllant *et al.* 2001). In all the series of experiments, each treatment was assayed in quadruplicate.

Cortisol measurements

Cortisol concentration in the perfused fluid was determined by RIA (Rotllant *et al.* 2001). The antibody used for the assay was purchased from Biolink, S.L. (Costa Mesa, CA, USA) in a

Table 1 Effect of two fatty acid concentrations (50 and 150 µM) and two incubation times (1 h and 3 h) for three polyunsaturated fatty acids on cortisol secretion stimulation factor

	1 h	3 h	
Treatment			
Control	$14{\cdot}71 \pm 2{\cdot}41$	13·28	
EPA	50 µM	$29{\cdot}63 \pm 2{\cdot}59$	$7{\cdot}79 \pm 3{\cdot}29$
	150 µM	$7{\cdot}79 \pm 3{\cdot}29$	–
ARA	50 µM	$22{\cdot}26 + 6{\cdot}29$	$11{\cdot}75 \pm 4{\cdot}16$
	150 µM	$12{\cdot}25 \pm 1{\cdot}86$	–
DHA	50 µM	$35{\cdot}72 \pm 9{\cdot}28$	$2{\cdot}60 \pm 1{\cdot}16$
	150 µM	$4{\cdot}47 \pm 0{\cdot}28$	–

final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11·40% with 21-desoxycorticosterone, 8·90% with 11-desoxycortisol and 1·60% with 17α-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels are given as ng/g/h.

Statistical analysis

Significance of difference ($P<0·05$) between dietary treatments was determined by ANOVA, followed by Duncan's multiple comparison test (Sokal & Rolf 1995). Analyses were performed using SPSS software (SPSS for Windows 11·5; SPSS Inc., Chicago, IL, USA).

Results

The different incubation times and fatty acid concentrations assayed showed that 1 h of incubation time and a concentration of 50 μM of fatty acid were the best conditions to obtain the highest effect of fatty acid on cortisol secretion stimulation factor (Table 1). As expected, after the stabilisation period of 3 h, cortisol values remained at basal levels for these fish species and no significant differences were found among basal values for the different superfused tissues (Fig. 1).

The effects of supplementation with different HUFA on cortisol secretion are illustrated in Fig. 1. The production of cortisol by interrenal cells was modified when the medium was supplemented with HUFA in comparison with the control. Addition of n-3 fatty acids, DHA and EPA induced a higher and earlier cortisol response to ACTH than the control without fatty acid incubation. Addition of n-6 fatty acids did not modify the time of cortisol response in comparison to the control, but induced a higher response. Cortisol response was higher when ARA, EPA or DHA was added to fatty acid and lower when DHGLA was used. Such response expressed as stimulation factor was significantly ($P<0·05$) higher with EPA (33·71±4·5 basal secretion) and ARA (28·7±4·47) incubation than control and DHGLA treatment groups (Fig. 2). With DHA incubation, no significant differences were found in the stimulation factor. By contrast, DHGLA showed the lowest ($P<0·05$) stimulation factor with an increase of only 8·95±2·17.

Supplementation of INDO, a COX inhibitor, induced the stimulation of cortisol production by EPA and ARA observed in the former set of experiments, with cortisol absolute values not being different from those of the control (Fig. 3). However, a significantly higher ($P<0·05$) cortisol peak was obtained when the tissue was incubated with DHA, despite the addition of INDO (Fig. 3).

Comparison of cortisol stimulation factors when INDO was added showed a significantly higher ($P<0·05$) cortisol secretion in the tissue supplemented with EPA, ARA and DHA (Fig. 4). Thus, the stimulation factor of cortisol was 7·83±3·31 when tissue was supplemented with EPA, 6·97±4·56 with ARA, 13·67±2·66 with DHA and only 1·58±0·45 for control.

In addition, the comparison of cortisol stimulation factors between experiments with or without INDO showed that the addition of INDO significantly decreased ACTH-stimulated cortisol secretion in all the treatment use of this COX inhibitor (Fig. 5). However, this impaired stimulation of cortisol production was lower in the DHA-supplemented group in which INDO caused a 40·84% reduction in cortisol secretion, giving values that were significantly different ($P<0·05$) compared to EPA treatment where INDO caused

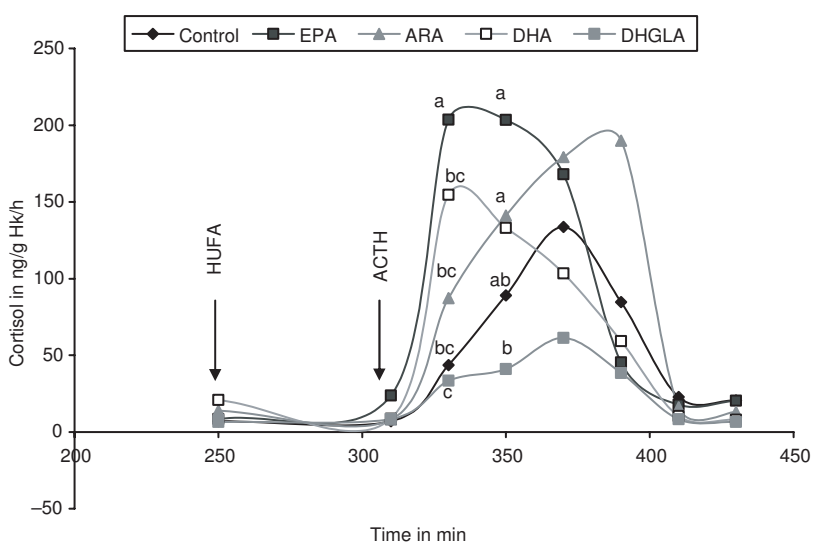

Figure 1 Absolute cortisol secretion (ng/g Hk/h) by sea bream head kidney (Hk) after ACTH stimulation following incubation with HUFA (different letters for a given time indicate significant difference, $P<0·05$).

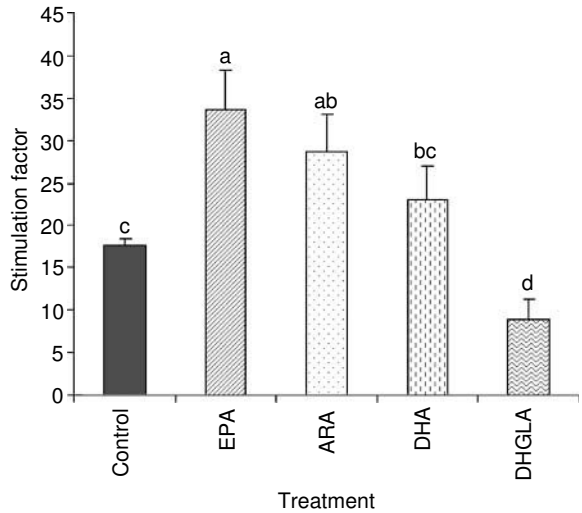

Figure 2 Cortisol stimulation factor in sea bream head kidney after ACTH stimulation following incubation with different HUFA (different letters for different treatments indicate significant difference, $P<0{\cdot}05$ ANOVA).

a 76·76% reduction in cortisol secretion and a 75·71% reduction with ARA treatment.

Discussion

The present study showed the first evidence of the effect of HUFA on cortisol production by ACTH-stimulated interrenal cells in fish. These results are in agreement with the observed modulating effect of dietary fatty acids in sea bream plasma cortisol levels (Montero *et al.* 1998, 2001) and confirm the hypothesis of these authors about the effective action of these fatty acids on the cortisol secretion by the interrenal cells in gilthead sea bream.

Both ARA and EPA promoted ACTH-induced cortisol production in sea bream interrenal cells used in the present experiment. Dietary EPA has been shown to affect fish stress resistance in several species. Although it promoted the growth and survival of the red sea bream (Watanabe *et al.* 1989), gilthead sea bream (Liu *et al.* 2002) and Japanese flounder (Furuita *et al.* 1998), its effects on larval stress resistance seem to depend on species and dietary levels. For instance, elevation of dietary EPA increased red sea bream handling stress resistance (Watanabe *et al.* 1989) and gilthead sea bream resistance to air exposure and temperature shock, but not to salinity stress (Liu *et al.* 2002). On the contrary, too high EPA levels reduced stress resistance to air exposure in Japanese flounder (Furuita *et al.* 1998). ARA has also been shown to affect stress resistance in several fish species. Dietary ARA levels of about 1% dry weight feed are necessary not only for optimum growth and survival of sea bream larvae (Bessonart *et al.* 1999), but also for improved stress resistance after handling (Koven *et al.* 2003, Van Anholt *et al.* 2004). Dietary ARA levels close to those used by these authors did not affect the handling of stress resistance in Japanese flounder, whereas higher ones reduced larval stress resistance (Furuita *et al.* 1998).

These differences of the effects of dietary EPA or ARA on stress resistance in different species may also be related to different ratios among these fatty acids, since both are competing substrates for cyclooxygenase enzymes (Izquierdo *et al.* 2001). For instance, in Atlantic salmon, alteration in the dietary ratio of n-3/n-6 fatty acids has been shown to prevent stress susceptibility to transport (Bell *et al.* 1991). The present

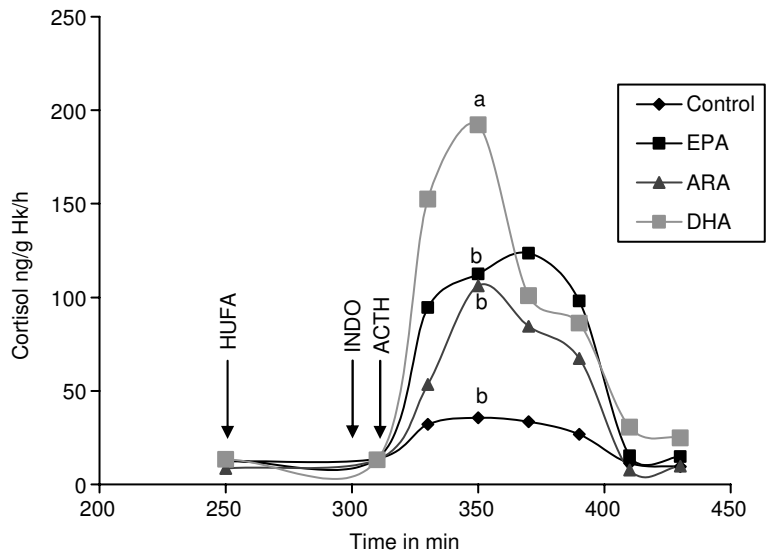

Figure 3 Absolute cortisol secretion (ng/g Hk/h) by sea bream head kidney (Hk) after ACTH stimulation following incubation with different HUFA and INDO supplementation (different letters for a given time indicate significant difference, $P<0{\cdot}05$).

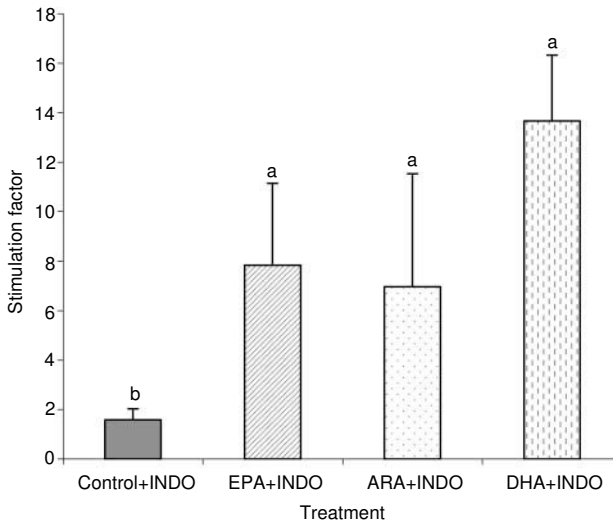

Figure 4 Cortisol stimulation factor in sea bream head kidney after ACTH stimulation following incubation with different HUFA and INDO supplementation (different letters for indicate significant differences among treatment).

study shows the first evidence found in fish that cyclo-oxygenase-derived metabolites are involved in ACTH-induced cortisol release by interrenal cells. The strong reduction of cortisol release caused by INDO addition in EPA and ARA supplemented groups suggested that the effect of these fatty acids was, at least partly, mediated by their cyclooxygenase-derived metabolites. Both fatty acids have been found to be good precursors of cyclooxygenase-derived PG in fish (Bell *et al.* 1994*a*, Ganga *et al.* 2005). In turn, cyclooxygenase-derived PG have been shown to increase *in vitro* cortisol release in interrenal tissue of female frogs during

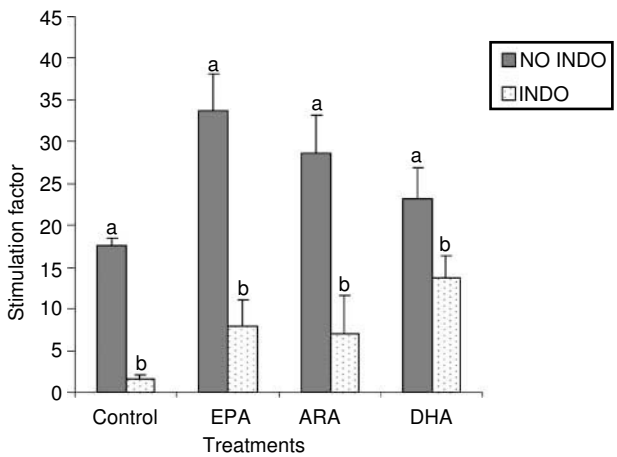

Figure 5 Comparison of cortisol stimulation factor in sea bream head kidney after ACTH stimulation following incubation with different HUFA and including (filled bars) or not (dotted bars) INDO supplementation (different letters for control or each fatty acid incubation indicates significant differences by INDO addition).

ovulation (Gobbetti & Zerani 1993) and in human adrenal cells as well (Vakharia & Hinson 2005).

Interestingly, DHA stimulation of ACTH-induced cortisol production was lower than that caused by EPA or ARA. Besides, the inhibitory effect of INDO in the DHA-supplemented group was not so marked as in the other treatments, suggesting that the action of DHA in cortisol release from ACTH-stimulated interrenal cells is less dependent on COX metabolites in gilthead sea bream. Indeed, this fatty acid is a poorer substrate for COX than EPA or DHA. The action of DHA on interrenal cells, whether it is direct or mediated by its lipoxygenase derivatives, still has to be elucidated since lipoxygenase metabolites have been shown to modify the hormone-induced release of cortisol in mammal adrenal tissues (Wang *et al.* 2000, Yamazaki *et al.* 2001). Using nordihydroguaiaretic acid, a lipoxygenase inhibitor, cortisol secretion was inhibited in response to ACTH in bovine adrenocortical cells (Wang *et al.* 2000).

DHA has long been known for its high value as an essential fatty acid for marine fish (Watanabe 1982), particularly during larval stages (Izquierdo *et al.* 1989) when it invariably promotes growth, survival and stress resistance to a higher extent than EPA or ARA in all the studied species (Watanabe *et al.* 1989, Kanazawa 1997, Rodríguez *et al.* 1997, Furuita *et al.* 1999, Izquierdo *et al.* 2005). In gilthead sea bream, dietary deficiencies of n-3 HUFA and especially DHA have been shown to increase plasma cortisol levels after both acute (net chasing) and chronic (high stocking density) stress (Montero *et al.* 1998, 2001). Besides, imbalances in the dietary n-3/n-6 fatty acids ratio induced by the inclusion of vegetable oils in the diet have been shown to alter the release of cortisol after stress in this species (Montero *et al.* 2003) and in other species such as chinook salmon (Welker & Congleton 2003). The role of dietary oils on stress response in fish remains unclear, but results indicate that dietary fatty acids could be regulating the *in vivo* stress response through the mechanisms discussed above. Moreover, vegetable oils in fish diets have been shown to regulate COX-derived eicosanoids directly (Ganga *et al.* 2005). Dietary supplementation of other fatty acids such as ARA seems to be affecting plasma cortisol levels after stress (Van Anholt *et al.* 2004), although the effect on cortisol release *in vivo* is dose dependent, since high levels of ARA in diet seem to be detrimental to chronic stress resistance in larval gilthead sea bream (Koven *et al.* 2003).

Concentrations of fatty acids used in the present study were those providing the maximum cortisol stimulation factor (50 μM). However, higher concentrations reduced and even inhibited cortisol secretion (Acerete L, Ganga R, Tort L & Izquierdo MS. unpublished results), suggesting a concentration dependency in the type of effect of these fatty acids. This is the case in other organs such as testicles where medium concentrations of ARA (3-30 μM) induced testosterone production in testicular cells of male sea bass, whereas high concentrations (300 μM) inhibited it (Asturiano 1999). Despite the fact that no previous data has been published on *in vitro* exposures of fish interrenal cells to fatty acids, cytotoxic

effects at the membrane level have been found in mammalian tissues, including renal cells (Zager *et al.* 1997). Particularly, excess of ARA and its derived eicosanoids has been found to cause apoptosis associated with oxidative stress in human leukocytes (Pompeia *et al.* 2002). Studies are being conducted at present to elucidate the effect of low and high physiological concentrations of fatty acids on ACTH-induced cortisol release by fish interrenal cells. Although this superfusion method is widely used for studies of interrenal tissue in fish (Rotllant *et al.* 2001), it prevents the possible control of blood flow by COX-derived prostanoids. Nevertheless, new results have shown the vasoregulatory function of COX-derived products in fish (Stensløkken *et al.* 2002), none of them relate though to interrenal tissues.

In summary, the role of ARA and EPA in the ACTH-induced release of cortisol from gilthead sea bream interrenal cells seems to be partly related to COX-derived metabolites dependent on ARA and EPA, whereas the role of DHA seems to be dependent on other factors. Both EPA and ARA as single supplemented fatty acids increased the ACTH-induced cortisol release from gilthead sea bream interrenal cells whereas the effect of DHA was weaker. A well-balanced supplementation of these three fatty acids could be necessary to regulate cortisol release from interrenal cells in gilthead sea bream.

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Chapter 7

ACTH-stimulated cortisol-release by the head-kidney interregnal tissue from seabream (*Sparus aurata*) fed with linseed and soybean oil

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ACTH-stimulated cortisol-release by the head-kidney-interrenall tissue from seabream (Sparus aurata) fed with linseed and soybean oil

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Abstract

The mode of action of highly unsaturated fatty acids (HUFA) in the regulation of gilthead seabream (S*parus aurata*) interrenal cortisol production was studied through *in vitro* trials using a dynamic superfusion system. Fish were previously fed with different diets containing several levels of inclusion of linseed oil (LO) or soybean oil (SO) for a period of 26 weeks. Five diets were tested, with anchovy oil as the only lipid source for the control diet (fish oil, FO), two different substitution levels (70 and 100%) were tested using either linseed or soybean oils (70LO, 70SO, 100LO, 100SO). Fatty acid compositions of the head kidney reflected the dietary input, thus EPA, DHA, ARA and n-3 HUFA were significantly ($P<0.05$) reduced in fish fed VOs compared to the FO diet. Feeding 70 % or 100% LO increased significantly ($P<0.05$) cortisol release in head kidney after stimulation with adrenocorticotrophic hormone (ACTH), while feeding SO decreased but not significantly this response. Cortisol stimulation factor was increased in fish fed 70LO and 100LO diets compared to control. Moreover, inhibition of eicosanoid production by incubating the head kidney tissue with indomethacin, (INDO) as a cycloxygenase inhibitor, or nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor, significantly reduced ($P<0.05$) the cortisol release after ACTH stimulation in 70LO, 100LO and 100SO. Cortisol stimulation factor was reduced when incubating the head kidney from FO, 70LO, 100LO with INDO or NDGA, while it was increased in 70SO. This experiment described the modulation of cortisol release by dietary HUFAs and the mediation of cyclooxygenase and/or lipooxygenase in their mechanism of action.

Key words: Gilthead seabream, HUFA, LO, SO, INDO, NDGA, cortisol, head kidney, superfusion

Introduction

Marine teleosts have requirements for the essential long chain highly unsaturated fatty acids of the n-3 series (HUFA), docosahexaenoic acid (DHA, 22:6*n*-3) and eicosapentaenoic acid (EPA, 20:5*n*-3) (Watanabe *et al.,* 1983a,b; Izquierdo *et al.,* 1989, Izquierdo, 1996). Other studies have also pointed out the importance of the long chain *n*-6 HUFA, arachidonic acid (ARA, 20:4*n*-6), as essential for marine fish (Castell *et al.,* 1994; Bessonart *et al.,* 1999). These three fatty acids, as components of phospholipids, constitute a critical part of the cell membrane of most tissues and are responsible for maintaining an adequate physiological response of the cells (Gurr and Harwood 1991; Sargent *et al.,* 1993a), being particularly important to promote stress resistance and defence against pathogenic challenge (Montero *et al.,* 1998).

Stress in fish is monitored by plasma cortisol levels which is a general indicator of stressful conditions in vertebrates (Pickering and Pottinger 1989) and its release into the circulation is controlled by the hypothalamus–pituitary–interrenal axis. The cortisol release is preceded by the stimulation of the interrenal tissue by secretion of pituitary hormones, in particular the adrenocorticotrophic hormone (ACTH) (Donaldson, 1981). Although some other hormones have been shown to stimulate cortisol release from interrenal tissue, ACTH is the dominant secretagogue (Schreck *et al.,* 1989, Arnold-Reed and Balment, 1991, Lamers *et al.,* 1992).

The understanding of the pathways and intracellular messengers that regulate cortisol synthesis in fish is very limited. The cortisol synthesis by ACTH has been found to be dependent on cAMP as an intracellular second messenger in the coho salmon (*Oncorhynchus kisutch*) (Patiño *et al.,* 1986**).** In mammals, the main pathway leading to corticosteroid synthesis by ACTH stimulation involves a signalling cascade integrating G-proteins, adenylyl cyclase, cAMP and protein kinase A (PKA) (Schimmer, 1995). Other pathways, involving protein kinase C (PKC) via stimulation by angiotensin II (AII) or acetylcholine, both known secretagogues of cortisol in fish (Kloas *et al.,* 1994), share a role in the regulation of corticosteroid synthesis (Bird *et al.,* 1990). Moreover, PKA has been suggested to be a crucial stimulatory component in the ACTH-mediated signalling pathway in fish adrenal steroidogenesis (Lacroix and Hontela 2001), whereas PKC has been shown to have an inhibitory role in the acute cortisol response in fish adrenocortical cells (Lacroix and Hontela 2001).

How lipids and mainly HUFA could modulate cortisol release in vertebrates is not well known. In a previous study, we showed that free HUFA modulate cortisol secretion in seabream head kidney (HK) maintained in superfusion (Ganga *et al.,* 2006). ARA, EPA and DHA stimulated cortisol release by interrenal tissue while dihomogamalinolenic acid (DHGLA; 20:3n-6) inhibited release Besides, it was demonstrated that ACTH induced cortisol release is partly mediated by cycloxygenase (COX) metabolites (Ganga *et al.,* 2006). Moreover, PGE2 derived from ARA has been shown to modulate the sensitivity of the hypothalamus–pituitary–adrenal (HPA) axis, which is responsible for the release of cortisol in response to stress in mammals (Lands, 1991; Nye *et al.,* 1997), and possibly also in the homologous hypothalamus–pituitary–interrenal (HPI) axis in fish (Gupta *et al.,* 1985).

Fish oil (FO) has been the main source of HUFA in aquafeeds, but in recent years, due to the concerns about FO sustainability and cost, alternative oil sources are frequently included in fish feeds. FO partial replacement by vegetable oils (VO) does not affect fish growth or feed utilisation in several species (Torstensen *et al.,* 2000; Bell *et al.,* 2001, 2002; Caballero *et al.,* 2002; Izquierdo *et al.,* 2003, 2005; Montero *et al.,* 2005). Nevertheless, high substitution levels by VO have been shown to alter fish resistance to stressful conditions by increasing their cortisol levels (Montero *et al.,* 2003). However, little is known about the physiological mechanisms involved in the regulation of stress by fatty acids.

Since vegetable lipids lack HUFA, its dietary inclusion may modify tissue composition, altering cell membrane fluidity, receptor mediated cortisol response and finally leading to a reduced functionality of the target organ. In addition, vegetable lipids can reduce the availability of eicosanoid precursors ARA, EPA, DHGLA and DHA leading to dysfunctional eicosanoid signalling (Balfry and Higgs, 2001; Tocher *et al.,* 2000) which may affect fish response to stress.

The aim of the present study was to clarify the effect of replacing FO by linseed and soybean vegetable oils, in the gilthead seabream (*Sparus aurata*) stress response to ACTH stimulation. For this purpose, the HK tissue was maintained in a superfusion system and incubated with inhibitors of eicosanoid production. Accordingly, five diets with different levels of FO substitution (0, 70 and 100), either with linseed oil (LO) or soybean oil (SO) were fed to gilthead seabream juveniles.

Materials and Methods

Animals

The study was carried out at Instituto Canario de Ciencias Marinas (ICCM, Canary Islands, Spain), and fish were purchased from a local fish farm (ADSA, Canary Islands. Spain). A total of seven hundred and fifty gilthead seabream (*Sparus aurata*), with an average initial body weight of 45 g were randomly distributed in 500 l polyethylene circular tanks (45 fish/tank, 3 tanks per diet). Tanks were supplied with continuous seawater (36 ‰) flow and aeration. Fish were fed under natural photoperiod conditions of approximately 12 h/12 h dark-light. Water temperature and dissolved O_2 concentration during the experimental period ranged from 20.0 to 24.2 ºC, and from 5.04 to 8.7 ppm respectively.

Diets

Fish were fed the experiment diets (Table 2.3) until apparent satiation (3 times/day, 6 days/week), until they reached commercial market size after 26 weeks. Five iso-energetic and isonitrogenous experimental were diets formulated to provide a lipid content of 16 % were formulated. Anchovy oil was the only added lipid source in the FO diet (100% fish oil). All the other diets contained vegetable oils: to substitute either 70% of the anchovy oil by linseed oil (LO) in diet 70LO, by soybean oil (SO) in diet 70SO or 100% in diets 100LO (with LO) and 100SO (with SO). Fish oil was included in diets 70LO and 70SO at a level high enough to meet the EFA requirements of this species (Kalogeropoulos *et al.,* 1992).

Biochemical analysis

Lipid from the experimental diets and fish HK were extracted with chloroform: methanol (2:1 v:v) as described by Folch *et al.,*(1957). The fatty acid methyl esters were obtained by transesterification with 1 % sulphuric acid in methanol and purified by absorption chromatography on NH_2 Sep-pack cartridges (Waters. S.A. Milford, Massachusetts) and separated and quantified by gas-liquid chromatography (Christie, 1982).

Preparation and stimulation of head kidney tissue

At the end of the feeding period, 2 fish were randomly taken from each tank (6-8 per treatment) in less than 1 minute, immediately anaesthetized with 2-phenoxyethanol (1:1000 v/v) and blood collected with a hypodermic syringe from the caudal vein to minimize haemorrhage during the extraction of the tissue. HK tissue was removed from two fish in each superfusion trial, and cut into very small fragments in Hepes Ringer Medium, which was used as the superfusion medium. Afterwards, HK homogenates were pooled and distributed in 8 superfusion chambers (volume: 0.2 ml) in order to obtain a homogeneous aliquot in each of them. Tissues were superfused with a Hepes (pH 7.4) Ringer's solution containing 171 mM NaCl, 2 mM KCl, 2 mM $CaCl_2H_2O$, 0.25 % (w/v) glucose, and 0.03 % (w/v) bovine serum albumin (Rotllant *et al.,*2001). The system was temperature-controlled at 18°C and superfusion medium was pumped through the chamber at a rate of 75 ml/min by a Masterplex L/S^R multichannel peristaltic pump (Cole Parmer Intrument Co., Vernon Hills, Illinois).

Trials were started after 3 h of superfusion when cortisol reached a stable baseline level (Rotllant *et al.,* 2000a, b), in order to avoid deviations due to the different dispersion of interrenal cells in the perfusion preparation and the individual differences or the pre-stress level of each fish. After the stabilisation period of 3 h, tissues were stimulated with ACTH at a concentration of 5 nM $hACTH_{1-39}$ (Sigma) for 20 min. Subsequently, perfusion was maintained for another 170 min, fraction samples being collected every 20 min during this period. In a second series of experiments to clarify the action mechanisms of HUFA and the implication of eicosanoids in this process, tissues were incubated with a COX inhibitor (Indomethacin, INDO), or lipoxygenase (LOX) inhibitor (Nordihydroguaiaretic acid, NDGA) for 20 min at a concentration of 25 µM diluted in superfusion medium, and subsequently the tissues were stimulated with ACTH as explained before and maintained for another 170 min collecting samples every 20 min. In all series of experiments each treatment was assayed in quadruplicates. Cortisol stimulation factor (SF) was calculated by comparison of maximum cortisol released after ACTH stimulation with baseline cortisol released (maximum release-baseline release)*100/(baseline release) (Rotllant *et al.,* 2001).

Cortisol measurements

Cortisol concentration in the perfused fluid was determined by radioimmunoassay (RIA) (Rotllant *et al.,* 2001). The antibody, Biolink, S.L. (Costa

Mesa, California), was used in a final dilution of 1:6000. This antibody cross reactivity is 100% with cortisol, 11.40% with 21-desoxycorticosterone, 8.90% with 11-desoxycortisol and 1.60% with 17a-hydroxyprogesterone. The radioactivity was quantified using a liquid scintillation counter. Cortisol levels are given as ng g HK^{-1} h^{-1}.

Statistical analysis

Significance of difference (P<0.05) between dietary treatments was determined by one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test (Sokal and Rolf 1995). Analyses were performed using SPSS software (SPSS for windows 13).

Results

Effect of vegetable oil inclusion on fatty acid compositions

Lipid peroxidation products were determined as thiobarbituric acid reactive substances (TBARS) and they showed no significant difference between the diets, ranging between 8.56 and 3.85 mmole of malonaldehyde (MDA)/kg of wet diet. No dietary effect was observed on TBARS concentration (P<0.05).

Fatty acid composition of the diets reflected the inclusion of plant oils (Table 2.6). Total saturated fatty acids ranged from 16.28% in 100LO to 36.68% in FO diet. Total monoenoics fatty acids (mainly oleic acid (18:1n-9, OA)) ranged from 22.62 % in 100LO to 29.09 % in 100SO diet. α-Linolenic acid (18:3n-3, LNA) increased with increasing inclusion of LO, from 0.48 % in FO to 37.63% in diet 100LO. Similarly, linoleic acid (18:2n-6, LA) increased, particularly with the increasing inclusion of soybean oil, from 4.02 % in FO to 38.51% in 100SO. EPA and DHA were reduced by the increased content of plant oils in the diets ranging from 10.05% in FO to 2.06 % in 100SO diet and from 7.82% in FO to 2.91% in 100SO, for EPA and DHA respectively. ARA was also decreased by the plant oil inclusion ranging from 1.11% in FO to 0.24% in 100LO diet.

Fish HK fatty acid profile of total lipids reflected the dietary lipid composition (Figure 7.1). N-3 fatty acids were significantly increased in fish fed LO diet, while n-6 content was significantly increased in SO fed diet. N-9 was also increased in fish fed the VO diet compared to control, due mainly to OA increase. However, the content in HUFA

(EPA, ARA and DHA), mainly n-3 series, was significantly ($P<0.05$) reduced in fish fed the two vegetable oils, compared to control diet fed fish oil, which were significantly reduced. Then, a 45 % reduction was found in DHA from HK of fish fed 70LO, 55 % in fish fed 100LO, 58 % in fish fed 70SO and 61 % fish fed 100SO in comparison to fish oil fed fish. A 64 % reduction was found in EPA from HK of fish fed 70LO, 76 % in fish fed 100LO, 73 % in fish fed 70SO and 80 % in fish fed 100SO in comparison to fish fed fish oil. Regarding ARA, a 47 % reduction was found in fish fed 100LO, 16% in fish fed 70LO, 88% in fish fed 70SO and 86% in fish fed 100SO. As a consequence, the ratio ARA/EPA was also markedly affected by the inclusion of vegetable oils and differed depending on the dietary oil fed. Thus, lowest ARA/EPA ratio was found in HK of fish fed 70SO, followed by 100SO, FO, 70LO and, finally, 100LO.

Fish fed LO have significantly higher content of OA, LA and mainly LNA compared to fish fed the control diet. The fish fed SO showed significantly higher levels of OA, LNA and mainly LA. However, the contents in HUFA, mainly EPA, ARA and DHA, were significantly reduced in fish fed vegetable oils. In comparison to FO fish, DHA was decreased by 45% in HK of 70LO fish, 55 % in 100LO fish, 58 % in 70SO fish and 61 % 100SO fish. EPA was decreased by 64 % in HK of 70LO fish, 76 % in100LO fish, 73 % in 70SO fish and 80 % in 100SO fish.

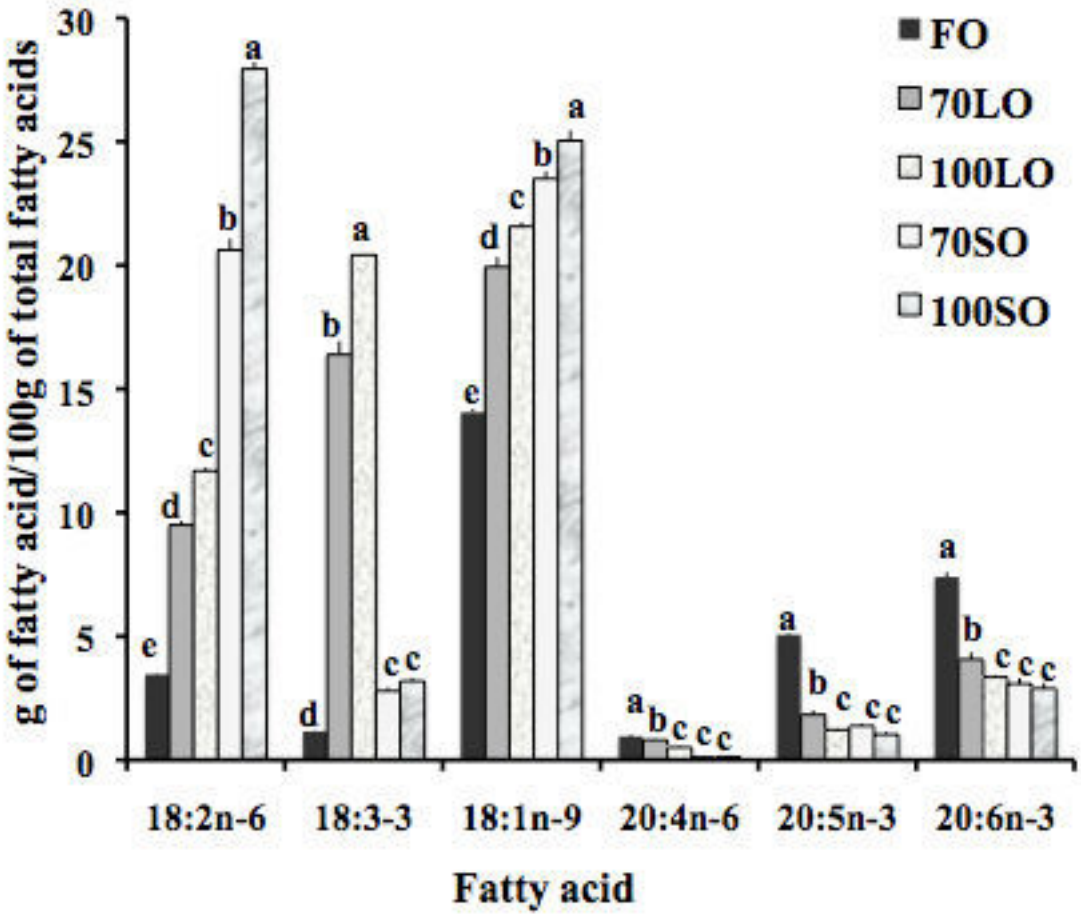

Figure 7.1: Effect of feeding vegetable oils on HK fatty acids profile (g fatty acid/100g total fatty acids).

Effect of feeding vegetable oils on cortisol response to ACTH stimulation

Feeding fish with LO increased cortisol release in HK tissue after ACTH stimulation; fish fed 100LO showed the highest cortisol release en two different peaks. Fish fed 70SO and 100SO showed slightly lower, but not significant, cortisol response after ACTH challenge (Figure 7.2). Therefore, fish fed SO diets rich in LA showed a slight inhibition of cortisol response, whereas fish fed LO diets rich in LNA showed a significant enhancement of cortisol release.

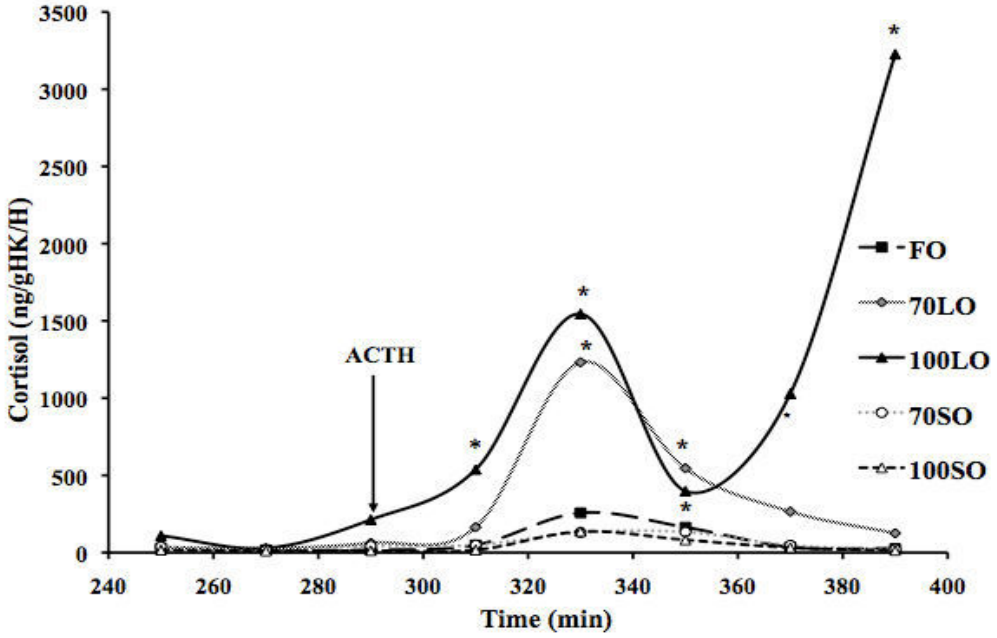

Figure 7.2: Effect of Feeding LO and SO on HK cortisol release after ACTH challenge.

The overall cortisol release after ACTH challenge expressed as Cortisol SF (Figure 7.3), was significantly highest in 100LO followed by 70LO. No significant differences were found in cortisol SF of fish fed SO and FO diets. The correlation factor between SF and ARA/EPA in HK was 98% reflecting the importance of both EPA and ARA as eicosanoid precursors on cortisol secretion.

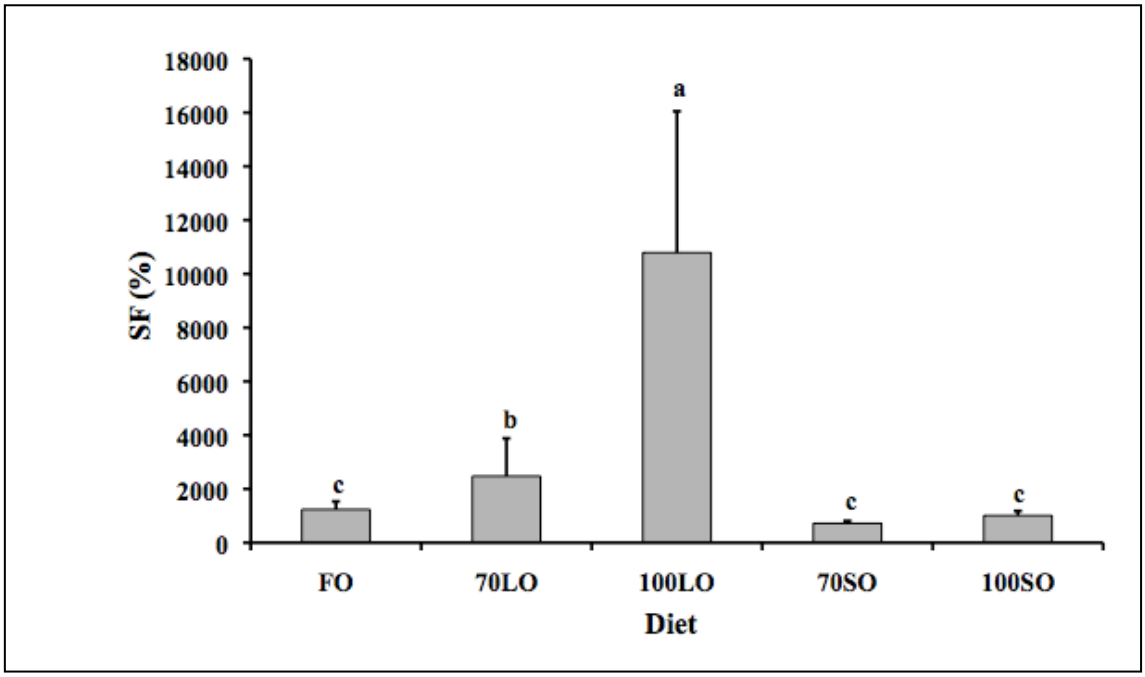

Figure 7.3: Cortisol stimulation factor from fish fed different vegetable oils, after challenging HK with ACTH.

Implication of COX and LOX metabolites in cortisol release

Cortisol release from HK after ACTH challenge was affected by the incubation with eicosanoid inhibitors. Control fish decreased cortisol release after ACTH stimulation when the HK tissue was incubated with both inhibitors (INDO or NDGA) (Figure 7.4), denoting the implication of metabolites from both COX and LOX enzymes in cortisol secretion.

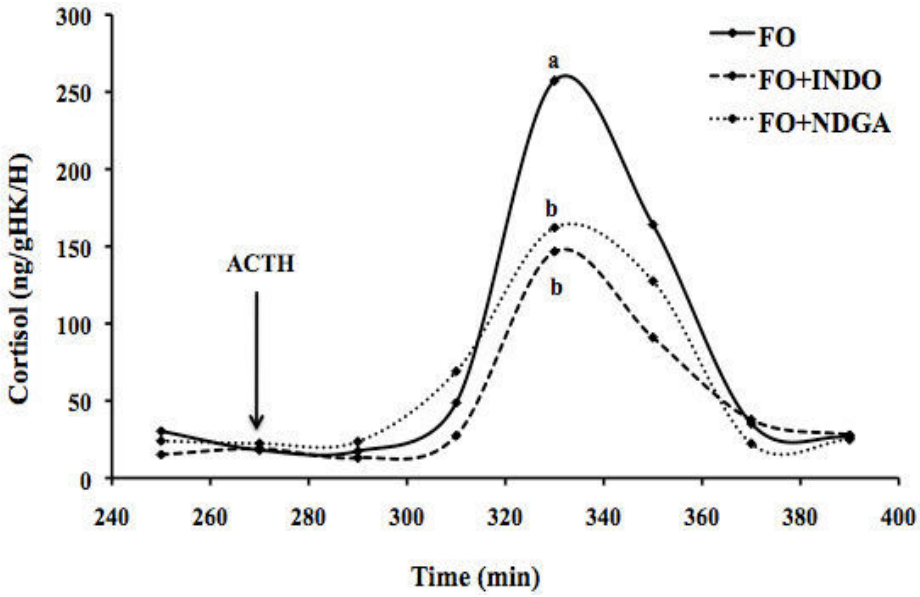

Figure 7.4: Effect of incubating head kidney tissue with INDO or NDGA on cortisol release in control fish (FO).

When the HK was incubated with INDO and NDGA, the cortisol release was significantly decreased in fish fed 70LO diet, indicating the modulation of the cortisol secretion by these two pathways, with this decrease being more pronounced when NDGA was the used as inhibitor compared to INDO incubation (Figure 7.5).

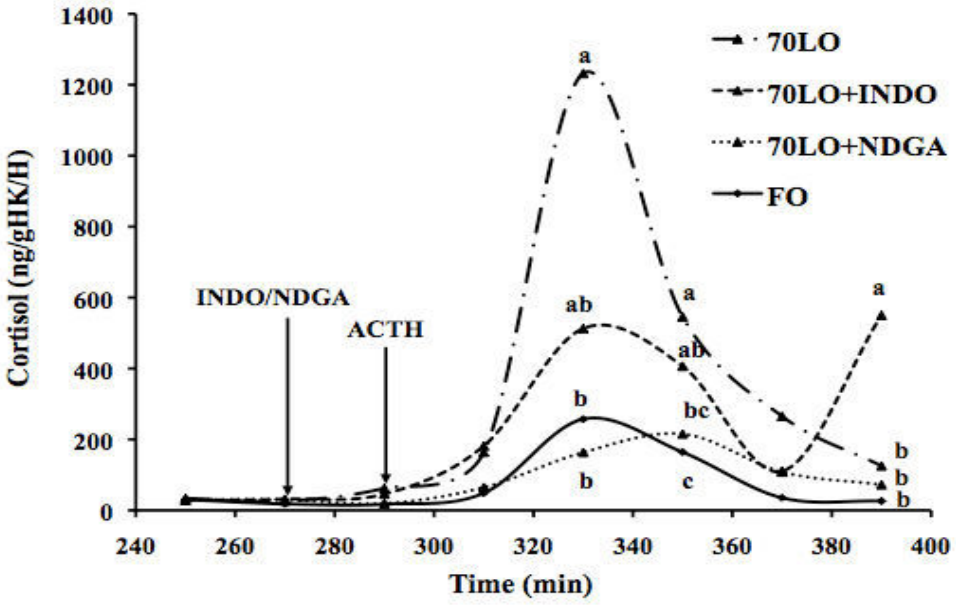

Figure 7.5: Effect of incubating head kidney tissue with INDO or NDGA on cortisol release in 70LO fed fish.

In fish fed 100 LO, incubating tissue with INDO decreased the cortisol release. Incubating tissue with NDGA decreased the cortisol release from this tissue, proving again the COX and LOX role in the stress secretion. Also in this case, the response was delayed and the use of NDGA was more effective in inhibiting the cortisol release than INDO (Figure 7.6).

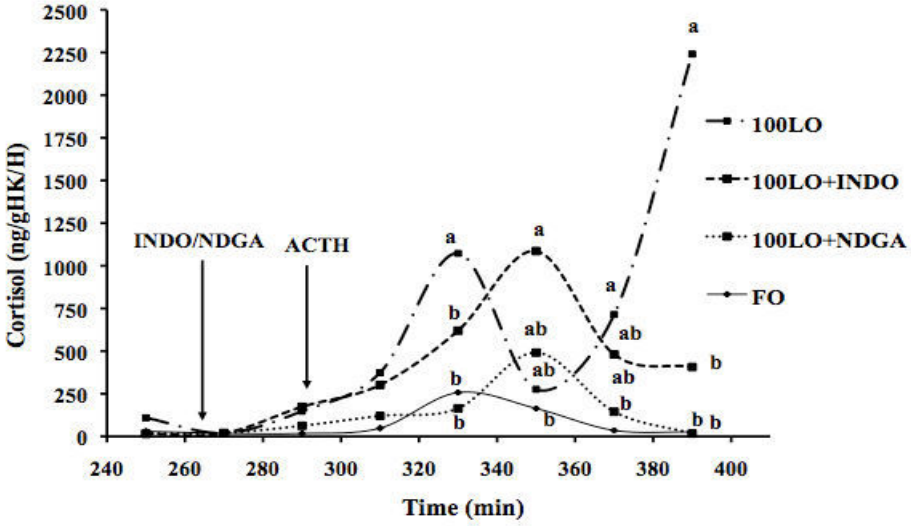

Figure 7.6: Effect of incubating head kidney tissue with INDO or NDGA on cortisol release in 100 LO fed fish.

In fish fed 70SO incubating HK tissue with INDO increased cortisol release, indicating another pathway of action in this fish species. NDGA incubation did not affect cortisol release after ACTH stimulation (Figure 7.7). HK from fish fed 100SO diet

showed also significantly decreased cortisol release after ACTH challenge when incubated with INDO, there was also a slightly decreased cortisol release, but not significant when the tissue was incubated with NDGA (Figure 7.8).

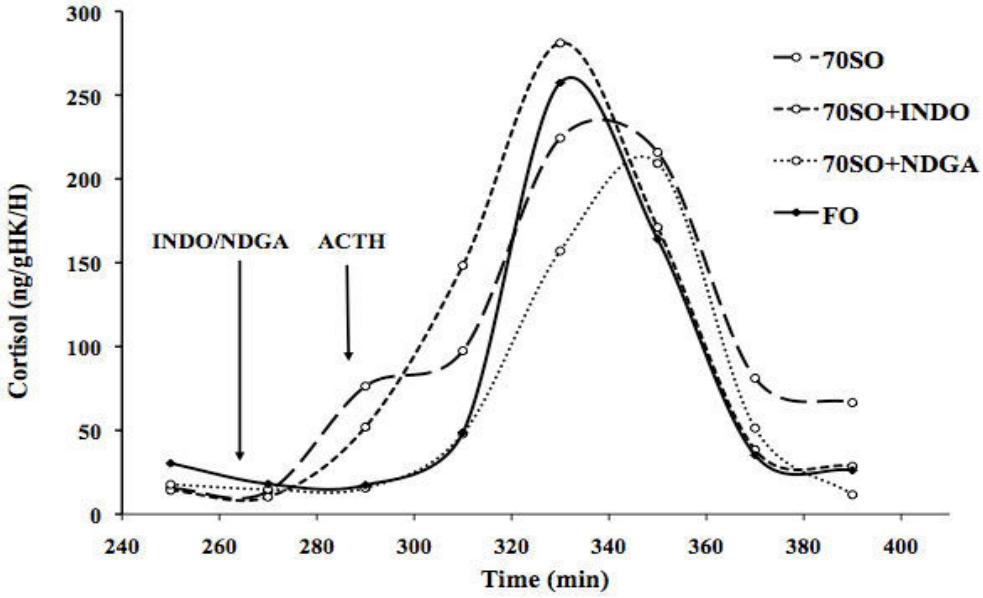

Figure 7.7: Effect of incubating head kidney tissue with INDO or NDGA on cortisol release in 70 SO fed fish.

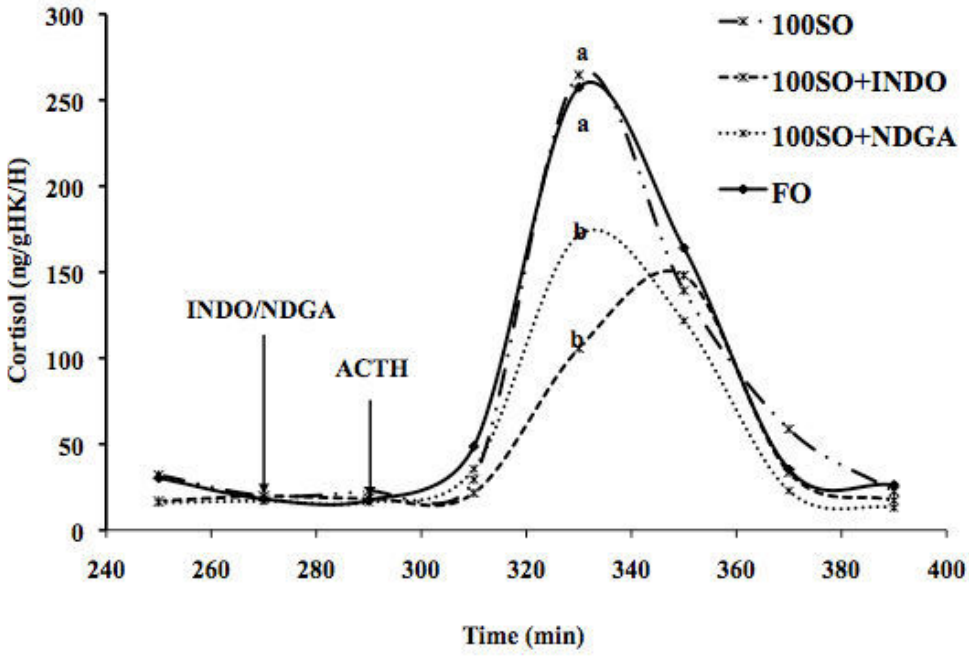

Figure 7.8: Effect of incubating head kidney tissue with INDO or NDGA on cortisol release in 100 SO fed fish.

Cortisol stimulation factor (SF) from the experiment using INDO and NDGA (Figure 7.9) showed that incubating HK from FO fed fish with INDO reduced the cortisol SF to 26 %, and when it was incubated with NDGA the SF was reduced to a 42 %. Also when the 70LO fed fish HK was incubated with INDO or NDGA, the cortisol SF level was decreased by 39 % and 66 %, respectively. HK from 100LO fed fish showed apparently the same level of decrease of the cortisol SF when incubated with INDO or NDGA corresponding to 46 %. By contrast, in fish fed SO the action of the COX and LOX inhibitor was different, such that incubating HK from fish fed 100SO, with INDO, reduced the cortisol SF to a 20 % and the incubation with NDGA decreased the cortisol SF to only 10 %. By comparison, the fish fed 70SO showed the opposite response to the use of COX and LOX inhibitors, as when the tissue was incubated with INDO the cortisol SF increased by about 3 fold and when it was incubated with NDGA, the cortisol SF was increased by 60%.

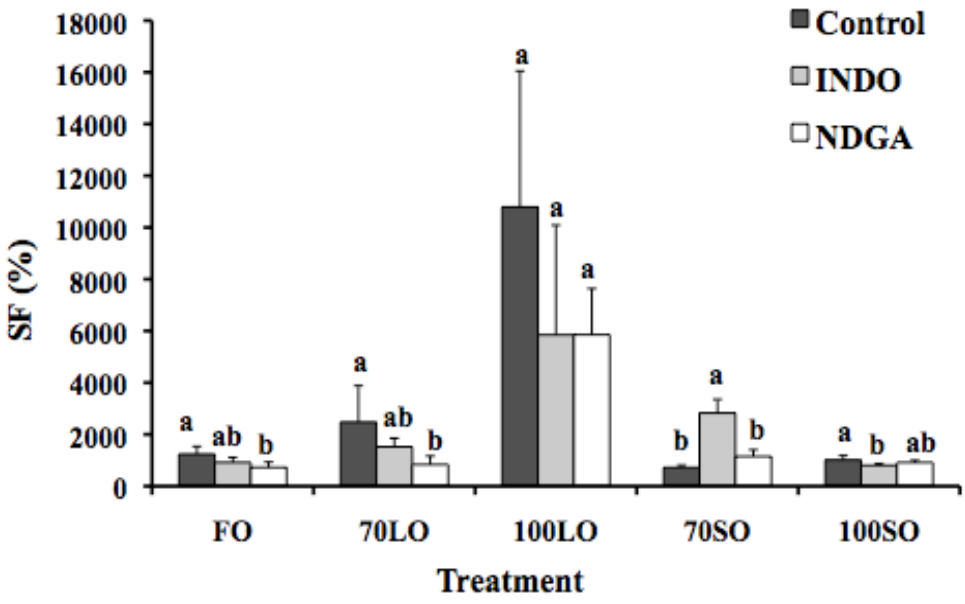

Figure 7.9: Cortisol Stimulation factor from all the treatment after incubating the HK tissues with INDO or NDGA.

Discussion

The EFA for marine fish comprise HUFA with carbon chain lengths of 20 and 22, for both the n-3 and n-6 series. Because the HUFA cannot be synthesised "*de novo*" by the fish (Sargent *et al.,* 1989) at a sufficient rate to meet the requirements, these EFA must be provided in the diet. HUFA are essential for normal fish growth, for its cellular

structure and function, including the maintenance of membranes and eicosanoid metabolism (Henderson and Tocher, 1987). Replacing FO, in commercial diets, with VO that contain very low levels of C20 and C22 PUFA, results in reduced tissue levels of ARA, EPA and DHA (Bell *et al.,* 1998; 2001, 2002; Montero *et al.,* 2003, 2005). In this experiment, at the end of the 26 week feeding period, the fatty acid profile from HK total lipids from gilthead seabream reflected the dietary input. Thus, fish fed the SO had significantly higher levels of LA and OA, and fish fed LO had significantly higher levels of LNA and OA. In addition, since LO and SO contain large quantities of C18 fatty acids, the n-6/n-3 fatty acid ratio was altered and this could affect fish health (Waagbo *et al.,*1993, Montero *et al.,*2003, 2005). The contents of DHA and EPA, decreased significantly in fish fed VO according to the level of inclusion, for instance EPA decreased by about 80 % in fish fed 100SO and 76 % in fish fed 100LO compared to controls, DHA was decreased by 61% in fish fed 100SO and 55% in fish fed 100LO. Saturated and monounsaturated fatty acids, particularly 16:0, 18:1n-9, 20:1n-9 and 22:1n-11 are easily catabolised in fish to produce energy, while DHA and EPA are less easily catabolised via ß-oxidation (Sargent *et al.,* 2002). Thus, an increase in the OA, LA and LNA contents in tissue from fish fed the VO could imply higher energy availability in these fish to deal with homeostasis disturbances.

Under stress conditions, cortisol is secreted from interrenal cells of the HK triggered by the corticotropin-releasing hormone (CRH) and ACTH hormonal cascade of the HPI axis in fish. ACTH is well known to induce the production and release of cortisol and is considered to be the major glucocorticoid stressor (Barton and Iwama, 1991), playing an important regulatory role in many important physiological processes (Mommsen *et al.,* 1999). Feeding gilthead seabream with 70LO and 100LO significantly increased cortisol release from HK tissue after ACTH stimulation, in accordance with previous results from *in vivo* studies (Montero *et al.,* 2003). The levels of cortisol found in this study for the control diet fed fish were similar to those reported in our previous study (Ganga *et al.,* 2006), whereas the results from HK of fish fed 100LO were 9 fold above the control response, indicating the negative effect of including high levels of VO on diets for gilthead seabream.

It is well established that HUFA provided by the diet play an important role in stress response in mammals as in fish. Enhancing the levels of dietary ARA promoted

survival and resistance to stress in gilthead seabream larvae (Koven *et al.,* 2001, 2003), and feeding the ARA-supplemented diet resulted in a diminution in cortisol response after net confinement compared to the fish fed a diet containing a low level of this fatty acid (Van Anholt *et al.,* 2004). In this study, the content of ARA in HK decreased by 55 % in fish fed 70LO and by 81 % in 100LO fed fish, which could be associated partly with the increased cortisol levels seen in these treatments. However, a number of authors have also demonstrated the importance of EPA and DHA in stress response and stimulating cortisol release in fish (Kanazawa, 1997; Montero *et al.,* 1998; Harel *et al.,* 2001; Liu *et al.,* 2002, Ganga *et al.,* 2006). In this experiment, the low dietary levels of these important HUFAs in the diet lead to a parallel decrease in the HK causing a significant modulation of cortisol release. These results demonstrated that feeding fish with diets poor in these EFA affected cortisol release from HK after challenging the tissue with ACTH, clearly denoting the importance of these nutrients in the correct function of HK in response to stress situations.

The apparent contradiction in cortisol release levels found between fish fed the LO or SO, could be explained according to the physiological mechanisms implicating different FA provided by these oils. Once released from the membrane, free FA not only acts as a precursor of eicosanoids but also acts directly as a ligand, affecting positively or negatively the binding of steroid hormones to their specific intracellular receptors (Sumida, 1995). In addition, FA can also co-regulate glucocorticoid-dependent gene expression by modulating the activity of protein kinases involved in the phosphorylating transcription factors (Sumida, 1995). Therefore, they may be playing a role in modulating the intracellular steroid hormone signalling pathway to co-regulate a glucocorticoid-sensitive promoter (Vallette *et al.,* 1995). In human platelets, unsaturated fatty acids such as palmitoleic acid (16:1), OA, LA, LNA and ARA were detected to inhibit PLA2 activity (Ballou and Cheung, 1985), which is the key enzyme responsible of liberating the COX and LOX substrate precursors from the membrane phospholipids. On the other hand, the decrease in cortisol release in fish fed SO could be explained by the higher content of OA and LA in their tissue. In rats, the maximal steroidogenic response to ACTH was inhibited approximately 50 % by OA, concluding that the modulation of steroidogenesis by these abundant naturally occurring lipids may be an important component of the control mechanisms within the HPA pathway in disorders of lipid homeostasis (Sarel and Widmaier, 1995). It is also demonstrated that OA and LA, inhibited the action of ACTH on the adrenal gland, and the mechanism of action of these

FA may be partly via inhibition of cAMP production (Matthys and Widmaier, 1998). By contrast, the increased cortisol in HK from fish fed LO could be due to the effect of higher content on LNA present in this tissue. It was demonstrated that reducing the LNA in the diet for postmenopausal women reduced the cortisol release during stress (Spence *et al.,* 2003), denoting the role of this FA in stimulating cortisol release.

The mechanism by which these FA could modulate cortisol release in fish is still a subject of many research studies and many hypotheses have been suggested. One of the most important roles of these EFAs, is that they provide the eicosanoids precursors, a well known series of hormones produced by the actions of the COX and LOX on these FA, and which modulate many physiological and immunological processes. The implication of the COX metabolites in cortisol release has been proved recently by in vivo studies in fish (Koven *et al.,* 2001, 2003; Van Anholt *et al.,* 2004), and in our previous in vitro study we demonstrated that incubating HK with a COX inhibitor, significantly decreased cortisol release (Ganga *et al.,* 2006). In mammals, there is clear evidence that prostaglandins modulate the release of hypothalamic CRH and/or pituitary ACTH (Abou-Samra *et al.,* 1986; Zacharieva *et al.,* 1992; Nasushita *et al.,* 1997). For example, it is known that prostaglandins, and PGE2 in particular, modulate the sensitivity of the mammalian HPA axis and consequently change the stress response (Nye *et al.,* 1997; Bugajski *et al.,* 2001). In turn, COX derived protaglandins have been shown to increase in vitro cortisol release in interrenal tissue of female frogs during ovulation (Gobbetti and Zerani, 1993) as well as in human adrenal cells (Vakharia and Hinson, 2005).

In this study, incubating HK with INDO decreased cortisol release in control, 70LO, 100LO and 100SO fed fish, proving again the implication of prostanoids in cortisol release in these fish. The implication of the LOX metabolites in cortisol release was recently investigated but as far as we are aware, the present study is the first report of the implication of LOX derivatives in the modulation of cortisol secretion by HK in fish. Incubating the HK tissue with NDGA decreased the cortisol release in the majority of the treatments. This is in accordance with the results from other studies proposing the implication of the LOX pathway in stimulating ACTH release when ARA was added to fish cells *in vitro* (Koven *et al.,* 2003), and that LOX metabolites of ARA were reported to stimulate GH (growth hormone) release in rat anterior pituitary cells (Abou-Samra *et*

al., 1986; Won and Orth, 1994). More studies have pointed out the role of LOX products in ACTH secretion and adrenal steroidogenesis in mammals (Wang *et al.,* 2000, Yamazaki *et al.,* 2001).

Therefore, the two enzymes (COX and LOX) are both involved in cortisol release and stress response. The mode of action of each enzyme could depend on the availability of the precursor itself, and they may show antagonistic effects. Elsewhere, some studies suggested a different hypothesis to establish an explication of this relationship. Abou-Samra *et al.*,(1986) suggested an inhibitory effect of COX products on ACTH release, down-regulating cortisol release, while LOX derivatives may have the opposite effect, stimulating ACTH release and consequently up-regulating cortisol. In addition, the stimulatory effect of Interleukine-3 on cortisol release is dependent on the LOX pathway, while the effect of Interleukine-6 on adrenocortical cortisol secretion is mediated through the COX pathway in bovine adrenocortical cells in primary culture (Michl *et al.,* 2000). However, other eicosanoids such as epoxygenase metabolites could also be potential modulators of the HPI axis in fish, since they have been demonstrated to stimulate ACTH and β-endorphin secretion from rat pituitary cells (Cowell *et al.,* 1991, Won and Orth, 1994).

The understanding of the pathways and intracellular messengers that regulate cortisol synthesis in fish is limited, and the way by which HUFA could modulate its release in fish is a very new research target. Cortisol synthesis by ACTH has been studied over a considerable time period, demonstrating the importance of cAMP as an intracellular second messenger in the stimulation of cortisol production in the coho salmon (*Oncorhynchus kisutch*) (Patiño *et al.,* 1986). In mammals, it is well known that the main pathway leading to corticosteroid synthesis in the adrenal gland involves ACTH which stimulates a signalling cascade integrating G-proteins, adenylyl cyclase, cAMP and protein kinase A (PKA) (Miller, 1988; Schimmer, 1995). Other pathways involving protein kinase C (PKC) via stimulation by angiotensin II (AII) or acetylcholine, both known secretagogues of cortisol in fish (Perrott and Balment, 1990; Kloas *et al.,* 1994), at least share a role in the regulation of corticosteroid synthesis, during both chronic and acute stress (Bird *et al.,* 1990). Subsequently, PKA has been suggested to be a crucial stimulatory component in the ACTH-mediated signalling pathway in fish adrenal steroidogenesis (Lacroix and Hontela, 2001), and in fish ovarian follicular steroidogenesis in a similar cAMP-mediated pathway (Srivastava and Van der Kraak, 1994a,b). Although, PKC has been shown to have an inhibitory role in the acute

cortisol response in fish adrenocortical cells (Lacroix and Hontela, 2001), as activation of PKC caused a dose-dependent decrease in ACTH-stimulated acute cortisol synthesis. Studies with human and bovine adrenal cells have shown that stimulating PKC suppresses cAMP-stimulated steroidogenesis (Mason *et al.,* 1986; Naseeruddin and Hornsby, 1990).

LA was found to stimulate PKC in cultured B16 mouse melanoma cells, suggesting that the activation of PKC plays a pivotal role in the linoleic acid-induced inhibition of melanogenesis in B16 cells (Ando *et al.,* 1990). On the other hand, OA has been found to stimulate PKC in platelet homogenates (Khan *et al.,* 1992). Therefore, relating these findings to our results, we could suggest that OA and LA, could inhibit cortisol release in fish fed SO through stimulation of PKC which inhibits the cAMP-mediated pathway of cortisol release.

Finally, studies carried out with fatty acids require great care in interpretation because fatty acids can be hydrophobic or hydrophilic according to pH, quickly moving between cell compartments, such as membranes, lipid-binding proteins, cytoplasm and lipid droplets. Fatty acids can be found free or esterified and constantly move from one form to another. Moreover, the elongation of shorter chain fatty acids and the oxidation of fatty acids can also interfere with the levels of fatty acids in any given subcellular compartment. For this reason, it is hard to predict the effect of these fatty acids on cortisol release and have a clear explanation for the contradictory responses between LO fed fish and SO fed fish, depending only on the diets fatty acid content. Other physiologic processes could be implicated and interfere in this response. It is clear that VO affected cortisol release in HK from gilthead seabream, this is due partly to the action of COX and LOX enzymes converting the HUFA, present in the membrane, to a biologically active eicosanoids. The presence of higher levels of C18 FA in the HK from fish fed VO could activate other pathways related to cortisol release, due to the direct action of these FA on PKC, PKA or cAMP. More studies are needed to clarify these mechanisms involving all the pathways responsible for cortisol release in fish.

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Chapter 8

Conclusions

Conclusions

1- Up to 70% of the dietary fish oil can be substituted by vegetable oils in on-growing diets for gilthead seabream that contain South American fish oil and fish meal as lipid and protein sources and a lipid content of about 17%, without affecting seabream growth and survival. Higher percentage of substitution causes growth retardation.

2- Membrane lipids of plasma and total lipids of muscle, liver and gills of sea bream may be profoundly altered by giving diets with high content on VOs. N-3 HUFA content in fish fillet was markedly reduced by feeding VOs, what could negatively affect its nutritional value for human consumption. Nevertheless, feeding VOs affect fatty acid composition of liver in a higher extend than in other tissues, whereas gills fatty acids profiles showed double ARA content than the other tissues.

3- There was a selective retention of DHA and ARA in plasma phospholipids and total lipids from muscle, liver and gills, confirming their importance in those tissues.

4- Not only ARA, but also EPA was found to be an important precursor of prostaglandines, and the dietary ratio between both fatty acids altered prostaglandins production, what can affect numerous physiologic functions, some of them related to fish health.

5- Plasma leptin concentration reflects feed intake, and is not significantly affected by feeding VOs.

6- EPA and ARA as single supplemented fatty acids stimulate the ACTH-induced cortisol release from seabream interrenal tissue.

7- DHA effect moderately stimulated interregnal tissue, while DHGLA inhibits ACTH-induced cortisol release from the same tissue.

8- ACTH induced release of cortisol and the role of ARA and EPA is mediated by COX and LOX-derived metabolites.

9- ACTH induced release of cortisol secretion by HUFA seems to be dependant on the concentration and type of each fatty acid.

10- FO substitution by LO in diets for sea bream stimulated the ACTH-induced cortisol release from interrenals tissue, mediated at least partly by COX and LOX. In vivo, feeding LO increased basal and poststress plasma cortisol levels.

11- Feeding various types of VOs affect differently stress response in sea bream, with 70LO and 100LO increasing cortisol response after ACTH stimulation, while SO decreasing this response. A well-balanced supplementation of these dietary oils is important to regulate cortisol release and stress resistance.

12- Cortisol release is modulated by dietary HUFAs and this response is mediated by cyclooxygenase and/or lipoxygenase metabolites.

Chapter 9

Summary

Summary

The aim of this thesis was to investigate the effects of feeding different vegetable oils on fish growth and fish health of gilthead seabream (*Sparus aurata*).

Article 1 describes how feeding seabream with diets containing high levels of fish oil substitution by n-3 or n-6 fatty acids rich vegetable oils affects culture performances and fatty acid composition of different fish tissues. Triplicate groups of gilthead seabream were fed eight practical-type diets with a blend of LO and SO at different levels during a period of 26 weeks. Substitution up to 70% or more of fish oil by VOs reduced growth and affected negatively feed utilization. Fatty acid composition of muscle, liver and gills were affected and correlated with the dietary input. EPA, ARA, DHA and total n-3 HUFA were reduced in all the tissues while the C18 (LA and LNA) fatty acids were increased in parallel with increasing VOs in diet affecting the fish nutritional quality for the consumer. The elongase and Δ6 desaturase from C18 end products were also increased in all the tissues.

Article 2 was focused on studying the effect of fish oil substitution levels and n-3/n-6 fatty acids ratio on seabream welfare and resistance to stress. The fatty acids composition of the head kidney was affected by the different dietary treatments; fish fed VOs had increased amounts of C18 and lower amounts of n-3 HUFA. The physiological response to crowding was affected by the diet. Fish fed LO registered higher basal plasma cortisol concentration comparing with the other treatments. After 2h of crowding all the treatments showed higher cortisol production while fish fed LO showing dramatic levels. Cortisol pattern was influenced by the diet and LO fed fish showed higher and faster response and a recuperation of normal levels after 1 week of crowding. However, fish fed SO showed a lower and slower response but it takes more time to recuperate the normal levels even after 1 week of crowding. Then, there is an effect of feeding seabream with LO and/or SO on it response to stress.

In article 3, we studied the effects of different levels of substitution of fish oil by vegetable oils blends on gilthead seabream health and welfare in terms of plasma fatty acid composition and prostaglandin and leptin production. ARA, EPA and DHA were preferentially incorporated into plasma polar lipids, and DHGLA accumulated with increasing VO inclusion. ARA-derived PGE_2 production in plasma was not affected by VOs, in agreement with similar eicosanoids precursor ratio (ARA/EPA) in plasma

phospholipids. Moreover, PGE_3 was the major prostaglandin produced in plasma of fish fed fish oil diets pointing out the importance of EPA as a precursor of prostaglandin in marine fish. A negative correlation was found between plasma PGE_2 and leptin plasma concentration. Alteration in the amount and type of PG produced can partially be responsible for the changes in the immune system and health parameters of fish.

Article 4 focuses on understanding the mechanisms implicated in the regulation of stress resistance by polyunsaturated fatty acids by studying the production and release of cortisol in gilthead seabream interrenal cells stimulated with adrenocorticotropic hormone and the effect of eicosanoids production inhibitors. ARA and particularly EPA promoted cortisol production in seabream interrenal tissue, while the DHA effect was moderated. Moreover, incubation with indomethacin reduced this increase in ARA and EPA treatment suggesting the implication of cyclooxygenase-derived products in this release, while in DHA treatment was not marked. In contrast, incubating head kidney tissue with DHGLA reduced cortisol production.

Article 5 describes the effect of feeding VOs along gilthead seabream on-growing period on welfare in terms of cortisol production and release by head kidney and the physiological pathways involved. Fatty acid compositions of the head kidney reflected the dietary input, thus EPA, DHA, ARA and total n-3 HUFA were reduced in fish fed VOs compared to FO diet. Feeding 70% or 100% LO, increased cortisol release in head kidney after stimulation with ACTH, while feeding SO decreased this response. However, inhibition of eicosanoids productions by incubating the tissue with indomethacin or nordihydroguaiaretic acids reduced the cortisol release after ACTH stimulation in 70LO, 100LO and 100SO. This study confirms the modulation of cortisol release by dietary HUFA, and the mediation of cyclooxygenase and/or lipooxygenase in their mechanism of action.

Resumen

El fin de la presente tesis era investigar los efectos de alimentar diversos aceites vegetales sobre el crecimiento y salud de la dorada (*Sparus aurata*).

El articulo 1 describe cómo alimentando la dorada con dietas que contienen niveles altos de substitución del aceite de pescado, por los aceites vegetales ricos en ácidos grasos de la serie n-3 o n-6, afecta al crecimiento y a la composición ácidos grasos de los diversos tejidos del pez. 3 grupos de dorada fueron alimentados con ocho dietas con una mezcla de LO y SO en diferentes niveles durante un período de 26 semanas. La substitución hasta el 70% o más del aceite de pescado con aceites vegetales redujo el crecimiento y afectó negativamente a la utilización del alimento. Las composiciones en ácidos grasos del músculo, el hígado y las branquias fueron afectadas y correlacionadas con el aporte dietético. El EPA, ARA, DHA y n-3 HUFA totales fueron reducidos en todos los tejidos mientras que los ácidos grasos C18 (LA y LNA) fueron aumentados paralelamente al aumento de los VOs en la dieta lo que afectaba a la calidad alimenticia de los peces para el consumidor. Los productos finales de la elongaza y la Δ6 desaturaza de los C18 también fueron aumentados en todos los tejidos.

El articulo 2 fue centrado en estudiar el efecto del cociente de los niveles de la substitución del aceite de pescados y de los ácidos grasos n-3/n-6 sobre el bienestar de la dorada y su resistencia al estrés. La composición en ácidos grasos del riñón anterior fue afectada por los diversos tratamientos dietéticos; los peces alimentados con los VOs habían registrado un aumento en las cantidades de C18 y cantidades más bajas de n-3 HUFA. La respuesta fisiológica al confinamiento fue afectada por la dieta. Los peces alimentados con las dietas LO registraron altos niveles basales de cortisol plasmático comparando con los otros tratamientos. Después de 2h de confinamiento todos los tratamientos demostraron una producción más alta del cortisol con los peces alimentados con LO demostrando unos niveles dramáticos. El patrón del cortisol fue influenciado por la dieta con los peces alimentados con LO demostrando una respuesta más alta y más rápida y una recuperación de niveles normales después de 1 semana de confinamiento, sin embargo, los peces alimentados con las dietas SO demostraron una respuesta más baja y más lenta pero tarda más tiempo para recuperar los niveles normales incluso después de 1 semana de confinamiento. Por lo tanto, hay un efecto de alimentar la dorada con LO y/o SO sobre la respuesta al estrés.

En el capítulo 3, estudiamos los efectos de diversos niveles de substitución del aceite de pescados por mezclas de los aceites vegetales sobre la salud y el bienestar de la dorada en términos de composición en ácidos grasos del plasma y la producción de las prostaglandinas y de la leptina. El ARA, EPA y DHA fueron incorporados preferencial en los lípidos polares del plasma, y el DHGLA fue acumulado con la inclusión creciente de los VOs. la producción de la PGE2 derivada del ARA en plasma no fue afectada por alimentar con los aceites vegetales, de común acuerdo con el cociente similar del precursor de los eicosanoides (ARA/EPA) en fosfolípidos del plasma. Por otra parte, la PGE3 fue la principal prostaglandina producida en el plasma de los peces alimentados con dieta con aceite de pescado, lo que precisa la importancia del EPA como precursor de la prostaglandina en peces marinos. Una correlación negativa fue encontrada entre la concentración en plasma de la PGE2 y de la leptina. La alteración en la cantidad y el tipo de las prostaglandinas producidas puede parcialmente ser responsable de los cambios en los parámetros del sistema inmune y de la salud en peces.

El capítulo 4 se centra en comprender los mecanismos implicados en la regulación de la resistencia al estrés a través de los ácidos grasos poliinsaturados estudiando la producción y la liberación del cortisol en las células interrenales de la dorada estimuladas con la hormona adrenocorticotrópica y el efecto de los inhibidores de la producción de los eicosanoides. El ARA y particularmente EPA promovieron la producción del cortisol en el tejido interrenal de la dorada, mientras que el efecto del DHA fue moderado. Por otra parte, la incubación con la indometacina redujo este aumento en el tratamiento del ARA y de EPA, lo que sugería la implicación de los productos derivados de la ciclooxígenasa en esta liberación, mientras que en el tratamiento con DHA no fue marcado. En cambio, la incubación del tejido del riñón anterior con DHGLA redujo la producción del cortisol.

El capítulo 5 describe el efecto de alimentar dorada con aceites vegetales durante el período de crecimiento sobre el bienestar en términos de producción y liberación de cortisol por el riñón anterior y de las vías fisiológicas implicadas. Las composiciones en ácidos grasos del riñón anterior reflejaron el aporte dietético, así EPA, DHA, el ARA y n-3 HUFA totales fueron reducidos en los peces alimentados con VOs comparando con la dieta FO. Alimentando el 70% o el 100% LO, aumentó el cortisol en el riñón anterior después del estímulo con ACTH, mientras que la alimentación con SO disminuyó esta respuesta. Sin embargo, la inhibición de la producción de los eicosanoides incubando el

tejido con indometacina o ácidos nordihydroguaiaretic redujo la producción de cortisol después de la estimulación con ACTH en 70LO, 100LO y 100SO. Este estudio confirma la modulación de la producción del cortisol por los HUFAs dietéticos, y la mediación de la cicloxígenaza y/o del lipoxígenaza en su mecanismo de acción.

ملخص

الهدف من هذه الأطروحة دراسة تاثير التغذية ببعض الزيوت النباتية على صحة ونمو اسماك الدنيس.

في البحث الاول تمت دراسة تاثير تغذية الاسماك باعلاف ذات مستويات مرتفعة من الزيوت النباتية المحتوية على احماض دهنية غنية ب N-6 و N-3 بدلا من زيت السمك، وتركزت التجربة على دراسة هذا التأثيرعلى نمو الاسماك وتركيزالأحماض الدهنية المذكورة في انسجتها باستخدام 8 انواع من الاعلاف التي تحتوي على نسب مختلفة من زيت بذور الكتان وزيت فول الصويا لمدة 26 أسبوعاً. نتج عن استبدال 70% او اكثر من زيت السمك بالزيوت النباتية انخفاض النمو والتأثير سلبيا على الاستفادة من العلف كما أن تركيبة الاحماض الدهنية في اللحم والكبد والخياشيم تأثرت بالأعلاف المستخدمة، كما لوحظ انخفاض نسبة EPA, DHA, ARA, n-3 HUFA في جميع الانسجة بينما زادت نسبة الاحماض الدهنية التي تحتوي على C18 (LA, LNA) طرديا مع زيادة نسبة الزيوت النباتية في العلف مما أثر على الجودة الغذائية للأسماك بالنسبة للمستهلك، بينما الأحماض الدهنية المنتجة من تاثير الانزيمات (المضيفة للرابطة الثنائية أو لذرتين من الكربون) على الأحماض الدهنية (C18) ارتفعت في جميع الانسجة.

البحث الثاني سلط الضوء على دراسة تأثير استبدال نسب مختلفة من زيت السمك و N-3/N-6 من الاحماض الدهنية بالزيوت النباتية على صحة السمك ومقاومته للإجهاد، فنتج عن ذلك أن تركيب الاحماض الدهنية للكلية الرئيسية تاثرت باختلاف نوعية الاعلاف. في الاسماك التي تمت تغذيتها بالزيوت النباتية ازدادت كمية الاحماض الدهنية المحتوية على C18 وانخفضت كمية الاحماض الدهنية n-3 HUFA . كما أن الأعلاف أثرت على الوظائف الحيوية للاسماك حين وضعت في الاقفاص ، فالاسماك التي غذيت بزيت بذور الكتان سجلت نسبا مرتفعة من الكورتيزول الطبيعي في بلازما الدم مقارنة مع الأعلاف الأخرى، فبعد ساعتان من الحبس في الاقفاص جميع الأعلاف أظهرت افرازات عالية من الكورتيزول في بلازما الدم ، بينما التغذية بزيت بذور الكتان سجلت اعلى نسب كورتيزول. سلوك افراز الكورتيزول أيضا تاثر بنوعية الاعلاف ، فنجد أن الاسماك التي غذيت بزيت بذور الكتان أظهرت افرازات سريعة و مرتفعة من الكورتيزول ثم عاد الكورتيزول لمستواه الطبيعي بعد اسبوع من الحبس في الاقفاص، بينما الاسماك التي غذيت بزيت فول الصويا أظهرت نسب بطيئة و منخفضة من افراز الكورتيزول وأيضا احتاجت وقتا أطول في العودة الى المستوى الطبيعي حتى بعد أسبوع من الحبس في الاقفاص. اذا هناك تاثير للاستبدال الجزئي لزيت السمك بزيت بذور الكتان او زيت فول الصويا على الاستجابة للاجهاد في اسماك الدنيس.

البحث الثالث درس تاثير استبدال زيت السمك بنسب مختلفة من الزيوت النباتية على راحة اسماك الدنيس بواسطة قياس تركيبة الاحماض الدهنية في البلازما وانتاج هرمونات بروستاجلاندين (PG) . نتج عن ذلك أن الأحماض الدهنية ARAوEPA و DHA اندمجوا بشكل أفضل في الدهون القطبية للبلازما أما DHGLA تراكم بزيادة نسبة الزيوت النباتية في الاعلاف . كما لوحظ أن انتاج PGE2 المنشقة من ARA في البلازما لم تتأثر بالاستبدال بالزيوت النباتية موافقة لثبات نسبة ARA/EPA التي تعتبر الممهد لانتاج الكورتيزول في الدهون الفسفورية في البلازما. علاوة على ذلك PGE3 كانت أعلى أنواع البروستاجلاندين المنتجة في بلازما الاسماك

المغذية باعلاف زيت السمك مما يبين أهمية EPA كمصدر لانتاج البروستاجلاندين في الاسماك البحرية . كما سجل تركيز PGE2 علاقة عكسية مع هرمون اللبتين في البلازما. أما التغير في كمية ونوعية PG المنتجة يمكن أن يكون الممسؤول بصفة جزئية عن التغيرات في قياسات جهاز المناعة وصحة السمكة .

البحث الرابع ركز على فهم الاليات المتدخلة في تعديل مقاومة الاجهاد بالاحماض الدهنية المتعددة غير المشبعة بواسطة دراسة انتاج وافراز الكورتيزول في خلايا الكلى الرئيسية عن طريق التحفيز بهرمون ادرينوكوتيكوتروبين ومثبطات انتاج eicosanoids في اسماك الدنيس. ونتج عن ذلك أن الأحماض الدهنية ARA وخاصة EPA يشجعان على افراز الكورتيزول في خلايا الكلى الرئيسية في الدنيس بينما تاثير DHA كان معتدلا. اضافة الى ذلك حضانة خلايا الكلى الرئسية مع indomethacin خفضت هذا الارتفاع في معاملات (ARA&EPA) مما يشير الى تدخل منتجات COX في هذا الانتاج بينما في معاملة DHA لم يسجل هذا التاثير. وبالعكس حضانة خلايا الكلى الرئيسية مع DHGLA خفضت انتاج الكورتيزول.

البحث الخامس درس تاثير تغذية أسماك الدنيس بالزيوت النباتية (خلال فترة النمو) على صحته وذلك بقياس انتاج الكورتيزول من طرف خلايا الكلى الرئيسية والطرق الفيزيولوجية المتدخلة في ذلك. أظهرت النتائج أن تركيب الاحماض الدهنية في خلايا الكلى الرئيسية يعكس تركيبتها في الاعلاف وبالتالي DHA، ARA ، EPA ومجموع n-3 HUFA انخفضوا في الاسماك المغذاة بالزيوت النباتية مقارنة مع المغذاة بزيت السمك. كما تبين أن استبدال 70% او 100% من زيت بذورالكتان يزيد انتاج الكورتيزول في خلايا الكلى الرئيسية بعد تحفيزها ب ACTH ، بينما التغذية بزيت فول الصويا يخفض هذا الرد. علاوة على ذلك ، تثبيط انتاج eicosanoids بحضانة خلايا الكلى الرئيسية مع NDGA خفض انتاج الكورتيزول بعد التحفيز ب ACTH في المعاملات 70LO,100LO, 100SO

إذن هذه الدراسة تؤكد تعديل انتاج الكورتيزول بالاحماض الدهنية المتعددة الغير مشبعة (HUFA) المكونة للاعلاف ووساطة LOX أو COX في آليات عملهم.

Chapter 10

Resumen ampliado

CAPITULO 10

Resumen ampliado

10.1. Introducción general (1)

Los lípidos como componente de las dietas de los peces

Los lípidos y ácidos grasos

Los lípidos son un grupo grande y diverso de compuestos orgánicos naturales que tienen en común una rápida solubilidad en tales solventes orgánicos (hidrocarburos, cloroformo, benceno, éteres y alcoholes) e insolubilidad general en agua. Hay una gran variedad estructural entre los lípidos, teniendo como forma básica cadenas de un hidrocarburo con un grupo carboxílico en un extremo y un grupo metílico terminal en el otro (carbón de n o de w). La cadena acil podía ser saturada o no saturada y puede ser esterificada a otras moléculas; entonces, los lípidos incluyen una gama diversa de compuestos, como los ácidos grasos y sus derivados, carotenoides, terpenos, esteroides y ácidos de bilis.

Los lípidos constituyen un grupo heterogéneo de compuestos, y diversas clasificaciones se utilizan para distinguirlos. Para los propósitos de la cromatografía, los lípidos se dividen en dos clases amplias incluyendo los lípidos complejos y los lípidos simples. Estos último consisten casi en todas las grasas y aceites comercialmente importantes de origen animal y vegetal (planta) incluyendo, los triacilgliceroles, los diacylglycerols, los 2 monoacyl-sn-gliceroles, los esteroles, las ceras, los tocoferoles y los ácidos grasos libres. Los lípidos complejos incluyen los glicerofosfolípidos, glucoglicerolípids, esfingomielina, glucosphingolípidos y eicosanoides. Si no, según su grado de polaridad, los lípidos se separan a los lípidos polares o neutros. Lípidos polares incluyendo los glicerofosfolípidos (actualmente nombrados fosfolípidos), glucoglycerolípidos, esfingomielina y glucosphingolípidos, mientras que los lípidos neutrales contienen los ácidos grasos y sus glicerolípidos, esteroles, ceras y tocoferoles derivados, entre otros.

Los ácidos grasos son el bloque hueco básico de los lípidos, algunos son componentes esenciales de la dieta, puesto que no pueden ser sintetizados por los

animales y tienen funciones fisiológicas y estructurales específicas. Los ácidos grasos poliinsaturados (PUFA) son ácidos grasos que contienen más de 18 átomos de carbono con dos o más dobles enlaces. Los PUFA lo más frecuentemente encontrados en la naturaleza son: el ácido docosahexaenoico (DHA; 22:6 n-3), el ácido docosapentaenoico (DPA; 22:5 n-3 o n-6), el ácido eicosapentaenoic (EPA; 20:5 n-3), el ácido araquidónico (ARA; 20:4 n-6), el ácido γ-linolenic (GLA; 18:3 n-6), el ácido α-linoleico (LNA; 18:3 n-3) y el ácido linoleico (LA; 18:2 n-6). Estos últimos son alimentos dietéticos importantes para los mamíferos incluyendo los seres humanos (Simopoulos, 2000). Los ácidos grasos altamente insaturados (HUFA), por ejemplo DHA, DPA, EPA y ARA son esos PUFA de 20 y más átomos de carbono en su cadena alifática con 3 o más insaturaciones, y los peces son la fuente dietética principal de los n-3 HUFA (Ackman *el al*., 1980; Sargent and Tacon, 1999). El ARA respresenta generalmente solamente 1-2% de los ácidos grasos totales en fosfoglicéridos de peces, con la excepción notable de los fosfatidilinositoles donde el ARA puede ser el principal PUFA (Sargent *et al.*, 1993; Izquierdo, 1996), así, los peces tienen altos requerimientos alimenticios para estos tres ácidos grasos (Sargent *et al*., 1997).

Los peces consumen naturalmente dietas ricas en (n-3) PUFA, sin embargo, la composición de ácidos grasos de los tejidos de peces cultivados es determinada generalmente por el tipo del lípido dietético ingerido y de la capacidad individual de la especie de peces de modificar esta entrada dietética por ambas vías del catabolismo y de la conversión incluyendo la desaturación y el la elongación (Henderson and Tocher, 1987; Sargent *et al*., 1989; Torstensen *et al*., 2000; Bell *et al*., 2001, 2002). Generalmente, los peces marinos son incapaces de estas conversiones (Ghioni and Tocher, 1999; Tocher *et al*., 1999) y requieren EPA ARA y DHA en sus dietas.

Funciones de los lípidos en peces

En común con otros vertebrados estudiados hasta ahora incluyendo los seres humanos, los peces tienen unos requerimientos dietéticos obligatorios para (n-6) y (n-3) PUFA. Para proveer los peces con dietas equilibradas y adecuadas, es necesario saber las funciones fisiológicas y metabólicas de los diversos nutrientes dietéticos. Además, los lípidos tienen funciones estructurales y fisiológicas múltiples en peces, puesto que los ácidos grasos son los componentes principales de la membrana celular. Además,

algunos ácidos grasos son también precursores importantes para una gama de mediadores altamente activos biológicamente en el metabolismo y la fisiología de los peces. Así, los ácidos grasos pueden actuar como segundos mensajeros requeridos para la traducción de señales externas, puesto que se producen rápidamente como consecuencia del atascamiento de agonistas específicos a los receptores de la membrana plasmática. Dentro de las células, los ácidos grasos pueden actuar para amplificar o para modificar de otra manera las señales de influir las actividades de las enzimas tales como de proteína quinazas, fosfolipasa, y muchos más. Están implicados en la regulación de la expresión génica, apuntando principalmente los genes que codifican las proteínas con papeles en el transporte o el metabolismo de los ácidos grasos vía efectos sobre factores de la transcripción, es decir los receptores proliferadotes de la activación del peroxisomo (PPARs) en los núcleos de las células, tales efectos pueden ser altamente específicos a los ácidos grasos particulares. De hecho, los fosfolípidos desempeñan unos papeles múltiples en las células con excepción de establecer barreras de la permeabilidad, proporcionan una matriz para el montaje y la función de una gran variedad de enzimas, participan en la síntesis de macromoléculas, y actúan como señales moleculares para influir en los acontecimientos metabólicos.

La inestabilidad de las bio-membranas, incluyendo la fragilidad mecánica y osmótica es afectada por su contenido en ácidos grasos. Por lo tanto, el ambiente puede cambiar la fluidez de la membrana, que actúa como receptor sensible que inicia la regulación celular (Beney and Gervais, 2001). Es importante que la fluidez de la membrana también es afectada por el contenido en los HUFA presentes en los fosfolípidos constitutivos, que debido a su estructura tridimensional y a las interacciones más bajas del hidrógeno-hidrógeno, aumentan la inserción de la proteína. Por ejemplo, la composición en ácidos grasos de los fosfolípidos de las membranas determina el nivel de su fluidez (Bell *et al*., 1986), y considerando que el pez es un organismo poiquilotermo, su contenido en ácidos grasos de sus membranas es crucial para adaptarse a los cambios ambientales de temperatura. Además, los cambios en la composición en ácidos grasos de las membranas celulares podrían afectar directamente a las actividades de membrana limitando las enzimas y los receptores, y perturbando proceso fisiológico por lo tanto diverso (crecimiento, reproducción, sistema inmune,… etc).

Los requerimientos en los ácidos grasos esenciales (EFA) de las especies de peces de agua dulce y marinos han sido estudiado extensivamente durante los últimos 20 años y varían cuantitativamente y cualitativamente (Sargent *et al*., 1989, 1995, 2002). En los peces de agua dulce, los requerimientos en los EFA pueden ser cumplidos suministrando el LA y/o el LNA, aunque mejores resultados puedan ser alcanzados suministrando las formas "bioactivas" de los n-3 HUFA, principalmente de EPA y de DHA (Kanazawa, 1985). Los lípidos dietéticos proporcionan los PUFAs esenciales necesarios para el crecimiento y el desarrollo de las células y de los tejidos (Sargent *et al*., 1995) y son también una fuente de energía importante (Sargent *et al*., 1989) produciendo 9 Kcalorias/gr, promoviendo su uso para ahorrar parcialmente el uso de la proteína en los alimentos para la acuicultura bajando el coste de las dietas (Vergara *et al*., 1996; Morrow *et al*., 2004). Además, los fosfoglicéridos de los peces son naturalmente ricos en DHA y EPA y puede ser asumido que estos ácidos grasos cumplen el mismo papel estructural en los fosfoglicéridos de la bio-membrane que el ARA hace en altos mamíferos terrestres (Sargent, 1995).

En peces, algunos informes demuestran efectos positivos de n-3 FA sobre la respuesta inmune, puesto que la alta actividad de los macrófagos principales del riñón anterior ha sido asociada a altos niveles de los ácidos grasos dietéticos n-3 en el siluro (Blazer, 1991; Sheldon and Blazer, 1991). Ashton y colaboradores (1994) han encontrado que el sobrenadante principal del riñón anterior derivado de la trucha de arco iris alimentada con una dieta enriquecida con los ácidos grasos n-3 tenía mayor migración que la capacidad estimulante que el sobrenadante de peces alimentados con una dieta enriquecida con ácidos grasos de la serie n-6. De tal modo, una cantidad adecuada de los ácidos grasos n-3 fue encontrada ser esencial para la correcta función del sistema inmune.

Mientras que PUFAs de larga cadena poseen una amplia gama de funciones celulares, una de las más importantes es suministrar los precursores para la producción de eicosanoides, que son los metabolitos bioactivos de ácidos grasos que pueden modular muchas funciones inmunes (Gershwin *et al*., 1985; Uhing *et al*., 1990). Los eicosanoides se producen a partir de los C20 PUFAs, especialmente ácido di-homo gamma linolenico (20: 3n-6; DHGLA), ARA y EPA, por la acción de las enzimas ciclooxígenasa (COX) y de la lipooxígenasa (LOX) para producir una amplia gama de compuestos bio-activos, incluyendo las prostaglandinas (PG) y los tromboxanos (TX)

producidos por la acción de COX. La LOX rinde una gama de los ácidos grasos monohidróxidos (e.g. 5 (S) - ácido hidroxi-eicosatetraenoico; 5-HETE derivado de ARA), mientras que los di- y los ácidos grasos tri-hidroxis, tales como leucotrienos (LT), los lipoxinos (LX), también se forman vía los intermediarios epoxy (Samuelson, 1983).

El ARA es el principal precursor de eicosanoides en las células de mamíferos, dando lugar, entre otros productos, a las prostaglandinas de la serie 2 (Horrobin, 1983). Otros C20 PUFAs, tales como DHGLA y EPA son también substratos para la producción de eicosanoides y producen las prostaglandinas de la series 1- y 3-, respectivamente. Aunque el DHGLA y el EPA son generalmente substratos más pobres para la síntesis de la prostaglandina que el ARA (Crawford, 1983), ambos compiten para el punto de enlace de la enzima y pueden reducir la producción de prostaglandinas derivadas del ARA (Willis, 1981; Bell *et al*., 1994).

Los eicosanoides tienen diversas acciones patofisiológicas incluyendo la respuesta inmune y los procesos inflamatorios. Así, estan implicados en la regulación del sistema inmune por sus efectos directos sobre las células tales como macrófagos y los linfocitos o sus efectos indirectos vía las cítokinas (Rowley, 1995). La PG, especialmente la PGE2 es importante en la modulación de la función de las células inmunes y mientras que PGE2 se requiere para la normal función inmune, su superproducción puede ser inmunosupresiva (Kinsella *et al*., 1990). Además, el producto LTB4 de la lipooxígenasa es un modulador de gran alcance de la función de la células inmunes, aumentando la proliferación de las células de tipo T y B, estimulando la liberación de las cítokinas de los monocitos y de las células T, actuando como un potente quimo-attractante e induciendo la actividad natural de la célula natural killer (NK) (Kinsella *et al*., 1990; Claesson *et al*., 1992). Los derivados de la LOX también han demostrado regular y mediar las interacciones entre la inmunidad natural y adaptativa para la defensa del anfitrión (Hedi and Norberto, 2004). Los estudios recientes indican que algunos patógenos inhabilitan la inmunidad de los invertebrados a través de la inhibición de las acciones de los eicosanoides en la inmunidad celular, indicando el papel clave de los eicosanoides en la defensa inmune (Dean *et al*., 2002; Stanley and Miller, 2006). Los niveles dietéticos de n-3 HUFA influyen sobre los patrones de la producción de los eicosanoides en tejidos de peces, posiblemente como

consecuencia de cambios en el cociente de EPA/ARA en los tejidos (Bell *et al*., 1993, 1997).

Fuentes de los lípidos en dietas para peces

La acuicultura ha usado tradicionalmente productos provenientes de la industria pesquera, como harina y aceite de pescado (FM y FO), para convertir la proteína y el aceite relativamente baratos en productos de elevado valor, una práctica que esta científicamente y comercialmente correcta (Bell *et al*., 2002). Sin embargo, el desarrollo rápido de la acuicultura, que está creciendo por el 10% por año (Tidwell and Allan, 2002), junto con las inclusiones del FO para las alimentaciones de los animales domésticos y las aplicaciones farmacológicas, afectó marcadamente el coste y la disponibilidad del FO. Por lo tanto, la demanda para estos productos está aumentando rápidamente y las nuevas estimaciones sugieren que los alimentos para acuicultura consumirán aproximadamente el 90% y el 60-70% de la producción de FO y FM respectivamente en 2010 (Figura 1.1) (Allodi, 2007) y esta previsto que para 2012 las necesidades de acuicultura en FO excederán la producción total. Como consecuencia, la disponibilidad del FM y del FO para los alimentos de peces es limitada (Anon, 2002) durante períodos estabilizados de pesca extractivas, y arriesgadas puesto que las producción pesquera disminuye como consecuencia de fenómenos naturales tales como "El Niño" (Anon, 2002; Naylor, 2004). Por otra parte, el uso continuo del FM y FO puede convertir la acuicultura en una presión agregada sobre los caladeros que en un contribuidor neto a suministrar pescado para el mundo, con las consecuencias desastrosas para el ecosistema (Staniford, 2002; Milewski, 2002; New, 2002; Allan, 2004). Por otra parte, uno de los continuos largos debates en acuicultura es el uso de FM y FO en los alimentos y la cantidad de pescados salvaje necesarios para producir peces de cultivo, llamados los cocientes de Fish In-Fish Out (FIFO) que se extienden de 3:1 al 10:1 (organización internacional del aceite y de la harina de pescado: IFFO, 2008); y la revisión más reciente por Tacon y Metian (2008) encontró un IFFO para los salmones de 4.9:1, significando que hay que usar 4.9 toneladas de pescados salvaje para producir 1 tonelada de salmón de cultivo. Además, los precios del FO se han duplicado durante los últimos 5 años (Figura 1.1), y continúan incrementándose haciendo más rentable el uso de estos ingredientes para el consumo humano. Por lo tanto, las restricciones en el coste y la disponibilidad impuestas sobre el FM y FO durante los

últimos 15 años han incitado a la substitución gradual y sostenible de estos ingredientes por fuentes alternativas de lípidos y proteínas.

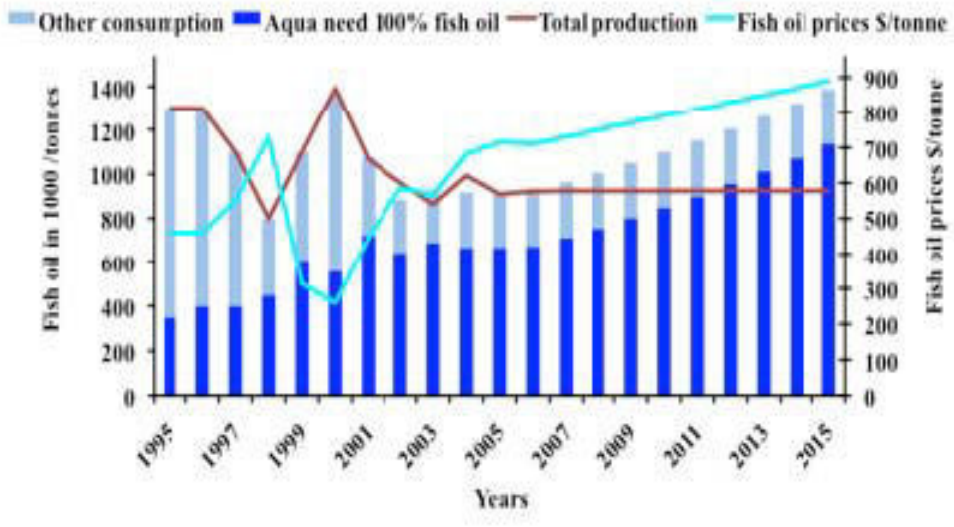

Figura 1.1: La evolución y la previsión de los usos y las necesidades en el aceite de pescado desde 1995 hasta 2015. Basado en datos de Allodi 2007.

Este gráfico demuestra claramente el aumento significativo del uso del FO para la acuicultura durante los 15 años pasados y su proyección de aumentar en los próximos 5 años. La sensible disminución en la producción del FO en 1998 era debido al fenómeno del "El Niño". Así, las aplicaciones del FO para los alimentos crecientes de menos del 30% hasta más del 80% de producción total del FO actualmente y esta previsto que todas las necesidades en el FO para alimentos de peces van a superar la producción total del FO en 2011. Como consecuencia de esta presión sobre los limitados recursos naturales, los precios del FO se han duplicado durante la última década, pasando de los 400 $/ tonelada en 2000 a más los 750 $/tonelada hoy, y de ella se espera que continuarán aumentando hasta 900 E.E.U.U. $/tonelada en 2015 (IFFO, 2009). Como consecuencia, se requieren ingredientes alternativos al FO de manera urgente, con los aceites vegetales siendo los primeros candidatos (Bell and Waagbø, 2008).

Los peces marinos son adaptados sobre todo a una dieta carnívora/piscívora que sea naturalmente rica en n-3 HUFAs, desarrollando una deficiencia relativa en las enzimas (los desaturasas y los elongasas) responsables de la vía de conversión del LA y

del LNA a sus productos finales de HUFAs (Ghioni *et al*., 1999; Tocher and Ghioni, 1999; Zheng *et al*., 2009). Igual como en los vertebrados, los PUFAs son esenciales en dietas para peces, pero hay unos requerimientos especiales en HUFAs tales como el EPA y el DHA, algo mas que PUFAs de cadena corta encontrados en los aceites vegetales (VO) (Tocher, 2003).

Actualmente, más de 100 millones de toneladas de aceites vegetales se producen por año incluyendo la palma, la soja, el lino, aceites de oliva y de girasol, con la palma y la soja demostrando ser las producciones más grandes (Figura 1.2) (O'Mara, 1998). Los aceites vegetales (VO) y las proteínas vegetales (VM) expresan con frecuencia producciones y precios más fiables que los FO y FM, lo que representa una ventaja en la producción de alimentos para peces.

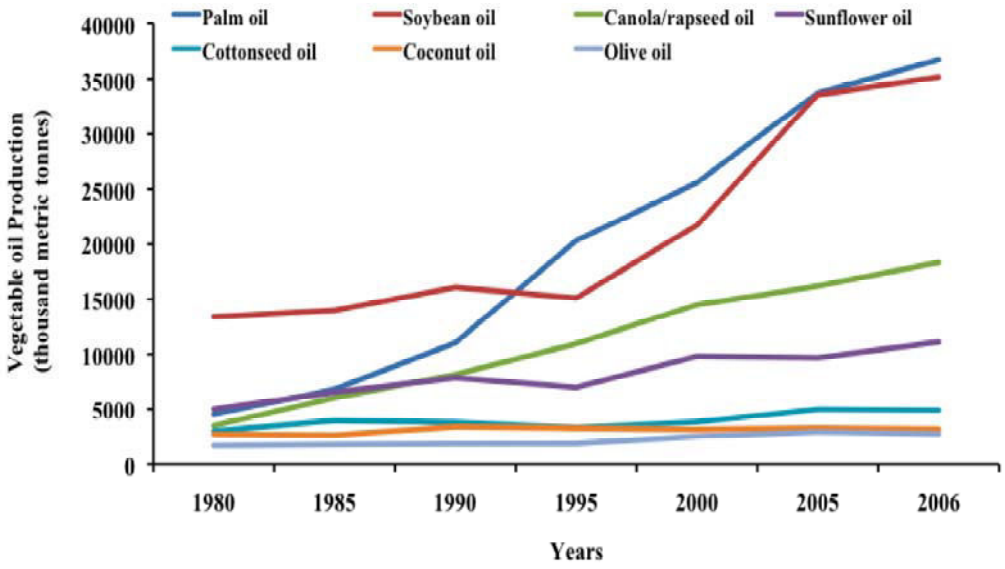

Figura 1.2: Evolución de la producción de los diferentes aceite vegetales.

Así, se podrían utilizar menos FM y FO sostenibles para las materias o como ingredientes estratégicos, su inclusión en dietas para peces dependiendo de la especie y los requerimientos de la etapa de la vida de peces así como de los precios relativos de los ingredientes. Por lo tanto los VOs se consideran actualmente como la única viable fuente alternativa competitiva de lípidos con costes competitivos en dietas para acuicultura y un número de trabajos ha utilizado como sustituto parcial y completo para FO (Caballero *et al*., 2002, Izquierdo *et al*., 2003, 2005; Montero *et al*., 2003, 2005; Bell *et al*., 2005a).

Las alternativas sostenibles de las fuentes de la proteína a FM incluyen varias harinas vegetales con los contenidos proteínicos crudos de 20 a el 50% (Hertrampf and Piedad-Pascual, 2000), con la harina de soja representando actualmente la opción predominante. Sin embargo, los peces más carnívoros tienen una capacidad limitada de tolerar alto contenido de la harina de soja (SBM) en sus dietas (Kaushik *et al*., 1990; Torstensen *et al*., 2008) debido a la pobre digestibilidad de la misma, una disponibilidad más baja de algunos aminoácidos esenciales y la baja palatabilidad de SBM con respecto a FM así como la presencia de factores antinutritivos. Con respeto a la substitución del FO, varios estudios han precisado la inclusión acertada de diversas mezclas de VOs en las dietas para diversas especies sin afectar el crecimiento o la utilización del alimento, con niveles de inclusión dependiendo de la especie (Bell *et al*., 2001; Caballero *et al*., 2002, Montero *et al*., 2003; Izquierdo *et al*., 2003, 2005; Torstensen *et al*., 2005; Mourente and Bell, 2006). Por ejemplo, se ha demostrado que es posible substituir hasta el 60% de las FO con aceites vegetales (VO) en las dietas para la dorada sin afectar al crecimiento, la supervivencia, la utilización del alimento o las características organolépticas del filete, cuando los peces se alimentan durante un periodo medio (3 meses, Izquierdo *et al*., 2000, 2003) o largo (8 meses) (Menoyo *et al*., 2004; Izquierdo *et al*., 2005). Sin embargo, el efecto de estos o niveles más altos de la substitución sobre la salud de los peces y resistencia al estrés no se ha determinado claramente.

Aceites vegetales y salud de los peces

La inclusión de las fuentes vegetales de lípidos y de las proteínas en dietas para peces de cultivo se considera como una solución sostenible a la industria de la acuicultura para hacer frente a la problemática del FO y FM. Para satisfacer este propósito, la investigación de la industria debe no sólo determinar el efecto de substituir los dichos ingredientes sobre los parámetros estándares de cultivo como el crecimiento, las tasas de supervivencia o el cociente de conversión del alimento (FCR), pero también sobre las características nutricionales, sensoriales, de proceso, y de seguridad de los productos de la acuicultura (Barlow and Pike, 2001). Además, es imprescindible que el impacto de potenciales sustitutos de FM y FO en otros factores tales como la salud y el bienestar serán determinados exactamente (Barlow and Pike, 2001).

La nutrición es uno de los factores principales que determinan el estado de salud y de bienestar en peces de cultivo. El suministro dietético de aminoácidos, de PUFAs, de cofactores de la enzima y de energía, es esencial para mantener el sistema inmune de los peces incluyendo la proliferación de linfocitos y la síntesis de los determinantes (e.g. inmunoglobulinas, lisozima y complemento) y de las moléculas de la comunicación (e.g. los cítokinas y los eicosanoides). Por otra parte, las dietas adecuadas mejoran la salud de peces convalecientes, mientras que alimentando peces con dietas desequilibradas causan la desnutrición y aumentan la susceptibilidad al estrés y a las enfermedades. Por lo tanto, los brotes de enfermedades en los peces ocurren comúnmente cuando los peces están estresados debido a una variedad de factores incluyendo la malnutrición (Lall, 2000). Así, la substitución inadecuada de FM y del FO por otras fuentes de origen vegetal puede llevar a los desequilibrios alimenticios que podrían afectar a la salud y al bienestar de los peces.

Algunos estudios han precisado alteraciones negativas en ciertos parámetros de la salud de los peces y la resistencia al estrés en la alimentación de los peces con VO (Bell *et al*., 1993, 1996; Montero *et al*., 2003; Jutfelt *et al*., 2007). Por ejemplo, los salmones alimentados con VOs tienen reducciones significativas en varios parámetros inmunes no específicos incluyendo el hematocrito, los glóbulos blancos totales y RBC, y la actividad respiratoria del macrófago (Good *et al*., 2001). Por lo tanto, el efecto de la inclusión de los VOs parece depender del tipo del VO, del nivel de la substitución del FO, de la especie de peces o de las condiciones de cultivo. En dorada, el aumento en los niveles de substitución de FO del 60 hasta el 80% por los aceites vegetales reduce significativamente el crecimiento y los índices de conversión del alimento (Izquierdo *et al*., 2007) alterando la morfología normal del hepatocito y del enterosito (Caballero *et al*., 2003), y afectando negativamente las funciones inmunes y la respuesta post estrés del cortisol plasmático (Montero *et al*., 2003). Además, la inclusión de la soja o del aceite de colza con el 60% de substitución reduce la actividad fagocitaría del macrófago (Montero *et al*., 2003) mientras que la reducción del EPA y del DHA en la dieta se ha demostrado inhibir significativamente la actividad alternativa de la vía del complemento del suero (Montero *et al*., 1998). Sin embargo, poco se sabe sobre los mecanismos implicados en el efecto de PUFA sobre la resistencia al estrés y a las enfermedades en peces de cultivo, que parece ser mediado, por lo menos en parte, por la producción de eicosanoides en tejidos. De hecho, los cambios en la composición de los ácidos grasos

(AG) de los fosfolípidos afectan a la cantidad y al espectro de la síntesis de los eicosanoides (Bell *et al*., 1992, 1993, 1994, 1995a, b, 1996; Ashton *et al*., 1994; Lall, 1998; Balfry and Higgs, 2001) y como en vertebrados superiores, la producción alterada de los eicosanoides puede afectar a la composición y el funcionamiento de las células inmunes (Kinsella and Lokesh, 1990; Kelley and Daudu, 1993). Por otra parte, a pesar de estos estudios sobre el efecto de los VOs dietéticos sobre la producción de las prostaglandinas en diversos tejidos se han conducido en peces de agua dulce y en ciertas especies marinas nórdicas (Bell *et al*., 1992, 1994, 1996a), ningunos estudios han sido conducidas hasta ahora en la producción de eicosanoides en dorada, o su alteración por los lípidos dietéticos.

Puesto que los diferentes aceites vegetales empleados como fuentes de lípidos para substituir al FO se diferencian marcadamente por su perfil en ácidos grasos, incluyendo el contenido relativo en los ácidos grasos n-3 y n-6, su introducción en dietas de peces modifican claramente la composición en ácidos grasos de los diferentes tejidos (Torstensen *et al*., 2000; Bell *et al*., 2001, 2002, Caballero *et al*., 2002; Montero *et al*., 2003; Izquierdo *et al*., 2003, 2005, 2007), esto a su vez puede llevar a las diferencias en la producción de los eicosanoides y en la respuesta inmune (Balfry and Higgs 2001; Thompson *et al*., 1996; Balfry *et al*., 2006). Así, Bell y colaboradores (1992, 1993, 1996) divulgaron que el salmón del Atlántico alimentado con dietas que contienen niveles crecientes de girasol (rico en n-6, por lo tanto con n-3/n-6 disminuido), producen más eicosanoides derivados del ARA comparados con los peces alimentados con dietas que contenían el aceite de la lino o de pescado (con un cociente más alto de n-3/n-6) que produjo más eicosanoides derivados de EPA. Además, la actividad creciente de los macrófagos principales del riñón se ha asociado con niveles más altos de los ácidos grasos dietéticos n-3/n-6 en el pez gato (Sheldon and Blazer, 1991). Por otra parte, se ha publicado que los eicosanoides derivados de n-3 HUFAs tienen una influencia más represiva en el sistema inmune, y son más frecuentes en las dietas que contienen altas concentraciones del ácido linolenic y de los n-3 HUFA (Higgs *et al*., 1995). De hecho, algunos investigadores han advertido que los cocientes relativos de ácidos grasos y de precursores de los eicosanoides son la clave para entender el efecto de los lípidos dietéticos sobre el sistema inmune (Ashton *et al*., 1994).

Además, alimentando peces con niveles bajos de n-3/n-6 fue asociado con alta actividad respiratoria realizada de los leucocitos periféricos de la sangre (PBLs) (Balfry *et al*., 2006), y a la actividad bactericida creciente de macrófagos del pez gato (*Ictalurus punctatus*). Además, una actividad significativamente más alta de la Natural-killer-like-cell también ha sido observada en los leucocitos aislados de la trucha arco iris (*Onchorhynchus mykiss*) (Kiron *et al*., 1995), y de los salmones del Atlántico (Balfry *et al*., 2006) que fueron alimentados previamente con una dieta enriquecida con n-3. Otros informes anteriores han sugerido que la alta producción del anticuerpo está asociada a alto n-3 HUFA dietético (Erdal *et al*., 1991; Waagbo *et al*., 1993). De hecho, los altos cocientes dietéticos n-3/n-6 dieron lugar a respuestas crecientes del linfocito B y mejoraron la supervivencia de los peces después de un desafío a la enfermedad (Thompson *et al*., 1996), y a números más elevados de trombocitos en el pez gato, y consecuentemente tenían bajo nivel de eicosanoides derivados de los ácidos graos de la serie (n-6) (es decir, tromboxano) implicados en la coagulación de sangre, y compensan esto con números elevados de trombocitos (Klinger *et al*., 1996). Hay evidencias que sugieren que cambiando la composición en ácido grasos de las células inmunes pueda influir en la función inmune cambiando la fisiología de la membrana celular pero quizás más importantemente influenciando la producción de porstaglandinas y de los leucotrienos. Esto es apoyada por un número de estudios que demuestran una reducción en la producción de PGE2 y del leucotrieno B4 (LTB4) por los macrófagos estimulados del riñón anterior de salmones alimentados con una dieta con un cociente dietético n-3/n-6 más alto (Bell *et al*., 1993, 1996). Montero y colaboradores (2003) encontraron que alimentando la dorada con un n-3/n-6 más alto incluyendo LO en la dieta (rica en LNA, cociente cada vez mayor n-3/n-6), disminuyó los números de eritrocitos en la circulación. También se ha demostrado que los lípidos dietéticos determinan el perfil de los ácidos grasos de macrófagos y de las células inmunes en el bacalao (Waagbø *et al*., 1995), la lubina (Farndale *et al*., 1999) y dorada (Montero *et al*., 2003).

Sin embargo, los impactos que los ácidos grasos dietéticos tienen en la respuesta inmune son complejos y dependen de varios factores que influencien la producción de eicosanoides incluyendo la competición entre los ácidos grasos de tipo n-3 y n-6 durante el metabolismo para la elongación y la desaturación de cadena. Por lo tanto, hay una sugerencia clara que los cocientes de n-6 a los ácidos grasos n-3 en los lípidos dietéticos pueden desempeñar un papel importante en el equilibrio de la composición en

PUFAs de la membrana celular de peces (Bell *et al*., 1993, 1996, 2001; Caballero *et al*., 2002; Izquierdo *et al*., 2005), modulando posteriormente sus parámetros inmunes (Balfry *et al*., 2006).

Los efectos beneficiosos de los lípidos de peces (ricos en n-3 PUFA) en el control de la enfermedad cardiovascular y del desarrollo neurológico están bien documentados en la literatura de la nutrición humana (Herbaut, 2006; Simopoulos, 2008). Por lo tanto, los altos cocientes dietéticos de n-6 PUFA y de n-6/n-3 PUFA se han sugerido para desempeñar un papel en muchas enfermedades crónicas occidentales (Simopoulos, 2008; Yam *et al*., 1996). Así, al substituir el aceite de pescado con una mezcla de aceites vegetales, hay que tener cuidado para establecer una correcta mezclas no sólo para asegurar el buen crecimiento de los peces, pero también proveer al animal los cocientes adecuados de los ácidos grasos n-3/n-6 para la correcta función de sus procesos fisiológicos y considerar las ventajas alimenticias del producto final para el consumidor (Bell *et al*., 2001).

Nutrición lipídica y resistencia de los peces al estrés

Los estudios anteriores han demostrado la importancia de alimentar los peces con niveles adecuados de diferentes nutrientes para garantizar la capacidad de los mismos de adaptarse al estrés crónico o agudo del medio (Salte et al., 1988). Entre varios nutrientes, los n-3 HUFA dietéticos aumentan la resistencia al estrés crónico y agudo en juveniles de dorada (*Sparus aurata*) (Montero and Izquierdo, 1998; Montero *et al*., 2001). En la anguila Europea (*Anguila anguila*) y el esturión Adriático (*Acipenser naccarii*) n-3 dietético HUFA aumentan la tolerancia a las condiciones de hipoxia (Mckenzie, 2001). Por otra parte, en adultos de dorada, alimentando los peces con dietas deficientes en n-3 HUFA aumenta el cortisol plasmático basal antes del estrés por el confinamiento, demostrando el efecto de la deficiencia alimenticia sobre el estrés (Montero and Izquierdo, 1998). Se ha demostrado también que el DHA y el EPA son esenciales para mejorar la resistencia al estrés causado por el manejo en las larvas de varias especies de peces tales como la dorada roja (*Pagrus major*) (Izqueirdo *et al*., 1988; Watanabe *et al*., 1989), platija japonesa (*Paralichthys oliváceo*) (Izquierdo et al., 1992; Furuita et al., 1999), también como al estrés debido al cambio de temperatura en lubina (*Dicentrarchus labrax*) (Persona-Le Ruyet *et al*., 2004). Generalmente, la manipulación de la composición dietética en ácidos grasos afecta a la capacidad de las

larvas de peces de resistir a varios factores estresantes, por lo tanto influenciando la supervivencia después del estrés (Izquierdo *et al*., 1989; Watanabe *et al*., 1989; Tuncer *et al*., 1993; Kanazawa, 1997; Gapasin *et al*., 1998; Koven *et al*., 2001, 2003; Liu *et al*., 2002). De hecho, alimentando con n-3 HUFA también se ha visto mejorar la resistencia de los crustáceos a diferentes condiciones estresantes, tales como el choque osmótico (Palacios *et al*., 2004; Sui *et al*., 2007), fluctuación de la temperatura (Chim *et al*., 2001) y amoníaco (Martins *et al*., 2006).

La elevación del ARA en la dieta, también se ha demostrado mejorar la supervivencia total (Bessonart *et al*., 1999) y la resistencia al estrés por el manejo en larvas de dorada (Koven *et al*., 2001). Una concentración óptima del ARA en la dieta también fue encontrada eficiente para mejorar la resistencia al estrés causado por un desafío de híper-salinidad en las larvas de la platija de verano (Willey *et al*., 2003) y la lubina estriada (Harel *et al*., 2001).

Varios estudios han sugerido que el ARA está implicado en la liberación del cortisol en peces, aunque los mecanismos reales no se hayan investigado (Gupta *et al*., 1985; Harel *et al*., 2001; Koven *et al*., 2003). Koven y colaboradores (2003) encontraron que la ingestión dietética del ARA conjuntamente con cambios diarios de la salinidad fue asociada con niveles elevados de cortisol en el cuerpo entero de las larvas de dorada al final del período experimental. Los autores sugirieron que el ARA mejora la sensibilidad del eje HPI, y conjuntamente con el estrés diario éste dio lugar a niveles más altos de cortisol. Así, el ARA se ha encontrado ser importante para mejorar la resistencia al estrés en larvas de la dorada (Koven *et al*., 2001a,b), y la alta ingestión dietética del ARA para la misma especie bajó la sensibilidad de los peces al estrés agudo, según lo evidenciado por los niveles del cortisol y la baja osmolalidad del plasma (Van Anholt *et al*., 2004).

Sin embargo, los mecanismos fisiológicos por los cuales estos ácidos grasos regulan la resistencia al estrés en los peces o los niveles de cortisol en plasma no están claros. En mamíferos, ciertos estudios sugieren que las prostaglandinas desempeñen un papel importante en la mediación de la acción corticosteroidogenasa de la Hormona adrenocorticotrófica (ACTH) (Kocsis *et al*., 1999), sugiriendo que el papel de los ácidos grasos en la respuesta al estrés es mediado por la producción de eicosanoides. En peces,

hay evidencias que sugieren también la mediación de estas moléculas en la liberación del cortisol (Koven *et al*.,, 2001; Van Anholt *et al*., 2004).

El estrés en los peces

Respuestas al estrés

La definición del estrés varía entre diversos autores, pero puede ser descrita como una serie de cambio fisiológicos que ocurren en el organismo para recuperar la homeostasis amenazada por los factores internos o externos (estresantes). El estrés se puede también describir como una respuesta hormonal interna de un organismo vivo causada por el medio o otros factores externos que mueven ese organismo fuera de su homeostasis (Selye, 1973) forzando una reasignación de la energía dentro de su sistema. El estrés es inevitable en acuicultura y es uno de los grandes problemas crecientes para los sistemas intensivos de la acuicultura moderna, donde los peces se exponen con frecuencia a varios factores estresantes, tales como clasificación, transporte y vacunación.

Las condiciones estresantes tienen consecuencias negativas en el funcionamiento de los peces en acuicultura, causando la reducción del crecimiento, la inmune-supresión y la susceptibilidad creciente a las enfermedades infecciosas, dando como resultado unas pérdidas económicas importantes para las granjas de acuicultura. La respuesta de peces a tales factores de estrés implica todos los niveles de organización desde la célula (Hightower, 1991) hasta el organismo individual (Barton and Iwama, 1991; Mommsen *et al*., 1999) y a la estructura de la población (Adams, 1990).

Bajo situaciones de estrés, el pez experimenta una serie de reacciones fisiológicas en un intento por recuperar la homeostasis que podría llevar a una respuesta adaptativa o a una respuesta mal-adaptiva lo que afecta negativamente a las características de los peces. Estas respuestas se distinguen en tres diversos tipos según el llamado síndrome general de adaptación (GAS; Selye, 1975):

a) Una respuesta primaria, la *reacción de alarma*, que incluye la percepción del nuevo estado alterado por el organismo y la liberación de las hormonas y de los neurotransmisores de estrés. Por lo tanto, las catecolaminas son liberadas a partir del tejido cromafino situado en el riñón anterior de los teleósteos y de los nervios adrenérgicos (Randall and Perry, 1992). El cortisol se libera del tejido interrenal,

situado en el riñón anterior, en respuesta a varias hormonas pituitarias, particularmente la ACTH que alternadamente es liberada del hipotálamo por la hormona de liberación corticotrófica (CRH) (Donaldson, 1981; Vijayan *et al*., 2005; Alsop and Vijayan 2008a).

b) Una respuesta secundaria o *etapa de resistencia*, durante la cual el organismo ajusta su metabolismo para hacer frente al disturbio sufrido. Así, las hormonas de estrés liberadas activan un número de vías metabólicas incluyendo aquellas que implican la movilización y reajuste de la energía, un aumento en el disturbio osmótico y en el ritmo cardiaco, un aumento en la absorción de oxígeno y su transferencia, dando por resultado alteraciones en la química y la hematología de la sangre (Barton and Iwama, 1991, Iwama *et al*., 2006).

c) Una respuesta terciaria o *etapa de agotamiento*, puede ocurrir cuando el organismo no puede adaptarse o superar los cambios causados por el factor estresante. Por lo tanto, un estrés severo puede causar mortalidad masiva, y el estrés subletal puede comprometer varias funciones del comportamiento y de la fisiología (Campbell *et al*., 1992; Agravio *et al*., 1996; Iwama *et al*., 1997; Wendelaar Bonga, 1997; Ortuño *et al*., 2002; Vijayan *et al*., 2005). Así, el crecimiento de los peces y las funciones de la reproducción se reducen en las situaciones de estrés (Barton *et al*., 1986; Mesa, 1994; Schreck *et al*., 2001), suprime la inmunidad celular afectando a las vías inflamatorias de la señalización (Ortuño *et al*., 2002; Holanda *et al*., 2003; MacKenzie *et al*., 2006; Aluru y Vijayan, en prensa), y también favorece enfermedades infecciosas e incluso causa la muerte de los peces (Pankhurst and Van der Kraak, 1997).

La regulación neuroendocrina de la respuesta al estrés en peces es similar a la de los vertebrados superiores e implica dos ejes reguladores (Figura 1.3) (Wendelaar Bonga, 1997; Barton, 2002):

• EL eje de las células hipotálamo- simpatetico-nervioso-cromafino (HSC), equivalente al sistema Comprensivo-Suprarrenal-Medular (SAM) en vertebrados. Consecuentemente a la activación de las fibras de nervio comprensivas, que inervan las células del cromafino, estimulando la liberación de catecolaminas (epinefrina y noradrenalina) vía los receptores colinérgicos (Reid *et al*., 1996). Las catecolaminas, como predominante la epinefrina en peces teleósteos, se liberan rápidamente a la circulación en respuesta a un estímulo del estrés (Randall and Perry, 1992) y se

transportan a los diversos órganos, alterando las funciones normales de los diversos procesos fisiológicos (respiración, reproducción, sistema inmune… etc.) (Wendelaar Bonga, 1997).

• Eje de las células Hipotálamo-Pituitario-Interrenal (HPI): Es equivalente al eje Hipotálamos-Pituitario-Suprarrenal, HPA en vertebrados superiores. El eje de HPI es el regulador de la respuesta al estrés en peces, una vez activado en respuesta a casi todas las formas de estrés (Wendelaar Bonga, 1997). Brevemente, en respuesta a una situación de estrés, el hipotálamo libera la Hormona Liberadora de la Corticotropina (CRH) que a su vez actúa en los *Pars distalis* de la adeno-hipófisis, cerca de la boca, accionando la liberación de la hormona adreno-corticotropica (ACTH), y, en menos cantidad, la hormona-estimulante de α-melanocyte (γ-MSH) y la hormona lipotrópica (β-LPH), que son productos intermedios de la síntesis de la ACTH. Luego, la ACTH induce la producción y la liberación del cortisol, a partir de las células interrenales situadas en el riñón anterior, a la circulación (Figura 1.3). La liberación del cortisol es controlado por una regeneración negativa en diversos niveles del eje HPI (Donaldson, 1981; Bradford *et al*., 1992; Wendelaar Bonga, 1997).

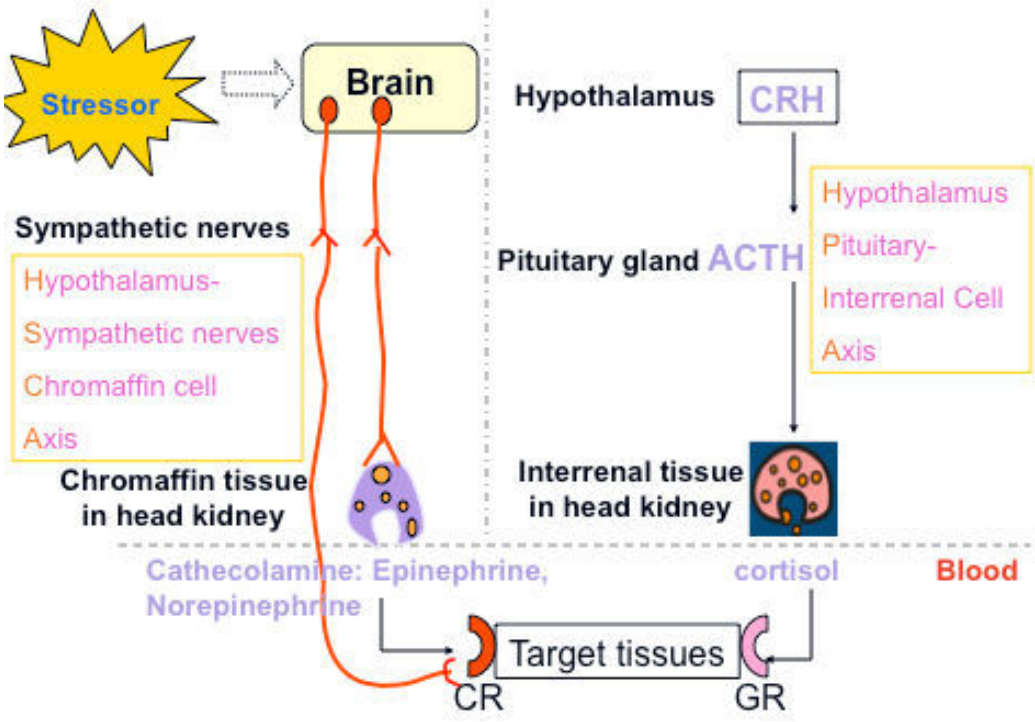

Figura 1.3: Una representación simplificada de los componentes centrales y periféricos de la respuesta al estrés en peces. Hormona liberadora de la corticotropina (CRH), hormona adreno-corticotropica (ACTH), receptor de la catecolamina (CR), receptor de glucocorticoides (GR).

Según la intensidad y la duración del desafío estresante, hay varios tipos de situaciones de estrés: el estrés agudo, crónico, repetitivo, irregular repetitivo, …etc. El estrés agudo es producido por un factor estresante intenso de duración corta, mientras que una situación crónica del estrés implica un disturbio de largo plazo que actúe como factor estresante intenso o suave.

El riñón anterior como órgano clave en la respuesta al estrés

Anatómicamente, los peces no poseen una glándula adrenal como en mamíferos. El riñón de teleósteos consiste en una parte anterior separada del resto del riñón (cuerpo del riñón), derivadas de los tejidos pronefros y mesonefros, respectivamente. La forma externa del riñón de los peces varía según las especies. El riñón anterior es un órgano encajonado en hueso, debe haber tomado su posición en la cabeza, y ha penetrado la vejiga de aire y el arco escapular, mientras que muchos nefrones y tejido linfoides intersticial constituyen el cuerpo del riñón entero. El riñón principal contiene la glándula interrenal (homóloga a la corteza suprarrenal en mamíferos) responsable de la liberación del cortisol y de las células de cromafin (homólogas a la médula suprarrenal), rodeando la vena y sus ramas (Milano *et al*., 1997) del postcardinal. Las células de cromafino se establecen solo o en racimos en las paredes de la vena del postcardinal rodeada por las células interrenales (Imagawa *et al*., 1995). La localización de las celulas interrenales y de las células de cromafino cerca de la vena del postcardinal facilita su regulación por el sistema endocrino vía la circulación sanguínea. El tejido interrenal exhibe la considerable variación morfológica entre los grupos taxonómicos (Nandi, 1962), y es considerado como el principal tejido endocrino, hematopoyético y linfático en peces (Takashima and Hibiya, 1995). Así, el riñón anterior es considerado como el tejido clave en la respuesta al estrés.

Hay una poca información disponible sobre las funciones del tejido del riñón anterior en peces, y los estudios recientes se han interesado en usar este órgano en diversas preparaciones experimentales para estudiar sus funciones. La variabilidad en el tamaño y el número de células del riñón anterior entre los peces individuales (Pottinger *et al*., 1995) y relativamente una gran cantidad de peces es requerida lo que imposibilita el uso rutinario de riñones anteriores enteros o en fragmentos en los sistemas estáticos o

de perfusión (Patiño *et al*.,, 1986; Benguira and Hontela, 2000, Rottlant *et al*., 2000) para las pruebas *in Vitro* (figura 1.4)

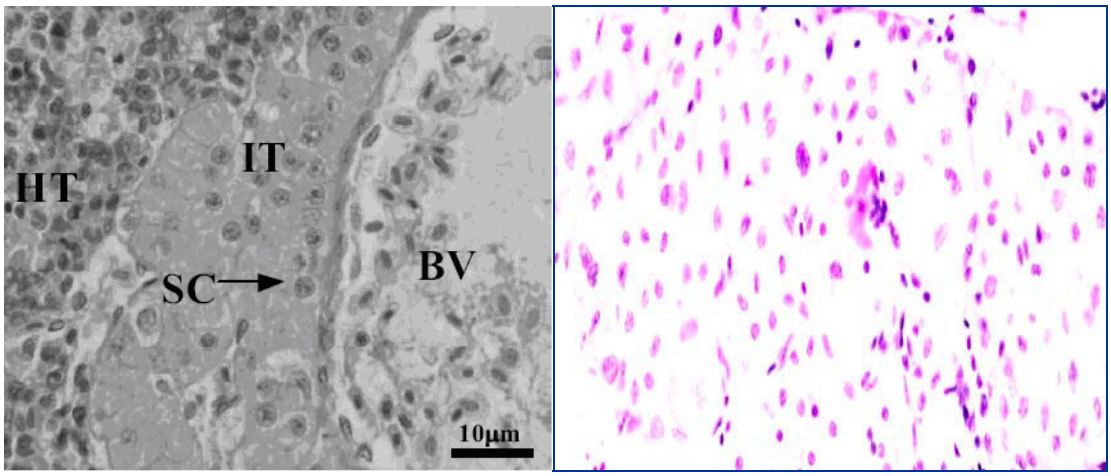

Figura 1.4: Cortes de parafina del riñón anterior de los peces teleósteos consistiendo en tejido hematopoyético (HT), venas sanguíneas (BV) y el tejido interrenal (IT) conteniendo las células esteroidogenas (SC)

El Cortisol: como indicador de la hormona del estrés en peces

El nivel de la circulación del cortisol en sangre es de uso general como indicador del grado del estrés en los peces experimentales (Barton and Iwama, 1991; Wendelaar Bonga, 1997) puesto que, 1) el cortisol es el mayor glucocorticoide en peces teleósteos y es demostrado que sus niveles en plasma aumentan en respuesta a una variedad de factores estresantes (Barton and Iwama, 1991), 2) se mide fácilmente y confiablemente usando el radioinmunoanálisis (RIA) y los análisis enzima-ligados del inmunosorbente (ELISA; Gamperle *et al*., 1994) y 3) desempeña un papel regulador crítico en muchos procesos fisiológicos importantes (Mommsen *et al*., 1999). La vía para la liberación del cortisol comienza en el eje HPI con la liberación del CRH por las células neurosecretoras hipotalámicas, lo que estimula las células corticotróficas de la adenohipofisis para secretar la ACTH. La ACTH de la circulación, a su vez, estimula las células interrenales encajadas en el riñón anterior para sintetizar y liberar los corticoesteroides en la circulación para su posterior distribución a los tejidos finales (Figura 1.3).

La producción de cortisol está bajo control del HPI (Wendalaar Bonga, 1997; Mommsen *et al*., 1999). Por ejemplo, el factor de liberación del cortisol (CRF) secretado de las neuronas hipotalámicas estimula la liberación de la ACTH de la pituitaria. Este péptido pituitario se une al receptor 2 de la melanocortina (MC2R) en las

células esteroidogenicas en el tejido interrenal que lleva a la corticosteroidogenesis (Aluru and Vijayan, 2008b). Se cree extensamente en mamíferos que la ACTH estimula la secreción del cortisol a través de la adenosina 3 ', ' - el monofosfato cíclico 5 (cAMP), que proporciona el colesterol del substrato activando la hidrólisis del éster de colesterol y facilitando el transporte del colesterol a la enzima de la hendidura de la cadena lateral (scc). Dempsher *et al* (1984) han establecido un modelo de liberación de cortisol (Figura 1.5), así la ACTH se une a uno o más receptores específicos en la membrana celular adrenocortical (MC2R, representado por A y B en la figura abajo) que tiene el efecto de activar la adenilato ciclaza (ACase). La concentración citosolica del cAMP aumenta, a su vez a) la hidroliza activadora del colesterol estera (CE), que cataliza la conversión del éster de colesterol a colesterol libre, y b) facilitando la transferencia del colesterol dentro de la mitocondria a un sitio accesible a la enzima scc. Por lo tanto, el receptor lipoproteína de baja densidad participa en la absorción del colesterol plasmático junto con la síntesis del colesterol citosolico proporcionando el substrato para la síntesis de esteroides, y ambos estos procesos pueden ser controlados por un pool intracelular de colesterol.

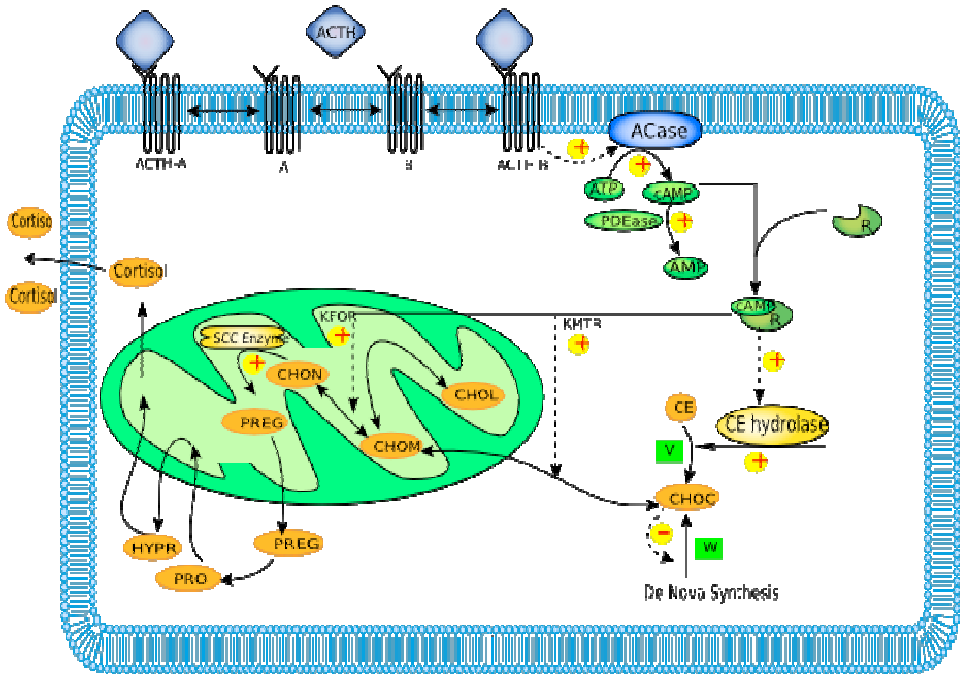

Figura 1.5: Un Modelo de la secreción del cortisol por la estimulación por ACTH en mamiferos (Dempsher et al., 1984). Adenylate cyclase (ACase), cholesterol ester (CE), adenosine 3',5'-cyclic monophosphate (camp), Adenosine triphosphate (ATP).

Otros estudios han precisado que el camino principal que lleva a la síntesis del corticoesteroide por el estímulo de ACTH implica una cascada de señales conteniendo las G-proteínas, la adenil ciclasa, el cAMP y la proteína kinaza A (PKA) (Miller, 1988; Schimmer, 1995). Hay evidencias que sugieren que el cortisol ejerza un efecto de regeneración negativa sobre la secreción del ACTH en la pituitaria y también suprime la síntesis de CRH en el hipotálamo. Además, hay también un efecto de la regeneración negativa de la ACTH sobre la secreción de CRH. Por lo tanto el proceso entero se puede considerar como sistema de auto-regulación, según lo resumido en la figura anterior. Además, el colesterol modula su propia síntesis inhibiendo la beta-hidroxi-beta-metilglutaril (HMG) – CoA reductasa en la célula adrenocortical (Dempsher *et al*., 1984).

Varios factores de estrés bióticos y abióticos son conocidos como moduladores de la biosíntesis del cortisol en peces alterando el patrón de la expresión de los genes que codifican las proteínas implicadas en el funcionamiento del eje HPI (Alsop and Vijayan, 2008a,b; Hontela and Vijayan, 2008). En teleósteos, varios receptores glucocorticoides (GRs) y un receptor de mineralcorticoid (SR.) están implicados en la señalización del cortisol (Vijayan *et al*., 2005; Prunet *et al*., 2006; Alsop and Vijayan, 2008a). La 11- deocycorticosterona fue identificada como liga para MR de la trucha usando análisis *in vitro*, mientras que todavía carece encontrar un papel fisiológico para esta liga *in vivo* (Sturm *et al*., 2005; McCormick *et al*., 2008). Un distinto papel fisiológico para los múltiples isoformas de GR en la señalización del cortisol no se ha establecido *in vivo* todavía (Bury *et al*., 2003).

Biosíntesis de los corticoesteroides por las células interrenales

Las hormonas esteroides son importantes reguladoras de numerosos procesos fisiológicos, e incluyen los glucocorticoids, mineralocorticoides y los esteroides sexuales, tales como estrógenos y andrógenos (Miller, 1988). Sin embargo, solamente los niveles bajos de aldosterona se han encontrado en teleósteos y la función de los mineralocorticoides parece ser realizada por los glucocorticoides (Wendelaar Bonga, 1997).

Las hormonas esteroides son estructuralmente similares y se sintetizan del colesterol en las células esteroidogenicas del riñón anterior. Así, el cortisol se forma del colesterol vía los intermediarios, la pregnenolona, la progesterona, la 17-

hidroxiprogesterona y el 11-deoxicortisol. La 17-hidroxiprogesterona se puede convertir a los corticoesteroides por la hidroxilaza 21 o a los esteroides sexuales por la hidroxilaza 17 que es así un paso de la rama en la vía de la biosíntesis esteroide. El 11-deoxicortisol formado por la 17-hidroxiprogesterona microsomal 21-hidroxilaza es metabolizada eventualmente al cortisol por la 11-hidroxilaza mitocondrial (Figura 1.6). En peces, las enzimas responsables de la producción del cortisol se han examinado muy poco. En mamíferos la 21-hidroxilación de la 17-hidroxiprogesterone es catalizada por el citocromo microsomal P450c21 al cortisol producido por las células adrenocorticales (Miller *et al*., 1997).

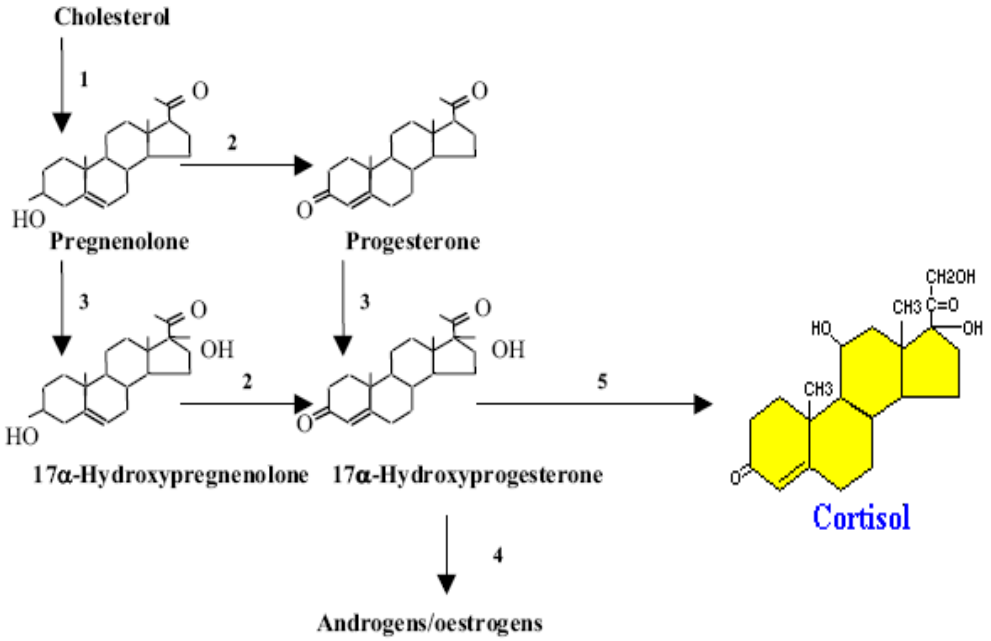

Figura 1.6: Representación de la vía esteroidogenica en peces teleósteos y el papel importante del P450c17 en la formación de los glucocorticoides y andrógenos/estrógenos.

Funciones del cortisol en peces

Mientras que las catecolaminas inducen la elevación rápida, a corto plazo de la glucosa en sangre sobre todo a través de la vía glicogenolítica (Vijayan and Moon, 1992), el cortisol tiene un papel clave en la regulación de muchas funciones fisiológicas importantes en los teleósteos. Así, está implicado a largo plaza en la movilización de las reservas de energía no procedentes de carbohidratos (Wendelaar Bonga, 1997) como la proteína y lípidos de los tejidos, lo que aumenta los ácidos grasos libres en plasma

(Mazeaud *et al*., 1977). Además de sus acciones en la regeneración en el eje corticotrófico, los elevados niveles crónico de cortisol son responsables de los efectos perjudiciales de estrés sobre las funciones fisiológicas vitales de peces, tales como la reproducción (Foo *et al*., 1993, Small, 2004), la osmoregulation (Reddeing *et al*., 1991; Mancera *et al*., 1994; McCormick, 2001), el crecimiento (Barton *et al*., 1987; Small, 2004) y el sistema inmune (Rottlant *et al*., 1997; Weyts *et al*., 1998, MacKenzi *et al*., 2006; Aluru and Vijayan, en prensa). En peces de agua dulce, la respuesta al estrés crónico puede también incluir: pérdida de electrólitos, disminuciones del hematocrito y de la hemodilución por el aumento de la permeabilidad de la branquia inducido por la catecolamina (Randall and Perry, 1992) o la pérdida creciente de iones a través de la orina (McDonald and Milligan, 1997).

Los niveles del cortisol varían y deben servir como pautas generales puesto que, las condiciones individuales, incluyendo diferencias entre las especies, las características genéticas de la cepa, el historial de cultivo anterior, y el ambiente local modifica los valores plasmáticos del control y de los estados estresados (Barton *et al*., 2002). Por ejemplo, los resultados del confinamiento de la trucha arco iris, Oncorhynchus mykiss, (Pottinger *et al*., 1992) y el salmón del Atlántico, *Salmo Salar* (Fevolden *et al*., 1991) han indicado que la respuesta del cortisol al estrés en peces de teleósteos es un rasgo altamente individualizado. Algunos individuos exhiben una respuesta al estrés constantemente alta del cortisol mientras que otros tienen una respuesta constantemente baja. Las razones de estas diferencias son actualmente confusas. Además, los programas selectivos de la cría han demostrado que la sensibilidad al estrés es hereditaria y que la sensibilidad individual es estable en el tiempo (Fevolden *et al*., 1991; Afonso *et al*., 1998; Pottinger and Carrick, 1999a).

La dorada como especie modelo para esta tesis

La dorada es una especie marina, que tiene una larga historia en la región mediterránea, con la evidencia de la captura y la cebadura de esta especie datados por mas de 2000 años. Pero, a partir de los años 80, la producción comenzó a crecer rápidamente, ampliándose desde 1.100 toneladas 1985 a 8400 MT en 1990 (FAO). Este aumento fue apoyado por el desarrollo de nuevas tecnologías y la disponibilidad de las ayudas económicas nacionales y de la UE. Hoy en día, la dorada constituye una de las

especies marinas más importantes cultivadas en el mediterráneo, y la producción europea total de la acuicultura en dorada llega aproximadamente a 140.000 toneladas en 2007, cerca de 40.000 toneladas más de 2005 (APROMAR, 2008) (Figura 1.7). En España, la dorada es una especie muy apreciada por el consumidor y tiene una alta importancia económica. Las técnicas de la producción de la dorada están por lo tanto bien desarrolladas y constituye un modelo excelente para los estudios fisiológicos en especies marinas de aguas calientes. Sin embargo, puesto que su cultivo es relativamente nuevo en comparación con los salmónidos y otras especies de agua fría, sigue habiendo muchos aspectos fisiológicos y alimenticios con todo sin estudiar.

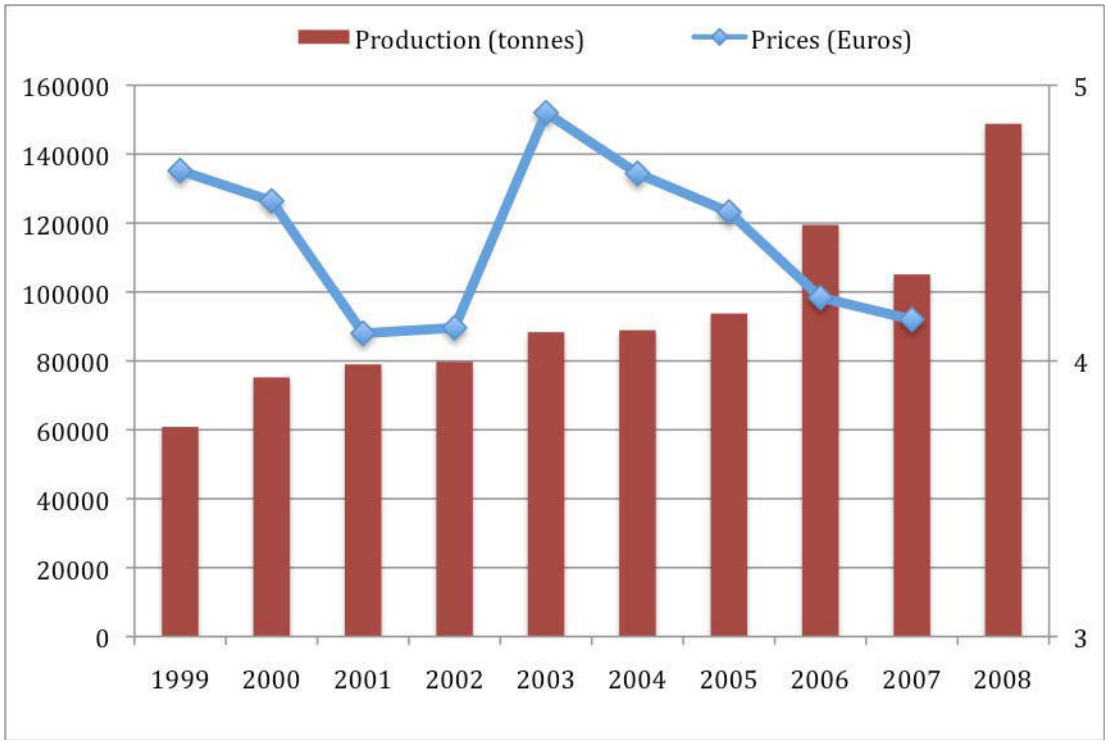

Figura 1.7: Evolución de la producción de la dorada en Europa durante la última década

10.2. Objetivos (2)

El objetivo general de esta tesis es promover la substitución del aceite de pescados por los aceites vegetales en las dietas de engorde para la dorada sin afectar el bienestar de los peces mejorando nuestro conocimiento en los mecanismos implicados en la regulación de la resistencia al estrés por los lípidos dietéticos. Para ese propósito varios objetivos fueron planteados:

1- Para determinar el efecto de niveles de la substitución del aceite de pescado por aceites vegetales ricos en los ácidos grasos n-3 o n-6 sobre las características del cultivo y la composición en ácidos grasos de diversos tejidos de dorada hasta el tamaño comercial.

2- Para estudiar el efecto de la sustitución del aceite de pescado y los cocientes entre los ácidos grasos n-3/n-6 sobre bienestar de la dorada y su resistencia a varios tipos de estrés.

3- Para investigar los efectos de diversos niveles de substitución del aceite de pescados por mezclas de los aceites vegetales sobre salud de la dorada y el bienestar en términos de composiciones de ácido graso del plasma y de los leucocitos y producción de la prostaglandina y de la leptina.

4- Para entender mejor los mecanismos implicados en la regulación de la resistencia al estrés por los ácidos grasos poliinsaturados estudiando la producción y la liberación del cortisol en células interrenales de la dorada incubados con la hormona adrenocorticotrópica.

5- Para aclarar el efecto de alimentar los aceites vegetales en dietas para engorde de dorada sobre el bienestar en términos de producción y liberación del cortisol por el riñón anterior y las vías fisiológicas implicadas.

Para alcanzar estas metas dos largas pruebas de alimentación a lo largo del período de engorde total y varios estudios "*in vitro*" fueron conducidas. Los resultados fueron organizados en cinco estudios científicos que se han publicado o se han presentado ya para la publicación. Algunos de esos estudios fueron incluidos en dos proyectos de investigación: RAFOA (2001-2005) financiado por la UE (Q5RS-2000-30058) y Linosalud (2005-2007) financiado por el gobierno español (AGL2004-08151-CO302).

10.3. Materiales y métodos generales (3)

Dos grandes experimentos de alimentación principales se incluyen en este capítulo, el primer experimento fue planteado para substituir el aceite de pescado en las dietas para la dorada por aceites vegetales, sin dañar a la salud, bienestar y el crecimiento de los peces, este experimento fue parte del proyecto RAFOA (Researching

Alternatives to Fish Oil for Aquaculture) Q5RS-2000-30058 de la UE. El segundo experimento fue parte del proyecto financiado por el Ministerio de Educación y Ciencias Español LINOSALUD (Efecto de la Sustitución parcial del aceite de pescado por aceite de Lino en la dieta sobre la salud y la resistencia al estrés en dorada (*Saprus aurata*)) (AGL2004-08151-CO3 02/ACU). En ambos experimentos los peces fueron cultivados desde juveniles hasta la talla comercial con dietas que contienen diversos cocientes del FO a los VOs, siendo los últimos el aceite de lino (LO), aceite de colza (RO) y aceite de palma (PO) durante un periodo de 281 días en el primer experimento, y el aceite de lino (LO) y el aceite de soja (SO) por un periodo de 240 días en el segundo experimento. La mezcla de aceites fue formulada para replegar al FO en términos de concentraciones en saturadas, monounsaturated y poliinsaturadas y cocientes del ácido graso excepto que no hubiera ningún HUFA encontrado en FO. Los dos experimentos de alimentación fueron realizadas en las instalaciones del Grupo de Investigación en Acuicultura (ULPGC&ICCM, España). Las prostaglandinas y el análisis de las leptinas fueron hechos en las instalaciones del departamento de la nutrición de peces, instituto de la acuicultura, universidad de Stirling (Escocia). Parte de los experimentos *in vitro*, y todo los análisis del cortisol fueron conducidos en las instalaciones del departamento de biología celular, de fisiología y de inmunología, Universitat Autonòma de Barcelona, Bellaterra, España.

Condiciones experimentales

Los juveniles de dorada usados en la presente tesis fueron suministrados por una granja local de peces (Alevines y Doradas, S.A.: ADSA, Las Palmas, España), después los juveniles fueron mantenidos durante 4 semanas de aclimatación en tanques de 1000 l (Figura 2.1) y distribuidos en tanques experimentales de 500 l (Figura 2.2).

Figura 2.1: Tanque de aclimatación de 1000 l

Los tanques cónicos cilíndricos con un volumen total de 500 l se colocaron en pares y fueron separados por un conducto de desagüe central. Los tanques tienen un diámetro de 1.5 m y una profundidad de 1.0m en la parte central (Figura 2.2). La entrada del agua se localiza en la parte inferior del tanque y la salida del agua se localiza en el fondo del tanque en el lado posterior. La entrada y salida laterales del agua se pueden modificar con diversos accesorios movibles, a través del período del cultivo según la secuencia de alimentación.

Figura 2.2: Una muestra de los tanques de cultivo intensivo de 500 l

Especie estudiada

La dorada (*Sparus aurata*, Linnaeus 1758) (Figura 2.3), es un teleósteos, perteneciendo a la familia de los esparidos, que tiene un total de 11 espinas dorsales, 13-14 de rayos suaves dorsales, 3 espinas dorsales anales; 11-12 rayos suaves anales. Es caracterizada por un cuerpo largo, con el punto negro grande en la cubierta de la branquia. Este pez tiene una distribución grande (Figura 2.4), así está presente en Atlántico del este (por las islas británicas), estrecho de Gibraltar, Cabo verde y alrededor de las islas Canarias, y está también presente en el mediterráneo. La dorada podría ser encontrado en capas de hierba del mar y partes inferiores arenosas así como en la zona de la resaca comúnmente a las profundidades de cerca de los 30m, pero los adultos pueden ocurrir a profundidad de hasta 150 m. Es un pescado sedentario, solitario o en pequeñas agregaciones. En primavera, se encuentran a menudo en lagunas y los estuarios costeros de las aguas salobres. Es una especie principalmente carnívora, pero puede ser herbívora, que alimenta con crustáceos, incluyendo mejillones y ostras (Bauchot *et al*., 1990). Éste es uno de los peces más importantes de la acuicultura salina y hipersalina.

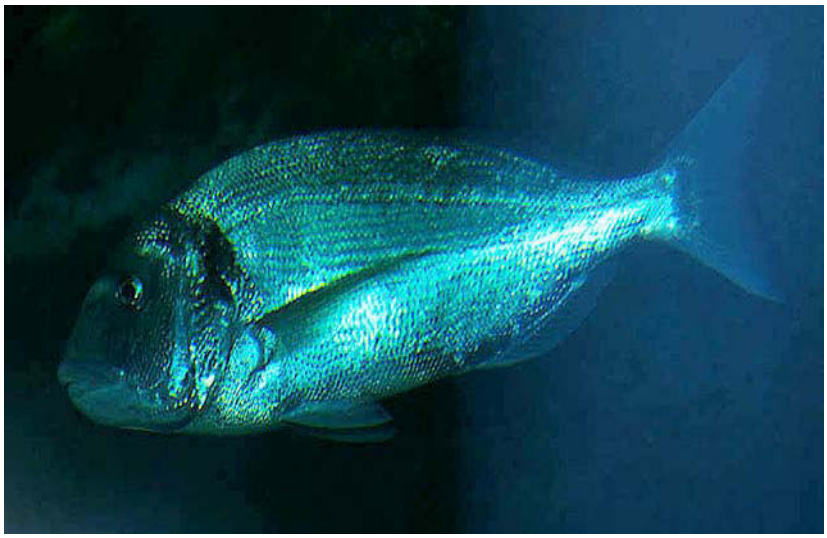

Figura 2.3: Un ejemplar adulto de dorada

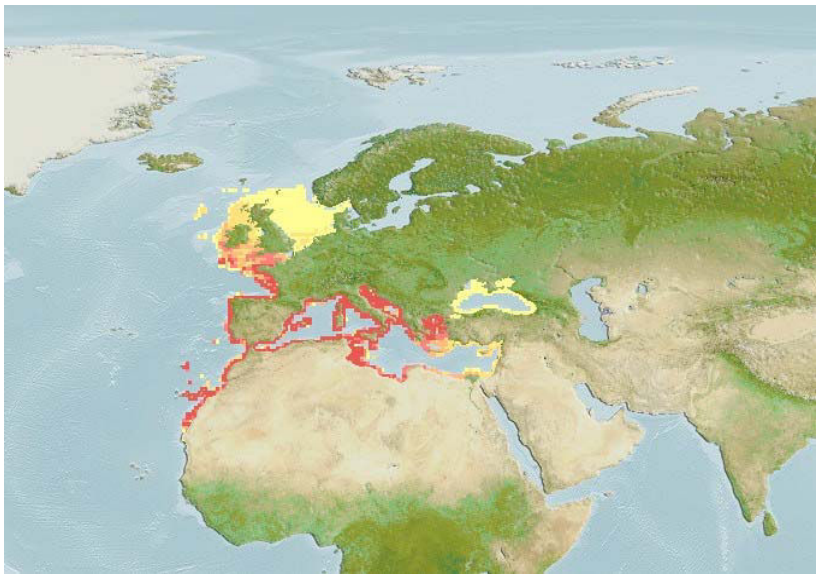

Figura 2.4: Distribución geografica de la dorada (Fish base, 2009)

10.3.1 - Experimento I: parte del proyecto RAFOA

Animales y dietas

Dos mil cuatrocientos juveniles de dorada con un peso inicial de 24 g fueron mantenidas en Instituto Canario de Ciencias Marinas (ICCM). Los peces fueron distribuidos aleatoriamente en 16 tanques circulares de polietileno de 1000 l (150 peces/ tanque, cada dieta probada en cuadruplicado) suministrados con flujos continuos de agua de mar (con una salinidad del 36‰) y de aire. Los peces fueron alimentados bajo fotoperiodo natural (aproximadamente 12:12 l/d). La temperatura del agua y el oxígeno disuelto durante el período experimental se extendieron entre 21.88-22.41ºC y 5.5-7.2 PPM, respectivamente. Las muestras iniciales para la composición de lípidos del hígado y músculo se recogieron a partir de 10 juveniles de peso medio aproximadamente de 24 g antes de empezar la alimentación con la dietas experimentales. Después de dos semanas de aclimatación de los peces a los tanques experimentales y a la dieta control, las dietas experimentales fueron suministradas a mano hasta la saciedad evidente tres veces por día a las 9:00, 12:00 y 15:00 h, seis días por semana. Todos los peces individuales en cada tanque fueron pesados una vez al mes y la ración fue ajustada. La tasa de alimentación fue determinado diariamente. Quince peces fueron muestreados para los parámetros bioquímicos tomados al principio y en el medio (98 días) del período experimental.

Cuatro dietas experimentales iso-energéticas y iso-proteicas fueron formulados con un contenido constante de lípidos de alrededor de 22%. Dos diversas mezclas incluidas en una substitución del 60% del aceite de pescado dietético en la dieta 1 fueron utilizadas para la dorada (Tabla 1), 15:60:25 por volumen en la dieta 2 y el 40:40:20 por volumen en la dieta 3, de los aceites de colza, de lino y de palma, respectivamente. Una cuarta dieta con 100% de substitución del aceite de pescado por la mezcla usada en la dieta 2 también fue incluida. Las dietas fueron preparadas y suministradas por Nutreco ARC. La composición de lípidos y de ácidos grasos de la dieta del tamaño de grano de 5 milímetros fue analizada y se demuestra en la tabla 2. La dieta control contuvo más saturados, DHA, EPA y ARA que las otras dietas, con la dieta 4 conteniendo los niveles inferiores de estos ácidos grasos. Considerando que, las dietas 2 y 3 (con el 60% de substitución) tenían niveles medios de EPA, DHA y ARA, pero de la dieta 3 fue más alta en ácidos grasos monoenoicos y más baja en n-3 (más baja en 18:3 n-3 y más alta en 18:2 n-6) para comparar la diferencia en la utilización de los ácidos grasos de la serie n-9 y n-3 por la dorada, y posteriormente los cocientes n-3/n-6 fueron más bajos que en la dieta 2. La dieta 4 era la más baja en EPA, DHA y ARA y la más alta en 18:2 n-6 y los monoenoicos (principalemente el ácido oleico).

El índice de conversión (CI), la tasa de crecimiento específica (SGR) y el índice hepatosomatico (HSA) eran calculados según las fórmulas siguientes:

CI= Tasa de alimentación / Ganancia de peso

SGR= [(Ln Peso final – Ln Peso incial)/ t)]*100

con t= el periodo del experimento (en días)

HSI = Peso del hígado / peso total

Tabla 2.1: Los diferente tipos y los % de los aceites usados en la dietas experimentales

	FO	**60 L**	**60 R**	**100 L**
Aceite de pescado	100	40	40	-
Aceite de Colza	-	10	24	17
Aceite de Lino	-	35	24	58
Aceite de Palma	-	15	12	25

Tabla 2.2: El contenido en los principales ácidos grasos de las dietas (g/100 g ácidos graos totales) (5mm)

	FO	**60L**	**60R**	**100L**
% Lípidos (p.s.)	20.24	21.36	22.79	25.14
14:0	5.92	3.05	2.93	0.79
15:0	0.50	0.28	0.24	0.08
16:0	19.30	14.99	15.95	15.92
16:1n-7	7.21	3.93	3.23	1.23
16:2n-6	-	-	-	-
17:0	0.26	0.15	0.13	0.02
16:4n-3	0.17	0.04	0.03	0.02
18:0	3.37	3.21	3.36	3.40
18:1n-9	11.71	24.61	29.89	32.98
18:2n-6	5.84	11.87	12.50	13.67
18:3n-6	0.07	0.10	0.08	-
18:4n-6	0.17	0.03	0.03	-
18:3n-3	1.62	14.36	12.25	23.02
18:4n-3	2.18	1.28	0.87	0.17
20:0	0.21	0.24	0.32	0.23
20:1n-9	2.38	2.05	1.98	2.23
20:1n-7	-	-	-	-
20:2n-6	0.15	0.10	0.08	0.03
20:4n-6	0.66	0.34	0.28	0.06
20:4n-3	0.54	0.27	0.21	0.05
20:3n-3	0.08	0.05	0.04	0.02
20:5n-3	11.90	6.10	4.86	1.06
22:0	0.08	0.10	0.24	0.13
22:1n-11	2.98	2.35	2.20	2.41
22:1n-7	-	0.02	-	-
22:4n-6	0.18	-	0.03	0.01
22:5n-6	0.24	0.12	0.11	-
22:4n-3	-	-	0.03	0.02
22:5n-3	1.17	0.56	0.47	0.08
22:6n-3	14.14	7.36	3.21	2.10
Saturados	30.01	22.22	23.33	20.63
Monoenoicos	27.70	33.12	37.44	38.85
Σ n-3	32.23	30.27	25.22	26.57
Σ n-6	7.37	12.59	13.13	13.78
Σ n-9	14.56	26.81	31.91	35.22
Σ n-3 HUFA	27.84	14.34	11.82	3.33
n-3/n-6	4.37	2.40	1.92	1.93

Procedimiento de muestreo

En el final del experimento de alimentación, después de 281 días, los peces fueron muestreados individualmente de cada tanque. La sangre fue recogida de las venas caudales en jeringas heparinizados a partir de 6 peces por cada tanque (18 peces por dieta) y transferidos a un tubo eppendorf cubriert con heparina de litio como anticoagulante. La sangre fue centrifugada inmediatamente a 3000 RPM durante 10 minutos para sedimentar las células. Un mililitro de plasma fue recogido, se le añadieron 50 µl/ml del ácido fórmico a 2M y las muestras acidificadas fueron congeladas en el nitrógeno líquido (- 80°C) para el análisis de los eicosanoides. 250 µl de plasma fueron recogidos y guardados al -80°C para el análisis de las leptinas y el resto de plasma fue mezclado formando1 pool por cada tanque y guardado a - 80°C para el análisis de los ácidos grasos.

La separación de los leucocitos de la sangre fue conducida en solamente 3 tratamientos dietéticos debido a que no se quedaron suficientes peces alimentados con la dieta 100 LO. Siete mililitros de sangre fueron recogidos de la vena caudal en jeringuillas heparinizadas a partir de 9 peces por dieta y transferidos a los tubos de cristal limpios guardados en el hielo. La sangre fue centrifugada a 500 g durante 10 minutos a 4 ºC. Después de la eliminación del sobrenadante, las células flotantes fueron suspendida de nuevo en 10 ml de HBSS sin Ca-Magnesio y entonces centrifugados a 500 g durante 10 minutos a 4 ºC, las células fueron separadas en 2 capas que fueron suspendidas de nuevo en 6 ml de HBSS sin Ca-Magnesio, y acodadas cuidadosamente sobre 6 ml de Percoll al 46% y centrifugadas a 450 g durante 40 minutos a 4 ºC. Las celulas de la capa intermedia blanca fueron recogidas y suspendidos de nuevo en 10 ml de HBSS sin Ca-Magnesio y centrifugados a 500 g durante 10 minutos a 4ºC. Las células recogidas fueron suspendidas de nuevo en 6 ml HBSS sin Ca-Magnesio, cuidadosamente acodados sobre 6 ml de Percoll al 46% y centrifugados a 450 g durante 40 minutos a 4 ºC. Los leucocitos (capa intermedia blanca) fueron recogidos y lavados con 10 ml de HBSS sin Ca-Mg. Los leucocitos obtenidos a partir de 3 peces fueron mezclados y suspendidos de nuevo en 4 ml de HBSS, 2 ml de cloroformo fueron agregados y la muestra fue guardada a -80 ºC antes de la extracción de lípidos.

Extracción y análisis de los lípidos

La extracción de lípidos totales de las muestras, dietas y de leucocitos del plasma fue realizada por el método de Folch *et al*., (1957) usando una mezcla de cloroformo: metanol (2: 1) (v:v) conteniendo 0.01% BHT, como un antioxidante, y KCl (al 0.88%); los 300 µl de plasma recogidos fueron utilizados. La extracción de los lípidos fue facilitada por el uso de vortex, mezclador vigoroso, seguido por la centrifugación lo que permitió la separación de cloroformo y las capas acuosas extrayendo los lípidos de plasma, dieta y leucocitos. Las capas más bajas se filtraron con el papel de filtro de Whatman y se secaron bajo flujo de nitrógeno, los lípidos totales fueron pesados. Las fracciones neutrales y polares fueron separadas por cromatografía de adsorción en los cartuchos Sept-pak de silicona, (las aguas, Milford, mA) según lo descrito por Juaneda y Rocquelin (1985).

Los ésteres metílicos de los ácidos grasos fueron producidos de las partes alícuotas de lípidos totales extraídos de dietas y de muestras de plasma por la transmetilación ácida-catalizada realizadas durante la noche en 50 °C según lo descrito por Christie (1982). Los ésteres metílicos de los diferentes ácidos grasos fueron separados y cuantificados por la cromatografía de gases (Shimadzu C-R5A, columna de 30m*0,32m m Silice con Supleco-10) según las condiciones descritas por Izquierdo *et al*., 1990. Los ésteres metílicos individuales fueron identificados por la comparación con estándares conocidos y datos publicados.

Extracción, separación e immunoensayo de los isómeros de las prostaglandinas

- Purificación de eicosanoides

El plasma congelada para el análisis de los eicosanoides fue deshelada y centrifugada a x 1000 g durante 5 min para precipitar los desechos. Los sobrenadantes fueron extraídos usando las minicolumnas de "Sept-Pak" del sílice de octadecyl (C18) (Millipor (Reino Unido), Watford) según lo descrito detalladamente por Bell et al, (1994). 200 µl del sobrenadante fue aplicada a la columna, que había sido prelavada con 5 ml de metanol y 10 ml de agua destilada. La columna fue lavada sucesivamente con 10 ml de agua destilada, 5 ml de el 15% (v/v) etanol y 5 ml de hexano/cloroformo (65: 35, v/v) antes de la elución de los prostanoides con 10 ml de etil acetato. Este extracto

fue secado debajo del nitrógeno y suspendido de nuevo en 100 µl de metanol y guardado en un pequeño frasco de cristal en al congelador (-4 ºC) antes de su análisis por immunoensayo.

- Separación de la PGE_3 con HPLC

La PGE_3 fue separado por HPLC a fase revertida usando una columna del C18 (ODS2) de Spherisorb 5 µm. El sistema cromatográfico fue equipado de bombas de agua modelo M-45 de 680 y los picos fueron vistos a 196 nanómetro usando un detector de Pye-Unicam LC-UV para determinar la elución de los estándares de las prostaglandinas. Un sistema isocratico fue utilizado que contenía 17 mM de ácido fosfórico/el acetonitrilo (70: 30, v/v) con un flujo de 0,75ml/min. Los 50 μl de eicosanoides sobrantes purificados del extracto de plasma fueron inyectados a la columna y las fracciones de 2.25 ml fueron recogidas usando un LKB 2112 "Redirac". Las fracciones que correspondían a PGE_3 fueron aplicadas al A.C. 18 "Sept-Pak" que había sido prelavado como se describe anteriormente, y la prostaglandina fue disuelta en 5 ml de etil acetato. Las muestras fueron secadas debajo de nitrógeno y redisueltas en 100 µl de buffer del immunoensayo. La cuantificación de la PGE_3 fue realizado usando los kits del immunoensayo de la enzima (EIA) para PGE_2 según el mismo protocolo descrito arriba (sur SPI-bio, del GIF Yvette, Francia). La especificidad de los anticuerpos del kit usados en este immunoensayo es de 100% con la PGE_2 y solamente 43% con PGE_3, se tomo en cuenta al calcular resultados.

- Immunoensayo de las prostaglandinas E_2 y E_3

Para las prostaglandinas E_2: 50 µl del extracto de metanol fue tomado, secado debajo del nitrógeno y disuelto en 500 µl del buffer de EIA y guardado en el refrigerador (a 4°C). Este análisis se basa en la competición entre PGE_2 y un PGE_2-acetilcolinesterasa (AchE) PGE_2 conjugado (el patrón) para una cantidad limitada de anticuerpo monoclonal PGE_2. Porque la concentración del patrón PGE_2 es constante mientras que la concentración de PGE_2 varía dependiendo de la muestra, así la cantidad del patrón PGE_2 que pueda atado al anticuerpo monoclonal PGE_2 y es inversamente proporcional a la concentración de PGE_2 en el posillo. Este complejo de anticuerpo PGE_2 se ata al anti-ratón policlonal IgG de cabra que se ha atado previamente al posillo. La placa se lava para quitar cualquier reactivo desatado y entonces el reactivo de Ellman (que contiene el substrato para AchE) se agrega al posillo. El producto de esta

reacción enzimática tiene color amarillo distinto y absorbe fuertemente a 412 nanómetro. La intensidad de este color, determinado espectrométricamente, es proporcional a la cantidad del AchE PGE_2 atada al posillo, que es inversamente proporcional a la cantidad de PGE_2 libre presente adentro bien durante la incubación. Todos los reactivos específicos de ensayo fueron preparados antes de comenzar el análisis.

- Buffer del EIA: el contenido del frasco del buffer se diluye con 90 ml de agua de UltraPura.

- Buffer para lavado: Diluyendo el contenido del frasco concentrado al 1:400 con agua UltraPura y se le añade del tween (0.5ml/liter del buffer para el lavado).

- Estándar de la prostaglandina E2: El concentrado del estándar PGE2 (10 ng) se reconstituye con 1ml del buffer del EIA. Ocho tubos limpios (#1-8) fueron preparados; poniendo de 360 µl del buffer del EIA al tubo #1 y 200 µl buffer del EIA a los tubos # 2-8. 40 µl del estándar bulk (10 ng/ml) se transfiere al tubo # 1 y se mezcla muy bien. En serie, el estándar fue diluido quitando el µl 200 de # 1 y colocándolo en tubo # 2, mezclándose. Después, quitando 200 µl del tubo 2 al tubo # 3, mezclándolo. El proceso fue repetido para los tubos # 3-8. Estos estándares diluidos no se deben almacenar por más de 24 horas.

- La prostaglandina E_2 AchE: El concentrado se diluye con 6 ml del buffer del EIA, la tinta se agrega en una dilución final del 1:100.

- Anticuerpo monoclonal de la prostaglandina E_2: El concentrado se diluye con 6 ml EIA, el tinte al antisuero reconstituido se agrega en una dilución final del 1:100. Una vez que los kits del immunoensayo fueron abiertos, cada kit contiene ocho en blanco (Blk), un posillo de liga no específica (NSB), un posillo de liga máxima (B0), y ocho puntos de la curva estándar (S1-S8). Una prueba con diversas diluciones de las muestras fue probada para elegir la mejor (µl 80).

Medir con una pipeta los diferentes reactivos: Se usaron diversas puntas para pipetear el buffer, el estándar, la muestra, el patrón, y el anticuerpo.

1- El buffer del EIA: 100 µl del buffer del EIA fueron agregados a los posillos de la liga no especifica (NSB) y 50 µl los posillos del ligad máxima (B0).

2- Estándar de la prostaglandina E_2: 50 µl del tubo # 8 al posillo estándar más bajo (S8). 50 µl del tubo # 7 fue agregado al posillo siguiente del estándar (S7). El mismo procedimiento fue utilizado hasta que todos los estándares fueron puestos.

3- Muestras: 80 µl de la muestra fue agregado por posillo.

4- el patrón de la prostaglandina E_2 AchE: 50 µl fue agregado a todos los posillos excepto a los blancos (Blk).

5- Anticuerpo monoclonal de la prostaglandina E_2: 50 µl fue agregado a todos los posillos excepto a la liga no específica (NSB), y al blanco (Blk).

6- La placa fue cubierta con la película de plástico e incubada durante 18 horas a 4°C lo que aumentan la sensibilidad del análisis.

7- Desarrollar la placa: Antes de desarrollar la placa, el reactivo de Ellman se reconstituye con 20 ml de agua ultrapura. Esto debe ser preparada y utilizado el mismo día que se prepara, y se protege contra luz al guardar. Los posillos fueron vaciados y aclarados cinco veces con el buffer de lavado. 200 µl del reactivo de Ellman fueron agregado a todos los posillos y 5 µl del patrón a los posillos de la actividad total. La placa se cubre con la película plástica y es desarrollada usando un agitador orbital en oscuridad durante 75 min.

8- Lectura de la placa: Es realizada en una longitud de onda de 405 nm.

9- Cálculo de los resultados: Los resultados fueron calculados manualmente como sigue: - Se sacó un promedio de las lecturas de la absorbancia de posillos Blk.

- Restar el Blk medio del resto de posillos.

- Restar NSB de todos los otros posillos.

– Se calculó el %B/B0 (% del estándar de la muestra ligada/máximo de liga) para los posillos restantes.

- Se desciño remonta la curva estándar (%B/B0 con la concentración estándar en PGE_2 en pg/ml).

- Finalmente, se calcularon las concentraciones de cada muestra en PGE_2 y PGE_3.

Immunoensayo de las leptinas

- Principio del análisis

Este análisis utilizó una técnica cuantitativa de immunoensayo enzimatico del emparedado. Un anticuerpo monoclonic para la leptina se aplicó a las placas. Un anticuerpo monoclonal específico (anticuerpo humano de la leptina) para la leptina había sido cubierto primero sobre un microplato. Los estándares y las muestras se metieron con pipetas en los posillos y cualquier letrina presente es atada al anticuerpo inmovilizado. Después de quitar cualquiera sustancia desatada, un anticuerpo monoclonal específico para la enzima del ligado para la leptina se agrega a los posillos. Después de un lavado para quitar cualquier reactivo desatado del anticuerpo de la enzima, una solución del substrato se agrega a los posillos y el color se convierte en proporción de la cantidad de límite de la leptina en el paso inicial. Se para el desarrollo del color y la intensidad del color se mide.

- Reactivos

- Microplato de la leptina: Un mircoplato de poliestireno de 96 posillos (12 columnas del posillo 8) con el anticuerpo monoclínico de la leptina.

- El conjugado de la leptina (parte 890574): 21 ml del anticuerpo monoclonico contra la leptina con su preservativo.

- Estándar de la leptina (parte 890575): 10 ng de ser humano recombinado de la leptina en proteínas del guardado con el preservativo.

- Análisis de los diluyentes RD1-19 (parte 895467): 11ml de la base protegida de la proteína con el preservativo.

- Concentrado del diluente RD5P (5x) del calibrador (parte 895151): 21ml del concentrado de la solución de la base protegida de la proteína con el preservativo.

- Concentrado de buffer de lavado (parte 895003): 21 ml del 25 veces concentrado de la solución buffer con el preservatif.

- Tinta de reactivif A (parte 895000): 12.5 ml de preoxidase estabilizada con hidrogeno.

- Tinta de reactivo B (parte 895002): 12.5 ml de cromógeno estabilizado (tetrametilbenzidina).

- Parador de la solución: 6 ml de ácido sulfúrico de 2 N.

- Preparación de los reactivos

Todos los reactivo se sacan a la temperatura ambiente antes del análisis.

- El buffer del lavado: El frasco del concentrado se mezcla suavemente y se diluye en 1:25 con agua desionizada. Se prepararon 500 ml del buffer para el lavado.

- Solución del substrato: El reactivo A y B del color fue mezclado junto en volúmenes iguales y puesto en obscuridad en un tiempo de 15 minutos antes de usar.

- Diluyente RD5P (1X) del calibrador: el concentrado se diluye a 1:5. 100 ml fueron preparados.

- Estándar de la leptina: Se preparó 15 minutos antes de usar. El concentrado se mezcla suavemente y se diluye con 1 ml de agua desionizada. Esta reconstitución produce una solución stock de 10.000 pg/ml. el estándar de 8 tubos se prepara según lo explicado arriba (estándar de la prostaglandina).

- Procedimiento del análisis

El plasma congelada fue deshelada y centrifugada a 1000 *g durante 5 minutos para precipitar los desechos.

- 100 µl del diluyente RD1-19 del análisis fue agregado a todos los posillos.

- 100 µl del estándar se agregaron a cada posillo estándar.

- 100 µl del control fue agregado a todos los posillos seguido por 200 µl de la muestra. La pipeta de varios canales fue utilizada.

- La placa se cubre y se incuba durante 2 horas a temperatura ambiente.

- Todos los posillos fueron aspirados y lavados con 400 µl del buffer de lavado. Este proceso se repite 4 veces. En el último lavado, los posillos fueron empernados contra el papel limpio para quitar cualquier buffer del lavado restante.

- 200 µl de la conjugación de la leptina fue agregado a todos los posillos, la placa fue cubierta con la nueva tira adhesiva e incubada para 1 hora a temperatura ambiente.

- Los posillos fueron aspirados y se lavaron como en el paso 5.

- 200 µl de la solución del substrato fue agregado a todos los posillos y la placa fue incubada durante 30 minutos a temperatura ambiente y protegida contra luz.

- 50 µl de la solución para parar la reacción fueron agregados a todos los posillos.

- La placa se desarrolló en el plazo de 30 minutos en un agitador y se leyó la densidad óptica de cada uno usando un lector de microplato fijado a 450 nanómetro.

- Se calcularon los resultados: La curva estándar fue dibujada poniendo la densidad óptica para los estándares contra la concentración de los estándares.

Los datos se linearon usando una transformación logarítmica. La ecuación de la curva estándar fue resuelta y la concentración final de la leptina en plasma fue determinada por el uso simple de su absorbencia en la ecuación estándar de la curva.

10.3.2. Experimento II: Parte del proyecto LINOSALUD

Animales y dietas

Los juveniles de dorada (*Sparus aurata*) (peso corporal inicial de 45 g) fueron distribuidos en 45 tanques de 500l (50 peces/tanque, cada dieta fue probada en triplicado) suministrando agua de mar con una temperatura que se extendía de 20ºC al principio del experimento a 24.2ºC al final, y la aireación, dos mil doscientos cincuenta animales fueron utilizados. Ocho dietas iso-energéticas y iso-proteicas con un contenido en lípidos cerca del 18% fueron formuladas. El aceite de la anchoa era la única fuente el lípidos en la dieta FO (aceite de pescado). El resto de dietas contuvieron una mezcla de aceites vegetales para substituir a FO con diversos niveles de substitución según lo mencionado en las tablas (2.3, 2.4, 2.5). La composición en ácidos grasos de los lípidos totales de las dietas experimentales se demuestra en tabla 2.6. Los peces fueron

alimentados con las dietas experimentales hasta la saciedad evidente (3 veces/día, 6 días por semana), hasta que alcanzaran el tamaño comercial después de 26 semanas.

La tasa de alimentación era diario determinado y todos los peces fueron pesados individualmente mensualmente. Los CI y SGR fueron calculados según las fórmulas descritas arriba. Las mortalidades fueron registradas diariamente y la supervivencia fue determinada mensualmente.

Tabla 2.3: Contenido principal de los ingredientes de las dietas experimentales (en %)

	% en peso seco
Aceites (Pescado[a]/lino/soja)	16.32
Harina de pescado suramericano	47.26
Trigo	7.00
Harina de Soja 47% [b]	25.00
Harina de Girasol	3.67
Premix de Vitaminas[c]	0.27
Premix de minerales[c]	0.48

a Suramericano, aceite de anchoa.
b Harina de Soja con 47% como proteína bruta, "no GMO"
c premixes de Vitaminas y minerales preparados según los estándares comerciales de Proaqua A/S.

Tabla 2.4: Contenido en vitaminas y minerales de las dietas experimentales

Vitaminas/ Minerales	Unidades
1- Vitaminas	
A-Retinol	11200.0 IU/kg
D3- Cholecalciferol	112.0 IU/kg
E- Tocopherol	280.0 mg/kg
C (Stay C)- Ascorbic acid	336.0 mg/kg
B1- Thiamin	9.0 mg/kg
B2- Riboflavin	15.7 mg/kg
B3- Nicotinic acid/Niacin	179.2 mg/kg
B5- Panthothenic acid	31.4 mg/kg
B6- Pyridoxin	13.4 mg/kg
B8- Biotin	0.5 mg/kg
B9- Folic acid	4.5 mg/kg
B12- Cyanocobalamin	0.036 mg/kg
K- Menadion	6.7 mg/kg
Inositol	44.8 mg/kg
2- Minerales	
I	4.5 mg/kg
Zn	44.8 mg/kg
Fe	67.2 mg/kg
Cu	3.6 mg/kg
Mn	14.6 mg/kg
Mg	136.1 mg/kg
Co	0.2 mg/kg
Se	0.06 mg/kg

Tabla 2.5: Tipo y % de los aceites usados en las dietas

	FO	70L	100L	70S	100S	20L50S	50L20S	50L50S
FO	100	30	-	30	-	30	30	
LO	-	70	100	-	-	20	50	50
SO	-	-	-	70	100	50	20	50

Tabla 2.6: La composición en ácidos grasos de las dietas (% total de los ácidos grasos identificados)

Ácidos grasos	FO	70L	100L	70S	100S	20L50S	50L20S	50L50S
14:0	9.23	3.11	1.57	3.32	1.59	2.67	2.60	1.37
15:0	0.26	0.10	0.06	0.11	0.06	0.32	0.23	0.06
16:0ISO	0.11	0.04	0.03	0.05	0.03	0.01	0.06	0.03
16:0	22.21	12.19	10.21	15.84	13.69	15.46	12.16	11.75
16:1n-7	11.25	3.91	1.98	4.06	2.01	3.07	3.24	1.73
16:1n-5	0.38	0.14	0.09	0.16	0.09	0.07	0.10	0.08
16:2n-4	1.83	0.61	0.28	0.61	0.29	0.11	0.51	0.24
17:0	0.85	0.38	0.25	0.41	0.28	0.52	0.31	0.25
16:3n-4	2.00	0.66	0.29	0.67	0.31	0.59	0.54	0.24
16:3n-3	0.15	0.07	0.05	0.07	0.05	0.57	0.03	0.05
16:3n-1	0.12	0.05	0.04	0.06	0.04	0.07	0.07	0.03
16:4n-3	0.72	0.26	0.13	0.24	0.11	0.04	0.05	0.09
16:4n-1	-	-	-	-	-	0.25	0.29	-
18:0	3.85	3.96	3.99	3.43	3.20	10.20	3.49	3.74
18:1n-9	9.10	13.75	15.31	18.60	21.48	15.30	18.48	19.04
18:1n-7	3.16	1.57	1.36	2.15	1.77	1.15	1.37	1.36
18:1n-5	0.14	0.08	-	0.12	0.11	0.09	0.10	0.10
18:2n-9	0.04	0.02	-	-	-	-	0.03	-
18:2n-6	4.02	12.36	16.21	29.93	38.51	22.25	18.47	27.25
18:2n-4	0.38	0.13	0.06	0.12	0.06	0.18	0.12	0.05
18:3n-6	0.36	0.13	-	-	0.07	0.13	0.12	0.09
18:3n-4	0.04	-	0.04	0.18	0.09	0.70	0.12	0.05
18:3n-3	0.48	31.94	37.63	5.63	6.01	11.38	20.67	23.03
18:4n-3	1.94	0.76	0.40	0.73	0.40	0.06	0.75	0.36
18:4n-1	-	-	0.03	-	-	-	0.08	-
20:0	0.28	0.21	0.19	0.28	0.28	0.72	0.26	0.24
20:1n-9	2.59	1.94	1.84	2.08	1.89	1.74	2.35	1.91
20:1n-7	0.26	0.13	0.09	0.13	0.10	0.11	0.15	0.09
20:2n-9	-	0.04	-	0.04	0.01	0.02	0.03	0.01
20:2n-6	0.19	0.11	0.08	0.11	0.09	0.10	0.04	0.08
20:3n-9	-	-	-	-	-	0.08	0.15	-
20:3n-6	0.25	0.09	0.04	0.06	0.03	0.08	0.08	0.03
20:4n-6	1.11	0.43	0.24	0.43	0.24	0.40	0.39	0.21
20:3n-3	-	-	0.07	-	-	0.06	0.08	-
20:4n-3	0.96	0.35	0.16	0.33	0.16	0.32	0.38	0.13
20:5n-3	10.05	4.00	2.07	3.77	2.06	3.85	3.99	1.73
22:1n-11	1.79	1.52	1.48	1.67	1.61	1.45	1.91	1.64
22:1n-9	-	-	0.42	-	-	0.33	0.52	-
22:4n-6	0.34	0.15	0.09	0.14	0.09	0.15	0.14	0.08
22:5n-6	-	-	-	-	-	0.64	0.75	-
22:5n-3	1.74	0.66	0.29	0.60	0.29	0.39	0.11	0.24
22:6n-3	7.82	4.16	2.92	3.87	2.91	4.35	4.69	2.63
Saturados	36.68	19.95	16.28	23.40	19.10	29.88	19.06	17.41
Monoenoicos	28.79	23.10	22.62	29.03	29.09	23.33	28.57	25.98
n-3	23.42	42.19	43.72	15.23	12.00	21.02	30.74	28.26
n-6	6.27	13.26	16.66	30.67	39.02	23.76	19.99	27.74
n-9	22.99	19.66	19.55	24.78	25.40	17.48	24.79	22.69
n-3 HUFA	20.57	9.17	5.50	8.57	5.42	8.97	9.24	4.73
n-3/n-6	3.74	3.18	2.62	0.50	0.31	0.88	1.54	1.02

Procedimiento del muestreo

Al final del experimento de alimentación (después de 26 semanas), 6 peces fueron muestreados individualmente de cada tanque. Los peces fueron anestesiados con el phenoxyethanol 2 (1:1000 v/v), y la sangre fue recogido de las venas caudales con jeringas heparinizadas a partir de 6 peces por cada tanque (18 peces por dieta) y transferido a un tubo de eppendorf cubierto con heparina de litio como anticoagulante. La sangre fue centrifugada inmediatamente a 3000 RPM durante 10 minutos para sedimentar las células, y el plasma fue guardado a -80 ºC para el análisis del cortisol basal. Las muestras de músculo, hígado, branquias y de riñón anterior fueron guardadas al -80ºC para el análisis bioquímico de todos los tratamientos dietéticos.

Preparación y estímulo del tejido de riñón anterior

En el final del período de alimentación, 2 peces fueron tomados aleatoriamente de cada tanque (6 por dieta) en menos de 1 minuto, anestesiado inmediatamente con el phenoxyethanol 2 (1: 1000 v/v) y se les saco sangre con una jeringuilla hipodérmica de la vena caudal para reducir al mínimo la hemorragia durante la extracción. El tejido del riñón anterior fue quitado a partir de dos peces por cada ensayo de perfusión, y cortado en fragmentos muy pequeños en el medio de Hepes Ringer, que fue utilizado como el medio de la perfusión. Luego, la mezcla de los riñones anteriores fue homogenizada y distribuidos en 8 cámaras de perfusión (con un volumen de 0,2 ml) para obtener una alícuota homogénea en cada una de ellas. La perfusión de los tejidos fue desarrollada con una solución de Ringer's Hepes (pH 7.4) que contenía 171 mM de NaCl, 2 mM de KCl, 2 mM CaCl2H2O, la glucosa 0.25% (peso/volumen), y la albúmina del suero bovino del 0.03% (peso/volumen) (Rotllant *et al*., 2001). La temperatura del sistema fue controlada a 18 °C y el medio de perfusión fue bombeado a través de una bomba peristáltica de varios canales de Masterplex L/SR con un flujo constante de 75 µl/min (col Parmer Intrument Co., Vernon Hills, Illinois).

Los ensayos fueron comenzados después de 3 h de perfusión cuando el cortisol alcanzó los niveles de una línea basal estable (Rotllant *et al*., 2000a, b), para evitar las desviaciones debidas a la diversa dispersión de células interrenales en la preparación de la perfusión y a las diferencias individuales o al nivel de la pre-estrés de cada pez. Después del período de la estabilización de 3 h, los tejidos fueron estimulados con la hormona adrenocorticotrófica (ACTH) con una concentración de 5 nanómetro

hACTH1-39 (sigma) durante 20 min. Posteriormente, las perfusión fue mantenido para otros 170 minutos, y las muestras de las fracciones fueron recogidas cada 20 minutos durante este período. En una segunda serie de experimentos para aclarar los mecanismos de la acción de los HUFAs y la implicación de eicosanoides en este proceso, los tejidos fueron incubados con un inhibidor de cicloxigenasa (COX) (indometacina, INDO), o el inhibidor de la lipooxigenasa (LOX) (ácido Nordihydroguaiaretic, NDGA) durante 20 minutos en una concentración de 25 μM diluido con el medio de perfusión, y los tejidos fueron estimulados posteriormente con ACTH como explicado antes y mantenidos para otros 170 minutos recogiendo muestras cada 20 min. En toda la serie de experimentos, cada tratamiento fue probado por cuadruplicado. El factor de estimulación del cortisol (SF) fue calculado por la comparación del cortisol máximo liberado después del estímulo con la ACTH con la línea basal (liberación máxima-liberación basal) *100/(liberación basal) (Rotllant *et al*., 2001).

Los paneles de estrés

Después del período de alimentación, 20 peces por tanque fueron expuestos al estrés por confinamiento. Así, los peces fueron asignados en grupos de hasta 4 mantenidos en cinco pequeñas jaulas flotantes (50x30x15cm) (figura 2.5). Se sacó sangre a 4 peces por tanque al principio de las pruebas y fueron considerados como controles. Las cinco jaulas, conteniendo 4 peces cada una, fueron mantenidas en cada tanque para diversos intervalos de tiempo, consistiendo para abarcar la respuesta "temprana" y la "tarde" al factor estresante, basado en las respuestas al estrés primarias y secundarias del cortisol plasmático. Así, después 2h una jaula por cada tanque fue extraída cuidadosamente, abierta y se sacó sangre a los peces en menos de 2 minutos de manejo, la sangre fue obtenido de la puntura caudal del sino con una jeringuilla de plástico de 1 ml. Las alícuotas de muestras de sangre fueron transferidas inmediatamente a un tubo de Eppendorf cubierto con heparina de litio como anticoagulante. El plasma fue obtenido por centrifugación a 3000 RPM durante 10 mínimos y guardado al -80º C para la determinación del cortisol y los peces fueron liberados. El mismo procedimiento fue repetido después de 5h, 24h, 48h y 1 semana. Este procedimiento fue tomado para evitar cualquier disturbio agudo a los peces restantes durante el muestreo de los peces. Así, el tiempo total de la captura fue menos

de 8 minutos por tanque para reducir al mínimo los efectos del estrés de la captura sobre los parámetros analizados (Sumpter, 1997).

Figura 2.5: Una muestra de la jaula usada para el panel de estrés

Análisis bioquímico

La humedad de las dietas y de los tejidos de peces, la ceniza y la proteína total fueron determinados en triplicados según la asociación de los métodos descritos por los analíticos oficiales de los químicos (AOAC). La humedad fue evaporada en un horno de 110 ºC hasta alcanzar un peso constante y la diferencia del peso inicial entonces era calculada como antes. El contenido proteínico fue calculado del contenido de N según el método de Khedjal. La muestra fue destilada con NaOH al 40% en el destilador del sistema de Kejeltek 1003 (Tecator, Höganäs, Suiza). Finalmente, la muestra fue validada con el ácido clorhídrico (HCl) (1N) medir el contenido de N y el contenido proteínico fue calculado utilizando un factor de conversión del nitrógeno de 6.75.

La extracción de los lípidos totales de las dietas, músculo, hígado, branquias y del riñón anterior fue realizada por el método de Folch *et al*., (1957) como esta explicado en el experimento anterior.

Análisis del cortisol

La concentración del cortisol en el líquido inundado fue determinada por radioinmunoanálisis (RIA) (Rotllant *et al*., 2001). El anticuerpo, Biolink, S.L. (Costa Mesa, California), fue utilizado en una dilución final del 1:6000. Esta reactividad cruzada del anticuerpo es 100% con cortisol, 11.40% con el desoxycorticosterone 21, 8.90% con el desoxycortisol 11 y 1.60% con 17α-hydroxyprogesterone. La radioactividad fue cuantificada usando un contador de centelleo líquido. Los niveles del cortisol se dan como ng/g/ h.

Análisis estadístico

La significación de la diferencia ($P<0.05$) entre los tratamientos dietéticos fue determinado por el análisis de varianza unidireccional (ANOVA) seguido por la prueba de comparación múltiple de Duncan (Sokal y Rolf 1995). Los análisis fueron realizados usando el software SPSS (SPSS para Windows 13).

10.4. Resúmenes de los experimentos (4)

Capitulo 3: Efecto de alimentar de la dorada (*Sparus aurata*) con una mezcla del aceite de soja y de lino sobre el crecimiento, la utilización del alimento y la composición en ácidos grasos del pez

Los grupos triplicados de dorada fueron alimentados con ocho dietas de tipo-práctico en las cuales los lípidos agregados fueron substituidos con una mezcla de aceite de lino (LO) y del aceite de soja (SO) en diversos niveles por un período de 26 semanas. El aceite de la anchoa fue la única fuente de lípidos en la dieta FO (aceite de pescado), mientras que las otras dietas contuvieron diversos niveles de aceites vegetales, 70L (el 70% LO y 30 FO), de 70S (el 70% SO y 30FO), de 100L (el 100% como LO), de 100S (el 100% como SO), de 50S20L (el 50% SO, el 20% como LO y el 30% como FO), de 20S50L (el 20% SO, el 50% con LO y el 30% con las FO) y de 50S50L (el 50% como SO y el 50% con LO). Los resultados demostraron que la substitución hasta el 70% o más del FO por los aceites vegetales en las dietas para dorada, redujeron

significativamente el crecimiento y afectaron la utilización del alimento. Las composiciones en ácidos grasos de los lípidos del músculo demostraron una correlación con la inclusión de SO o de LO en la dieta, así las proporciones del 18:2 n-6, del 18:3 n-3 y del 18:1 n-9 aumentaron todas con el aumento del % VO dietético. Las concentraciones del ácido eicosapentaenoico (20: 5n-3), ácido docosahexaenoico (22: 6n-3) y ácido araquidónico (20: 4n-6) en los lípido del músculo fueron significativamente reducidos junto con los ácidos grasos saturados totales, con el aumento del VO dietético, mientras que los últimos 2 ácidos grasos fueron reducidos menos en el músculo que en la dieta, indicando su retención selectiva. Los productos de la elongasa y de la Δ6 destaurasa del ácido linoleico (18: 2n-6) y ácido linolenic (18: 3n-3) también fueron aumentados con el aumento de VOs. La dieta indujo cambios, aproximadamente similar a los del músculo, en las composiciones en ácidos grasos del hígado y en las branquias con algunas excepciones. Las fuentes limitadas de aceites de pescados marinos requieren que los substitutos estén encontrados sin dañar el bienestar de los peces. Así, LO y SO se pueden utilizar con éxito en la alimentación de la dorada con niveles menos del 70% de los lípidos dietéticos sin afectar el crecimiento, pero las reducciones substanciales ocurren en el 20:5 n-3 del músculo, el 22:6 n-3 y el cociente poliinsaturado de los ácidos grasos n-3/n-6 (PUFA), que darán lugar a reducción de la calidad del producto final para el consumidor.

Capitulo 4: La respuesta al estrés en doradas (*Sparus aurata*) mantenidas bajo condiciones de confinamiento y alimentadas con dietas con diversos niveles de inclusión del aceite de lino y/o de soja

La respuesta fisiológica a los factores de estrés, incluyendo perfiles hormonales y sensibilidades asociadas del tejido. El objetivo de este estudio fue evaluar el efecto de alimentar la dorada (Sparus aurata) con dietas que contenían aceite de lino (LO) y el aceite de soja (SO) como substituto para el aceite de pescado (FO) y su efecto sobre el perfil de los ácidos grasos del riñón anterior y el efecto consiguiente sobre la respuesta al estrés después de una prueba de confinamiento. Los peces fueron alimentados con diversas dietas con diferentes niveles de substitución, el 0% (FO), el 70% (70LO, 70SO, 20LO50SO y 50LO20SO) y 100% (100LO, 100SO y 50LO50SO) durante 8 meses. Al final del experimento de alimentación, las muestras para el análisis bioquímico del

riñón anterior fueron recogidas y los peces fueron sometidos a un desafío de confinamiento. Las muestras de plasma para el análisis del cortisol fueron recogidas en diversas horas durante la semana de la prueba, de 0h, de 2h, de 5h, de 24h, 48 y 1 semana. Los niveles básales del cortisol fueron significativamente aumentados en los peces alimentados con 70LO, 100LO y 50LO50SO. La respuesta fisiológica al confinamiento fue afectada significativamente por la dieta. Después de 2 h de confinamiento, todos los tratamientos demostraron un cortisol más alto, con los peces alimentados con 100LO teniendo la máxima respuesta con 131.38 pg/ml comparados con 50LO20SO cuáles tenían la respuesta más baja del cortisol plasmático con solamente 18.73 pg/ml. Después de 5h y de 24h, el cortisol plasmático fue reducido en todos los tratamientos a excepción de 50LO20SO. Después de 48 h de confinamiento, el cortisol plasmático fue aumentado en todos los tratamientos con el valor máximo registrado en los peces alimentados con 100LO (72.12 pg/ml). Estos valores fueron disminuidos después de 1 semana confinamiento en los peces alimentados con FO, 70LO, 100LO y 50L050SO, pero fueron mantenidos en los peces alimentados con 70SO, 100SO, 20LO50SO y 50LO20SO. Los peces alimentados con LO fueron caracterizados generalmente por una respuesta al estrés más rápida y más fuerte y una recuperación rápida, mientras que los peces alimentados con SO tenían una respuesta lenta y una recuperación más larga.

Capitulo 5: Efecto de lípidos dietéticos sobre los perfiles en ácidos grasos del plasma y la producción de la prostaglandina y de la leptin en dorada (*Sparus aurata*)

El objetivo de este estudio fue investigar los efectos de diversos niveles de substitución del aceite de pescado por aceites vegetales ricos en ácido oleico, linoleico y linolénico sobre las composiciones en ácidos grasos del plasma y de los leucocitos de la dorada y la producción de la prostaglandina (PG) y de la leptina. Los juveniles de dorada con un peso inicial de 24 g fueron alimentados con cuatro dietas experimentales iso-energética y iso-proteicas durante 281 días. La composición en ácidos grasos de los lípidos del plasma fue muy afectada por la inclusión de los aceites vegetales (VOs). El ARA (arachidonate), EPA (eicosapentaenoate) y DHA (docosahexaenoate) fueron incorporados preferencial en los lípidos polares del plasma, y el DHGLA (di-

homogammalinoleate) fue acumulado con la inclusión creciente de los aceites vegetales. Los tratamientos dietéticos dieron lugar a alteraciones de los cocientes de DHGLA/ARA, pero no a ARA/EPA. La producción de la PGE2 derivada del ARA en plasma no fue afectada por la inclusión de los aceites vegetales, de común acuerdo con el cociente similar del precursor de eicosanoides (ARA/EPA) en lípidos de los leucocitos y fosfolípidos totales del plasma de los peces alimentados con los diferentes tratamientos dietéticos. La alimentación con aceites vegetales lleva a una disminución de la concentración del EPA lo que a su turno redujo la concentración de la PGE3 en el plasma. Por otra parte, la PGE3 fue la principal prostaglandina producida en el plasma de los peces alimentados con FO. Los presentes resultados precisan la importancia del EPA como precursor de las prostaglandinas en peces marinos, por lo menos para la función correcta de las células sanguíneas, y correlacionan muy bien con el papel predominante de este ácido graso en la regulación inmune en esta especie. Una correlación negativa fue encontrada entre la PGE2 del plasma y la concentración de ls leptina, sugiriendo que los niveles de circulación de leptina pueden actuar como una señal metabólica que modula la liberación de la PGE2. El actual estudio ha demostrado que la inclusión creciente de los aceites vegetales en la dieta para dorada puede afectar profundamente la composición en ácidos grasos del plasma y de los leucocitos, especialmente los HUFA (ácidos grasos altamente insaturados), y por lo tanto la producción de la PGE3, que puede ser una PG importante en plasma. La alteración en la cantidad y el tipo de PG producidos puede ser por lo menos parcialmente responsable de los cambios en el sistema inmune y los parámetros de la salud de los peces alimentados con dietas con la alta inclusión de VO.

Capitulo 6: Modulación de la liberación del cortisol a través de la inducción por la ACTH por los ácidos grasos poliinsaturados en células interrenales de dorada, *Sparus aurata*

Los ácidos grasos altamente insaturados son componentes esenciales de las membranas celulares de vertebrados y pueden modular diferentes procesos fisiológicos, incluyendo transporte a través de la membrana, la función del receptor y las actividades enzimáticas. En dorada, las deficiencias dietéticas en ácidos grasos esenciales para los peces marinos aumentan los niveles básales del cortisol y alteran el patrón del

liberación del cortisol después del estrés. El objetivo del actual estudio fue aclarar el efecto de los diversos ácidos grasos esenciales en la producción y la liberación del cortisol inducido por la hormona adrenocorticotrópica (ACTH) en peces, a través de estudios *in vitro* de las células interrenales de dorada mantenidas en perfusión y incubadas con diferentes tipos de ácidos grasos y de inhibidores de producción de los eicosanoides. Los resultados demostraron la primera evidencia del efecto de ciertos ácidos grasos sobre la producción del cortisol por las células interrenales estimuladas con ACTH en peces. El Tanto el acido araquidónico (ARA) y particularmente el ácido eicosapentaenoico (EPA) promovieron la producción del cortisol en células interrenales de la dorada. Por otra parte, la incubación con la indometacina (INDO) redujo la elevada producción del cortisol inducida por el EPA y ARA, sugiriendo la mediación por los productos derivados de la ciclooxigenasa. El ácido Docosahexaenoico estimuló la producción del cortisol en un grado inferior que lo causado por EPA o el ARA, pero el efecto inhibitorio de INDO no fue tan marcado como lo fue para los otros ácidos grasos. En cambio, la suplementación con el ácido dihomogamalinoleico redujo la producción del cortisol, denotando el efecto inhibidor de este ácido graso en la secreción del cortisol.

Capitulo 7: La liberación del cortisol después de la estimulación con ACTH por el tejido interrenal del riñon anterior de dorada (*Sparus aurata*) alimentada con aceites de lino y de soja

El modo de acción de los ácidos grasos altamente insaturados (HUFA) en la regulación de la producción por las células interrenales del cortisol de la dorada (*Sparus aurata*) fue estudiado con ensayos *in vitro* usando un sistema dinámico de perfusión. Los peces fueron alimentados previamente con diversas dietas que contenían varios niveles de inclusión del aceite de lino (LO) o del aceite de soja (SO) por un período de 26 semanas. Cinco dietas fueron probadas, con el aceite de anchoa como la única fuente de lípidos para la dieta control (aceite de pescado, FO), dos diversos niveles de la substitución (70 y 100%) fueron probadas usando los aceites de la lino o de soja (70LO, 70SO, 100LO, 100SO). Las composiciones en ácidos grasos del riñón anterior reflejaron la inclusión dietética, así EPA, DHA, el ARA y n-3 HUFA estaban perceptiblemente ($P<0.05$) reducido en peces alimentados con VOs comparados con la

dieta FO. La alimentación con el 70% o el 100% LO aumentó significativamente la liberación del cortisol en el riñón anterior después del estímulo con la hormona adrenocorticotrofica (ACTH), mientras que la alimentación con SO disminuyó pero no significativamente esta respuesta. El factor de estimulación del cortisol fue aumentado en los peces alimentados con las dietas 70LO y 100LO comparando con el control. Por otra parte, la inhibición de la producción de los eicosanoides incubando el tejido del riñón anterior con la indometacina (INDO) como inhibidor de la cicloxigenase, o ácido nordihydroguaiaretico (NDGA), un inhibidor de la lipooxigenasa, redujo significativamente ($P<0.05$) la liberación del cortisol después del estímulo con ACTH en 70LO, 100LO y 100SO. El factor de estimulación del cortisol fue reducido al incubar el riñón anterior de los tratamientos FO, 70LO y 100LO con INDO o NDGA, mientras que fue aumentado en 70SO. Este experimento describió la modulación de la liberación del cortisol por los HUFAs dietéticos y la mediación de la ciclooxigenasa y/o del lipooxigenasa en su mecanismo de acción.

10.5. Conclusiones (5)

1- Se pueden substituir hasta el 70% del aceite de pescado dietético por los aceites vegetales en las dietas de engorde para la dorada que contienen el aceite de pescado y harina de pescado de origen suramericano con un contenido en lípidos de cerca del 17%, sin afectar el crecimiento y la supervivencia. Un porcentaje más alto de la substitución causa el retraso del crecimiento.

2- Los lípidos de la membrana del plasma y los lípidos totales del músculo, del hígado y de las branquias de la dorada pueden ser profundamente alterados después de alimentar con dietas con altos contenidos en aceites vegetales. El contenido de N-3 HUFA en músculo de peces fue seriamente reducido cuando estan alimentados con VOs, lo que podría afectar negativamente a su valor alimenticio para el consumo humano. Sin embargo, la alimentación con VOs afectó la composición en ácidos grasos del hígado de manera mas alta que en otros tejidos, mientras que los perfiles de los ácidos grasos de las branquias demostraron el contenido doble del ARA que en los otros tejidos.

3- Hubo una retención selectiva de DHA y ARA en los fosfolípidos del plasma y los lípidos totales del músculo, hígado y branquias, confirmando su importancia en estos tejidos.

4- No sólo el ARA, pero también el EPA fue encontrado ser un precursor importante de las prostaglandinas, y el cociente dietético entre ambos ácidos grasos alteró la producción de las prostaglandinas, los que puede afectar a numerosas funciones fisiológicas, algunas de ellas relacionadas con la salud de los peces.

5- La concentración de la leptina del plasma refleja la tasa de alimentación, y no es afectada significativamente por la alimentación con los VOs.

6- El EPA y ARA como solos ácidos grasos implementados estimulan la liberación del cortisol estimulado por la ACTH del tejido interrenal de la dorada.

7- El DHA estimuló de manera moderada el tejido interrenal, mientras que el DHGLA inhibió la liberación del cortisol estimulado por la ACTH del mismo tejido.

8- La ACTH indujo la liberación del cortisol y el papel del ARA y de EPA es mediado por derivados de la COX y LOX.

9- La ACTH indujo la liberación del cortisol por los HUFAs parecen ser dependientes de la concentración y el tipo de cada ácido graso.

10- La substitución del FO por LO en las dietas para la dorada estimuló la liberación del cortisol inducido por la ACTH del tejido interrenal, y es mediado por lo menos en parte por la COX y LOX. In vivo, la alimentación con el LO aumentó los niveles básales y post estrés del cortisol en plasma.

11- La alimentación con diferentes tipos de VOs afectó de manera diferente a la respuesta al estrés, con una respuesta cada vez mayor del cortisol en peces alimentados con 70LO y 100LO después de la estimulación con ACTH, mientras que el SO disminuyo esta respuesta. Una suplementación bien equilibrada de estos aceites dietéticos es importante para regular la liberación del cortisol y la resistencia al estrés.

12- La liberación del cortisol es modulada por los HUFAs dietéticos y esta respuesta es mediada por los derivados de la COX y/o de la LOX.

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Curriculum Vitae

Rachid Ganga was born on December 6th, 1977 in Sidi Ifni, Morocco. He obtained his Agronomic Engineer diploma, in Agronomy specialized in marine resources at Hassan II Agronomic and Veterinary Institute, Rabat 2002. As an undergraduate student he developed a research study about the socio-economic impact of artisanal fisheries in the region of Sidi Ifni. After graduation, he was granted by Instituto Agronomico Mediterraneo de Zaragoza to begin a Masters Degree in Aquaculture at Universidad de Las Palmas de Gran Canaria. On June 2003 he visited the department of fish nutrition at the Institute of Aquaculture, University of Stirling (Scotland) for a training period of 10 weeks, analysing samples of sea bream plasma, to determine their PGE2, PGE3 and Leptins. This training was supervised by Prof. Gordon Bell, and granted by EU project (RAFOA). Once he finished his Masters on April 2004, Rachid Ganga began his first PH.D. experiment on June 2004 at the department of Cell Biology, Physiology and Immunology, Universitat Autonóma de Barcelona, Spain, and on October 2004 he began an aquaculture program at the University of Las Palmas about feeding vegetables oils in diets for sea bream and their effects on fish resistance to stress and fish health. He presented his DEA (Diploma de Estudios Avanzados) on December 2006. During his PH.D. work, he improved his laboratory skills with a short–course on Fish Welfare (Aqua TT grant) at the University of Varese (Italy) on September 2005 and a training of 3 months period (January to March 2007) on HPLC techniques at Meakines – Laboratories, McGill University, Montreal, Canada under the supervision of Prof. William Powell.

The thesis has been structured and supervised by Prof. Marisol Izquierdo. Through the entire research period Prof. Gordon Bell was a co-supervisor of the present thesis and Prof. Lluis Tort participated in the supervision of the *in vitro* experiment done at Universitat Autonóma de Barcelona, and in the discussion of all the cortisol results.

Departamento de Biología

Grupo de Investigación en Acuicultura