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Effect of feeding gilthead seabream (*Sparus aurata*) with vegetable lipid sources on two potential inmunomodulator products: prostanoids and leptins

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Trabajo realizado en el Instituto Canaria de Ciencias Marinas, España, bajo la dirección de la profesora, Marisol Izquierdo.

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Directora

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List of abbreviations

Ab : antibody

Ag : antigen

ANOVA: analyse of variance

ARA: arachidonic acid

BHT: Butylated hydroxytoluene

CMI :cell-mediated immunity

DHA: docosahexaenoic acid

DHGLA: di-homogamalinolenic acid

EFA: essential fatty acids

EIA: enzyme immunoassay

EPA: eicosapentaenoic acid

FABP: fatty acid binding protein

FO: fish oil

GC: gas chromatography

HBSS: Hank's balanced salt solution

HPLC: high-performance liquid chromatography

HSI: hepatosomatic index

HUFA: high unsaturated fatty acid

LA: linoleic acid

LNA: linolenic acid

LTs: leukotrienes

LXs : lipoxins

NL: neutral lipids

PCI : prostacyclines

PGE: prostaglandin E

PI: phosphatidyl inositol

PL: polar lipids

PLA_2: Phospholipase A_2

PUFA: polyunsaturated fatty acid

SD: standard deviation

SGR: specific growth rate

TLC: thin layer chromatography

TXs: thromboxanes

UV: ultraviolet

Abstract

Gilthead sea bream (*Sparus aurata*) were fed practical-type diets that contained different levels of substitution with a blend of linseed, rapeseed and palm oil (LO, RO, PO) for a 155 days period. The major PUFA in total, neutral and polar lipids from plasma were related to the dietary lipid intake. Levels of n-6 PUFA were highest while levels of n-3 PUFA were lower in fish fed vegetable oils compared with those fed fish oil. Fish fed blends of vegetable oils had significantly decreased 22:6n-3, 20:5n-3 and 20:4n-6 compared with fish oil fed fish in total, polar and neutral lipid classes. While fish vegetable oils had significantly higher levels of 18:2n-6, 18:3n-3, 18:1n-9, 20:2n-2 and 20:3n-6. There was also higher levels of 20:3n-3 and 20:3n-4. There was a constant level of 20:4n-6/20:5n-3 in plasma phospholipids among the four treatment, and reduced level of 20:4n-6/20:3n-6 in fish fed vegetable oils due to the increased 20:3n-6 in polar lipid of plasma from those fish. There was a little difference between the composition on fatty acids PI from leucocytes and phospholipids from plasma, PI incorporated more C_{20} PUFA and maintained a constant, but higher, ARA/EPA ratio and t a very small or inexistent content on DHGLA in this phopholipids.

Production of PGE_2 in plasma was not different among the four treatment which may be explained by the constant eicosanoids precursor. PGE_3 levels in plasma were different among the four treatments and was strongly correlated to EPA content in polar lipids, the highest value was enregistred in fish fed FO and the lowest in fish fed 100 % blend of vegetable oils. Plasma leptin was not different among the four treatments and correlate strongly with feed intake and conversion index.

INTRODUCTION

Introduction

Over the past two decades, high interest has been given to good nutrition practices in animal production systems in order to produce a healthy and high quality animals. In fish farming, nutrition is critical because feed represents around 40-50% of the production costs (Craig, 2002). Recently, fish nutrition has dramatically progressed, and more importance is given to feed fish with high quality diets providing all the nutrients required for it's optimum growth and health (Cho and Kaushik, 1990; Tacon, 1996).

Lipids, as an important component of the diet, have multiple functions in fish physiology. Dietary lipids provide essential polyunsaturated fatty acids (PUFA) for normal growth and development of cells and tissues (Sargent et al., 1995) and are also a major source of energy in fish diets (Sargent, Henderson and Tocher, 1989). They are high energetic nutrients that can be utilized to partially spare protein in aquaculture feeds enhancing diet profitability, thus when increasing lipids levels from 9 to 15% in diet increased growth in fingerlings gilthead sea bream (Vergara et al., 1996). Lipids supply about twice the energy of proteins and carbohydrates and serve as transporters for fat-soluble vitamins (Craig, 2002).

Despite a common practice in fish feeds is to use high levels of dietary lipids to partially spare protein in the feed, care should be taken to preserve fish health and market quality.

1- Lipid Sources

Aquaculture has traditionally used products from industrial fisheries, namely fish meal and oil, to convert relatively cheap protein and oil into high value products, a practice that is sound both scientifically and commercially efficient (Bell et al., 2002). Marine fish oils (FO) are naturally high (>30%) in omega 3 high unsaturated fatty acids (HUFA), are excellent sources of very digestible lipids for the manufacture of fish diets; and also can have beneficial effects on human cardiovascular health (Craig, 2002).

Marine lipids such as herring, menhaden, and anchovy oil have been employed as main sources of essential fatty acids (EFAs), non-protein energy, and other factors of nutritional importance in finfish diets. Commercial feeds are generally rich in oil, constituting for example between 25% and 35% of the diet at different stages of salmon growth (Bell et al., 2002).

Aquaculture production has grown by >10% per year since 1984 and is projected to at least double over the next decade and beyond (Sargent and Tacon, 1999). However, greater demands are being placed upon valuable fish oil and fish meal for expanding aquafeed markets and for direct use in human diet to prevent an array of health-related problems (Higgs et al., 1995). Meanwhile, there is a reduced supply of fish oil and fish meal due to stagnation in capture as a consequence of over fishing and events such as el Niño (Bell et al., 2002). Fish oil production knows a decrease and fluctuations which make higher its prices and uncertain its availability at the market. Some scientists expect that supplies of fish oils for aquaculture production become critical between 2005 and 2010 (Bell and Sargent., 2002). 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 ingredients as a potential substitutes in fish diets (Tacon and Jakson, 1985).

Recent studies have assessed the suitability of various alternate dietary lipid sources, such as vegetable oils, for finfish species in terms of growth, feed utilization, and flesh quality. By contrast, relatively little attention has been directed toward the possible adverse effects of novel lipid sources of varying lipid composition on the immune response(s) and disease resistance of fish. The latter effects could potentially occur, especially if tissue levels of eicosanoids precursors such as arachidonic acid (ARA, 20:4n-6) and eicosapentanoic acid (EPA, 20:5n-3) are altered (Balfry and Higgs, 2001).

2- Overview of the fish immune system

The fish immune system can be categorized into specific acquired immunity and nonspecific innate immunity. Both use cellular and humoral mechanisms to provide protection against infections. The specific immune system involves the recognition of a specific antigen on a pathogen, therby providing protection against that specific pathogen. Memory of the initial pathogen exposure is a vital feature to the specific immune response. It is affected by factors that influence antibody production, such as type and duration of antigen stimulation, age, temperature, and stress (Tatner, 1996). In contrast, the non-specific immune system provides an array of protection against a wide variety of pathogens, it is considered as the first line of defence (Ingram, 1980) and plays a vital role in preventing the establishment of infection. The non-specific defence does not alter on repeated exposure (Sigrun et al,. 2001). These complex networks of cellular and humoral factors of both the specific and non-specific immune systems provide fish with external and internal protection against infectious agents. The various factors comprising each of the systems can act alone or in combination to provide a range of protective mechanisms (Balfry & Higgs, 2001).

2-1 Nonspecific immune system

The non-specific immune system is composed by an array of organs and mechanisms including physical barriers & non specific humoral components. The physical barriers are formed by the mucus, skin, and scales which are very effective against external invading pathogens. Fish mucus also contains non-specific antimicrobial factors and specific antibodies that help to prevent the pathogens from attaching to and colonizing fish surface (Balfry and Higgs, 2001). The non-specific humoral components act in several ways to kill and /or prevent the growth and spread of pathogens. Many of the components use unique mechanisms to lyse the pathogen, others act as agglutinins (aggregate cells) or precipitins (aggregate molecules) (Secombes, 1996). There are also opsonins that

bind with the pathogen which facilitate its uptake and removal by phagocyte cells. Other non-specific humoral factors in fish have been studied recently (Yano, 1996; Balfry and Higgs, 2001), including various lytic substances (lysozyme hemolysins, chitinase, proteinases), agglutinins/precipitins (C-reactive protein, lectins, serum amyloid P-component, α-precipitin, natural precipitins, natural antibodies, natural hemagglutinins), enzyme inhibitors (serine proteinase inhibitors, cyseine-proteinase inhibitors, metalloproteinase inhibitors, α-macroglobulin), and pathogen growth inhibitors (interferon, transferrin, caeruloplasmin, metallothionein).

The process of phagocytosis is primordial in the immune system. Phagocytic cells are responsible for the clearance of foreign substances and senescent blood cells from the body. Monocytes, macrophages, neutrophils, eisonophils, and thrombocytes all appear to be phagocytic in fish. Neutrophils are the most active phagocytes, because they are highly mobile (Secombes and Fletcher, 1992). Phagocytosis is initiated with a passive process of attachment to the pathogen (which can be facilitated by opsonization by such substances as complement and antibody), followed by ingestion of the foreign substance into the phagocyte (include creation of phagosome within the cytoplasm of the phagocyte), and the killing of the pathogen which can involve both oxygen-dependent and oxygen-independent processes (Secombes, 1996). Phagocytes activity is tightly regulated by cytokines and eicosanoids (Secombes, 1996).

Cytokines are a variety of soluble factors released from cells that facilitate interactions among immune cells and therby modulate the immune response (Balfry and Higgs, 2001). They have an involvement in inflammatory reactions (Secombes 1996). Several cytokines have been described in fish (Manning and Nakanishi, 1996) including: Interleukin–2 (IL-2), IL-3, IL-4, and IL-6. Interferon, tissue necrosis factor (TNF), transforming growth factor β1, migration inhibition factor, and the platelet aggregation factor are also produced in fish (Secombes, 1996). Other cytokines that are important mediators of the inflammatory reponse are eicosanoids (Secombes, 1996). Cytokine play a key role in the immune response. Antigen stimulated macrophages to synthesize

interleukin-1(IL-1), process the antigen, and present it to T helper cells which in turn activate T cells to produce interleukin-2 and other lymphokines. These, in turn, activate B cells to produce antibodies. The production of growth promoting factors and both T and B cell proliferation is important in this sequence of events and PGE_2 is involved in various phases of these reactions (Kinsella et al., 1990).

Eicosanoids are oxygenated derivatives of polysunsaturated fatty acids formed by the metabolism of membrane phospholipids by the action of phospholipases (Rowley et al., 1995). In broad terms they 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) with a 20-carbon backbone that have a potent proinflammatory effects (Secombes, 1996), but also eicosapentaenoic acid (EPA; 20:5n-3), docosahexaenoic acid (DHA; 22:6n-3) and di-homogamalinolenic (DHGLA; 20:3n-6) are important substrates in fish due to their high presence in membrane phospholipids of these organisms (Henderson & Sargent, 1985). Once leucocytes are stimulated by a pathogen, the phospholipase enzymes located in their membrane become activated (Figures.1,2), and metabolize available phospholipids to generate nonesterified PUFA (ARA, EPA, DHA and DHGLA), which are converted by the actions of cyclooxygenases and lipooxygenases enzymes to produce an array of eicosanoids (Fig.3). The types and the amounts of the foregoing PUFAs produced, especially ARA and EPA, determine the types and amounts of eicosanoids released (Balfry and Higgs, 2001). It is reported that eicosanoids can be generated in a variety of different tissues in fish, including brain, gill, liver, spleen, and heart (Knight et al., 1995).

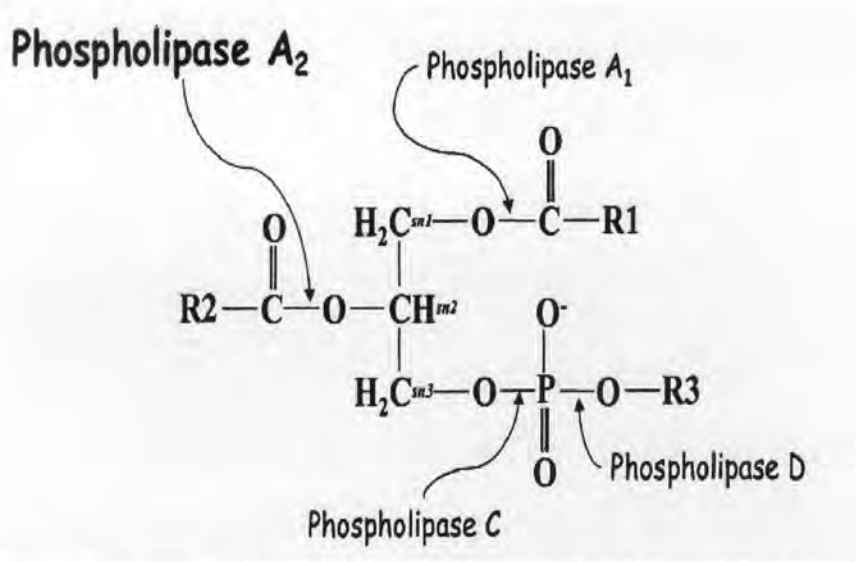

Fig.1: Sites of action for phospholipases in glycerol backbone of a phospholipid. The phospholipase A_2 releases the fatty acids occupying the *sn*-2 position (Capper and Marchall, 2001)

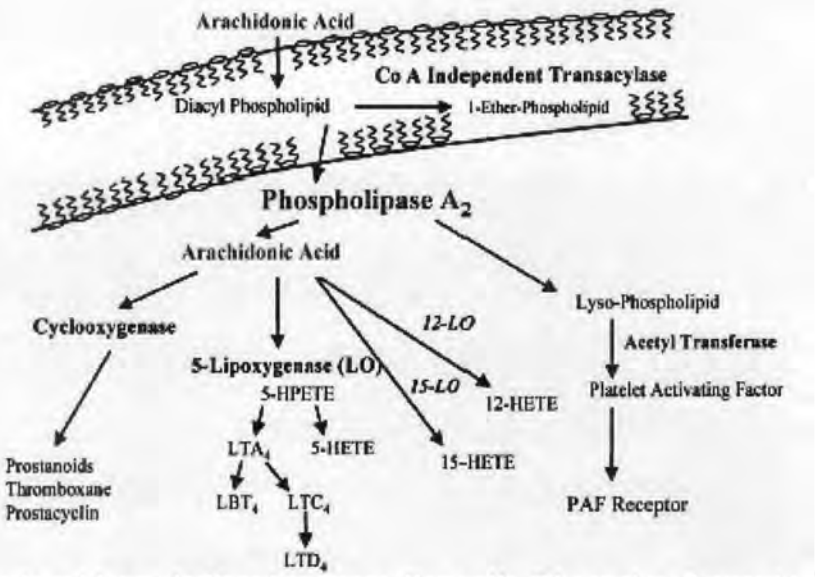

Fig. 2: Arachidonic acid metabolism in mammalian cells. The major players in ARA metabolism following PLA2 mediated acylhydrolosis (Capper and Marchall, 2001)

Eicosanoids have relatively a short half-life *in vivo* after their release (Rowley et al., 1995) and they are not stored in cell membrane but released soon after they are produced following cell stimulation and mobilization of phospholipase (fig.2). They include prostaglandins (PGs), prostacyclines (PCI) and thromboxanes (TXs) derived from cyclooxygenase activity and leukotrienes(LTs) and lipoxins (LXs) derived from lipoxygenase activity (Secombes, 1996).

Non esterified ARA, through the action of cyclooxygenase enzymes (Fig.3), yields 2-series prostanoids (Prostaglandins and thromboxanes) and, through the action of lipoxygenase enzymes produce 4-series leukotrienes and lipoxines (Fig.3). Alternatively, the metabolic derivatives produced from nonesterified EPA are 3-series prostanoids and 5-series leukotreines and lipoxines. Besides, the 1-series prostanoids are 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).

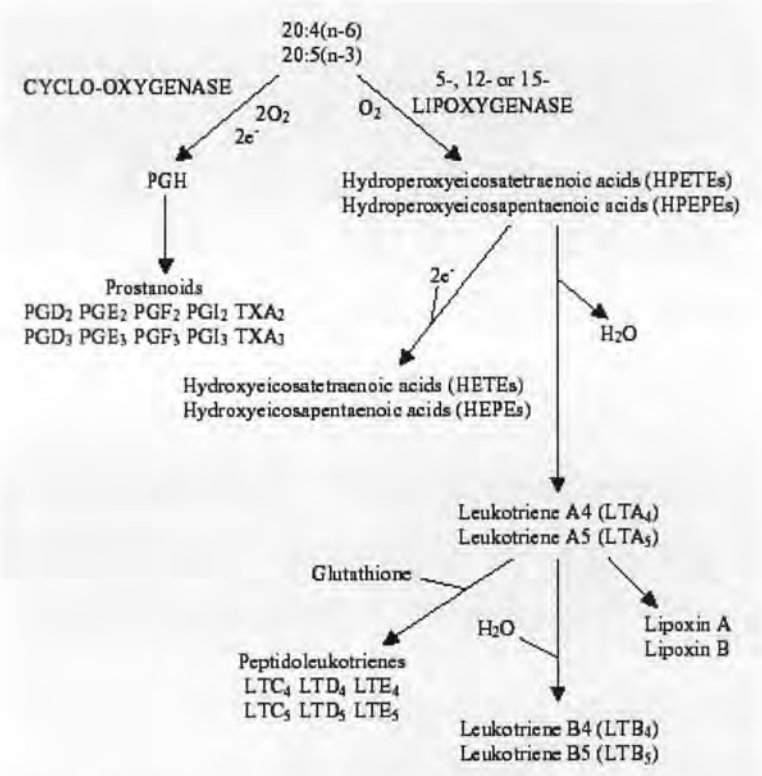

Fig. 3: Pathways of conversion of 20:4(n-6) and 20:5(n-3) to eicosanoids (Lall et al., 2000)

The amounts of synthesized eicosanoids depends on: the avalability of precursors, the activity of phospholipase A_2 (modulated by glucocorticoids and dexamethasone), the activity of cyclooxygenases (inhibited by non-steroidal anti-inflammatory drugs) and lipoxygenases (Kinsella et al., 1990). Eicosanoids are important intercellular signaling agents which affect cells behaviour and cell to cell interactions. They modulate secretory, smooth muscle (contraction and

relaxation), and cascade-type reaction which are essential to normal health (Kinsella et al., 1990). Some immune modulatory effects of eicosanoids operate via cytokine expression in the animals (Rowley, 1995). The nature of dietary lipids and the concentration of essential fatty acids have a direct effect on the eicosanoid metabolism and consequently on immune function (Lall et al., 2000).

The effect and the activity of eicosanoids depends on their relative concentration, PGE_2 at low concentration ($<10^{-9}$ M) activates certain cells of the immune system whereas high concentrations ($>10^{-8}$M) can suppress the immune system by providing a physiological feedback action (Kinsella et al., 1990). It has also been suggested that there are species differences in eicosanoid production (Rowley et al., 1995), thus, attention should be given when comparing eicosanoids results from different species. A deficiency of these compounds results in progressive impairment of function, while excessive or imbalanced production may result in a number of patho-physiological state (Kinsella et al., 1990).

The chemotactic activity of LT and LX is primordial in attracting more leucocytes to pathogens in the site of inflammation, this in itself may compromise bacterial clearance rates. Besides, PG act also in blood vessels dilatation to allow the increased migration of leucocytes to inflammation site (Kinsella et al., 1990). Leukotrienes also facilitate stimulation of lymphocyte proliferation and cytokine release, LTB_4 known to increase an activation marker (CD23) on resting B cells, to increase proliferation of intermediate B cells and increase Ig production from differentiated B cells, both enhancing antibody production (Balfry and Higgs, 2001). Lipoxins (LX) are relatively less produced in mammals, whereas in fish they are major eicosanoids products (Pettit, Rowley and Secombes., 1989).

Macrophages appear to be the major source of eicosanoids (Pettit et al., 1989; Blazer, 1992), although granulocytes, monocytes, and thrombocytes also contribute significantly. It is uncertain whether lymphocytes are capable of synthesizing eicosanoids, but their activity can be profoundly affected by eicosanoids generated by other leucocytes (Rowley et al., 1995). In the presence

of lymphocytes, macrophages produce increased levels of PGE_2 *in vitro* (Kinsella et al., 1990). It is also reported that PGE_2 affects the expression of certain antigen receptors on the surfaces of macrophages. Nevertheless, high level of PGE_2 is a suppressor of macrophage antigen presenting function, and of T cell expression via suppression of IL-2 production which is essential for T and B cell proliferation (Kinsella et al., 1990). Thus, PGE_2 should be considered as a regulators but not a universal suppressor of T cell functions (Kinsella et al., 1990). Prostaglandins are also known to mediate fluid and electrolyte fluxes in fish gill and kidney and are important in adaptation to changes in salinity (Mustafa and Srivastva, 1989). In other hand, previous studies showed that eicosanoids (particularly those of the E series) are potent stimulators of corticosteroid secretion (Wales and Toole, 1987). Injection of arachidonic acid and prostaglandin E_2 increased cortisol in plasma of hagfish (Wales, 1988) and are used to induce spawning in other fish species (Sargent et al., 1989, 1995). While, PGE_1 are known to inhibit aggregation of human platelets, relaxes uterine muscular arterioles, increases cAMP levels in several tissues, and is superior to PGE2 in suppressing the proliferation of synovial cells in vitro (Hummell, 1993).

The inflammatory response in fish has been well described by Secombes (1996). According to Suzuki and Lida (1992), three major events happen during inflammatory responses: first there is an increased blood supply to the infected area, followed by an increased capillary permeability, and lastly there is a migration of leucocytes out of the capillaries and into the surrounding tissue. Once in the tissue, they migrate toward the site of infection, attracted by a variety of host- and pathogen-derived molecules as described above. Neutrophils are the first to reach the site of inflammation, monocytes and macrophages joining later to remove and destroy the pathogen by phagocytosis (Balfry and Higgs, 2001). Eicosanoids and other cytokines play a primordial role in the development and control of inflammatory reactions (Secombes, 1996). First, the response is initiated by performed mediators such as vasoactive amines. Later, newly synthesized eicosanoids serve to attract and activate leukocytes. Once arrive to

the site of inflammation, leukocytes release mediators that regulate the response (Secombes et al., 1996). PGE_2 is apparently produced by macrophages in response to lectin stimulation of lymphocytes and may indicate that macrophages are under feedback control by the lymphocytes (Secombes et al., 1996).

In light of these reports, it appears that the balanced production of eicosanoids, many of which act antagonistically, modulates short-term local responses to injury and are required for normal health, tissue perturbations, and/or infection. Thus, knowledge of the factors regulating the eicosanoids synthesis is very important to improve fish immune system and health.

2-2 Specific immune response

The specific immune response is caracterized by the production of antibody (Ab) binding and neutralizing a specific foreign antigen (Ag), inhibiting its activity and virulence. Fish specific immune response seems to respond in a the similar manner to mammals, depending on the structure of the antibody (Ab) and the effector mechanisms involved. Ab/Ag binding serves to enhance the phagocytosis and clearance of pathogens from the body through the processes of opsonization, agglutination, and precipitation. It is also, characterised by production of memory cells which lead to fast and great response when there is subsequent exposure to the same antigen (Balfry and Higgs, 2001).

The specific immune responses that are independent of Ab are collectively termed cell-mediated immunity (CMI). They include, T lymphocytes as they become activated and differentiated into different functional cell types. In fish, there are lymphocytes analogues to mammalian T cells and B cells (Balfry and Higgs, 2001).

3 -Lipids nutrition and fish health implications

The influence of dietary components on fish health in cultured fishes has been recognized for many years. Nutritional status is considered one of the important factors that determine the ability of fish to resist. Fish diseases

commonly occur when fish are stressed due to a variety of factors including poor nutrition (Lall, 2000).

3-1 Dietary fatty acids, immune system and disease resistance

Fish require three long chain polyunsaturated fatty acids (PUFA) for their normal growth, health, development and reproduction: docosahexanoenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) (Sargent et al., 1995, 1997). In marine fish , EPA, DHA and , recently ARA are regarded as essential fatty acids (EFA) (Watanabe, 1993; Castell et al., 1994; Bell et al., 1995a,b; Izquierdo, 1996; Bessonart et al., 1999) due to the inability of marine fish to effectively synthesise them from their precursors linolenic acid (18:3n-3, LNA) and linoleic acid (18:2n-6, LA) by Δ^5&Δ^6destaurases and elongase (Castel et al., 1994; Sargent et al., 1995, Izquierdo et al., 1996) in sufficient amounts to cover their requirements for best growth and health. The enzymes performing the desaturation and elongation reactions are induced by the presence of precursors (LA and LNA), and inhibited by the presence of the end products (EPA, DHA and ARA). Hence, desaturase activity in salmon previously fed vegetable oil during the parr stage were reduced to very low levels upon feeding a diet containing fish oil simultaneous with transferring the smolts to seawater (Tocher et al., 2000). Increased hepatic fatty acid desaturase activities were also induced in rainbow trout fed a diet containing olive oil compared to a diet containing fish oil (Buzzi et al., 1997).

According to Balfry and Higgs (2001), there are three mechanisms by which dietary fatty acids may affect the fish immune system and disease resistance. The first is through their influence on cell membrane lipid composition, affecting cell permeability and function, which has profound effects on disease resistance because many immune reponses are based on leucocytes cell membrane interactions (e.g., phagocytosis, antigen-antibody binding, activation steps involving cytokine production). Some other studies report that changes in physical properties of membranes with aging on dietary lipids may reduce the sensitivity for normal responses. The second mechanism is by altering

signal transductions partioning between and within cells (Kinsella et al., 1990). Finally, a third mechanism is through the production of immunologically active eicosanoids from non esterified ARA, EPA, DHA and DHGLA, these mediators are important in modulating the immune response as described above.

The relationship between dietary levels of n-3 PUFAs and immunosystem could differ among marine and freshwater environments. The focus of recent studies has been on dietary fat modulation of macrophage function because of the primordial role of this cell in immune responses and tumoricidal activity (Jakson, 1997). Inadequate levels of dietary n-3 PUFAs are also known to reduce antibody production and in vitro killing of bacteria by macrophages in rainbow trout (Kiron et al., 1995) and depleted alternative complement pathway activity in gilthead seabream (Montero et al., 1998). Unbalances in fatty acids contents in the cell membrane could influence membrane physical properties which affect phagocytic activity (Montero et al., 2003). The work of Blazer and colleagues (1991) showed that increasing dietary levels of n-3 fatty acids lead to increased activity of head kidney macrophages. Furthermore, Thompson et al. (1996) observed that Atlantic salmon fed diets with high n-3 to n-6 PUFA ratios had more chemo-attractive supernatants and increased B lymphocyte response and survival following experimental challenges with *Aeromonas salmonicida* and *Vibrio anguillarum* indicating the immunostimulatory effects of dietary n-3 in these species. However, Bell et al. (1995a) have found that juvenile turbot fed diet deficient in n-6 PUFA had increased mortality and developed an extensive pathology in gill epithelium compared to those fed fish oil. In vitro, both enhancement and supression of T and B cell responses occur depending upon the concentrations and type of fat presented to the cells in culture (Johnston and Marshall, 1984). Changes in dietary fats can alter composition of lymphocytes (Kinsella et al., 1990).

ARA has been found to be preferentially retained in various species together with DHA during starvation, suggesting a metabolic priority for its conservation (Izquierdo, 1996); its importance in improving growth, survival, reproduction, stress resistance and regulation of immune function has been

studied (Izquierdo, 1996; Bessonart et al., 1999; Koven et al., 2001, Bell et al., 2002; Fountoulakis et al., 2003). Furthermore, ARA incorporation into leukocytes was even more marked (Farndale et al., 1999), denoting the importance of this fatty acid in this all either as a precursor of eicosanoids or intracellular messenger.

It is now well established that fatty acids released from membrane phospholipids play important roles in cellular signaling. This has been demonstrated from multiple cell types and especially in cells participating in an active immune response. Recent studies in various cell types suggest that fatty acids, especially unsaturated fatty acids, can modulate the activities of phospholipases in salmon (Bell et al., 1996) and ion channels and other biochemical mechanisms are involved in the coupling of stimuli to mammal cell responses (Massferrer, Rios and Schwartzman, 1990).

3-2 Dietary fatty acids and eicosanoids production

To date, there is accumulating evidence that alterations in the phospholipid composition led to changes in eicosanoid precursors, as was reflected by variations in amounts of derived eicosanoids (Bell, Sargent and Raynard, 1992). Therefore, by altering the dietary lipid content it is possible to alter the type and level of eicosanoids generated, which can have profound effects on fish health (Balfry and Higgs, 2001). Considering the fact that fish tissues are naturally abundant in n-3 PUFA (Henderson and Tocher, 1987), this offer a useful model system for studying the production and interaction of eicosanoids derived from both ARA and EPA (Bell et al., 1992).

Numerous studies have demonstrated the effect of dietary fats on the fatty acid composition of tissues and cells and the subsequent effects on eicosanoid production. For example, Bell et al. (1993) have found that Atlantic salmon postsmolts fed a diet which contain high linolenic acid content leads to decreased levels of ARA-derived PGE_2 and TXB_2, which resulted in incresed anti-inflammatory activity and marked reduction in the severity of cardiac lesions. Further, it was demonstrated that lipid composition, can be modified by changes

in dietary lipid composition and increased dietary ARA content resulted in significant increases in the production of PGE_2 and 6-ketoPGF$_{1\alpha}$ in tissue homogenates from juvenile turbot (Bell et al., 1995). They concluded that diet containing high levels of n-6 PUFAs will enhanced the immune response due to high levels of proinflammatory ARA-derived eicosanoids (Bell et al., 1994; 1995). The changes in phospholipid ARA and EPA could alter the spectrum and amount of eicosanoids produced (Bell et al., 1995). It was demonstrated also that DHA released after stimulation of gill cells from Atlantic salmon with A23187 was a substrate for 12-lipoxygenase (Bell et al., 1992). Analyses of turbot phospholipids have established that ARA is especially abundant in the PI fraction (Bell et al., 1995) which suggest that this phospholipids is the most provider of polyunsaturated fatty acids to cyclooxygenase and lipoxygenase enzymes.

The high dietary linolenic acid content also reduced the production of ARA-derived PGE_2 and TXb_2 (a stable metabolite of TXA_2). These responses resulted in increased anti-inflammatory activity and an attendant reduction in the severity of cardiac lesions in salmon (Bell et al., 1993). High n-6 PUFAs diet content is known to produce relatively higher levels of the proinflammatory 2-series PGs and 4-series Lts and LXs derived from ARA; by contrast, high n-3PUFAs diet content produce relatively higher levels of anti-inflamatory 3-series PGs and 5-series LTs and Lxs derived from EPA (Ashton et al., 1994). Also, feeding fish with a differents n-3/n-6 fatty acids ratio showed to affect profoundly the eicosanoids production (Bell et al., 1996a). Increasing dietary ARA level lead to significant increase in PGE_2 concentration in heart, brain and kidney in turbot (Bell et al., 1995), but high production of these homologues by leucocytes may be responsible for the severity of lesion development (Bell et al., 1993). Further, dietary lipid is found to profoundly influence production of LTB_4 and LTB_5 by salmon kidney macrophage-enriched preparations (Bell et al., 1996a).

4- Leptin and immune system

Leptin was first described as a hormone-like cytokine involved in the control of food intake. It is proposed to act as a 'fat-o-stat', being synthesized by fat cells to limit the intake of food and promote the breakdown of fat (Ahima and Flier, 2000; 0'Neill, 2001). It 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). Primary physiological function of leptin is to coordinate metabolic, endocrine and behavioral reponses to changing energy status (De Fanti, 2002). 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). Recently, Volkoff et al. (2003) demonstrated that administration of high doses of exogenous leptin reduce food intake in goldfish, they concluded that leptin plays an important role in the regulation of feeding and homeostasis in this specie. In humans, starvation or malnutrition is accompanied with decreased levels of leptin which results in immunosuppression (O'Neill, 2001).

The effect of leptin in immune system is still unclear, a recent reports indicate that leptin might be important in autoimmunity in mammals (Matarese et al., 2001, 2002). There are a similarities between the leptin receptor and hematopoietic cytokine receptors suggesting role of adipose tissue in immune function (Matarese, 2000). Other studies reported that leptin is required for the induction and propagation of autoimmune-mediated demyelination (O'Neill, 2001). Leptin may stimulates the secretion of IL-6, IL-18 and tumor necrosis factor *in vitro* and *in vivo* in mice (Fig.4)(Faggioni et al., 2000).

Exogenous leptin have been found to up-regulate macrophage phagocytosis and the production of proinflammatory cytokines (Loffreda et al., 1998), as well as enhance T-helper-cell responses (Lord et al., 1998).

Brunetti et al. (1999) have demonstrated that leptin stimulates production of prostaglandin E_2 and $F_{2\alpha}$ production in rat, and they suggested that these

prostaglandins may have a possible role as a mediators of leptin effects in the hypothalamus. They suggested that increased production of leptin could be involved in the signaling events that follow leptin binding to its receptor. Besides, Spanswick and colleagues (1997) demonstrated that leptin may activate ATP-sensitive potassium channels.

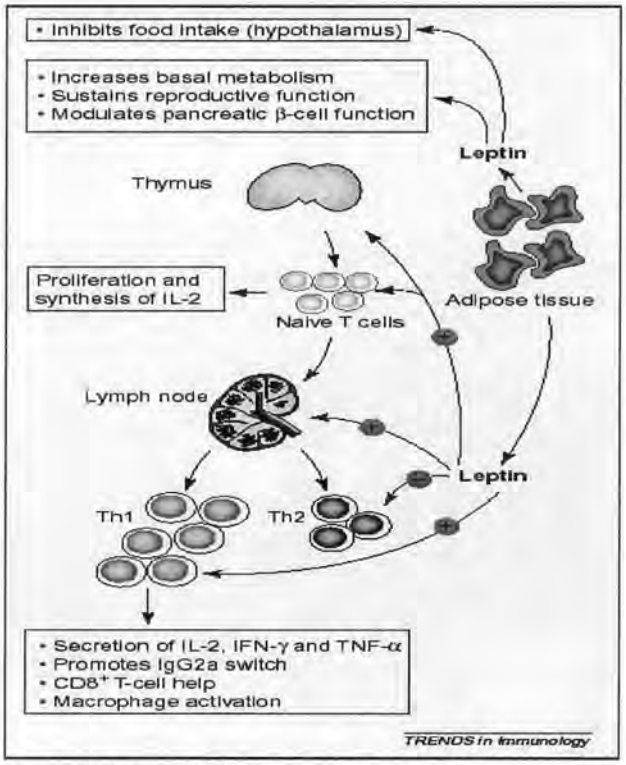

Fig. 4: Summary of effects of Leptin on Hypothalamus and immune system (Matarese et al., 2002)

Thus, modifying dietary fats in fish nutrition may have direct or indirect implications in fish health, despite the mechanisms are not yet clearly understood. When replacing marine fish oil with alternative lipid sources (vegetables oils), care should be taken to establish that the dietary essential fatty acids not only meets the requirements optimum growth and high utilization but also maintain proper immune function to prevent infectious diseases. Previous experiences on seabream have shown that it is possible to reduce a 60 % of the fish oil in diets for seabream without compromising growth survival, fillet organoleptic properties or fish feed utilisation, when fish are fed either for a medium (3 months, Izquierdo et al., 2000; Izquierdo et al., 2003) or for a long feeding period (Menoyo et al., in press; Izquierdo et al., submitted). However,

increase of substitution levels up to 80 %, significantly reduced growth and conversion indexes (Izquierdo et al., submitted). Nevertheless, significant alterations in hepatocyte and enterocyte morphology (Caballero et al., 2003), are observed together with a negative effect in immune parameters and poststress plasma cortisol response (Montero et al., 2003). Besides, reduction in the EPA and DHA content in diets for seabream significantly inhibited the plasma alternative complement activity (Montero et al., 1998) and 60% inclusion of soybean or rapeseed oil also reduced macrophage phagocytic activity (Montero et al., 2003). Despite the action mechanism of dietary HUFA on immune suppression seem to be mediated, at least partly, on the eicosanoid production in target tissues. The effect of dietary vegetables on eicosanoids production (in different fish organs) is well documented in freshwater and some Nordic marine fish species (Bell et al., 1991, 1992, 1993, 1994, Henderson et al., 1996), however, no studies have been conducted until now on the eicosanoid contents in sea bream or their alteration by dietary lipids.

5- Objectives

The aim of the present study was to investigate the effect of different levels substitution of fish oil with vegetables oils (rich in LA and LNA) on sea bream plasma and leukocytes fatty acids composition and it's effect on eicosnaoids and leptin production. To achieve this goal the following objectives were addressed:

1- To determine the effects of inclusion of vegetable oils on fish growth.
2- To analyse of the composition of the different diets on the fatty acid profile of blood plasma.
3- To develop a reliable technique to isolate seabream blood leukocytes.
4- To determine the fatty acid composition of seabream leukocytes fed different diets.
5- To measure the basal levels of prostaglanins E_2 and E3 in blood plasma.
6- To measure the basal levels of leptin in blood plasma.

MATERIALS & METHODS

1- Animals and diets

Two thousand four hundred juveniles sea bream obtained from a local fish farm (ADSA, Las Palmas, Spain) of initial weight 24g were maintained at Instituto Canario de Ciencias Marinas (ICCM). Fish were distrubuted randomly into 16 × 1000 L polyethylene circular tanks (150 fish / tank, each diet assyed 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. 88-22.41°C 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 the 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 diet 1 were used for sea bream (Table 1), 15:60:25 by volume in diet 2 and 40:40:20 by volume in diet 3, of rapeseed, linseed and palm oils, respectively. A 4th diet with 100% substitution of fish oil by the blend in diet 2 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 4 containing the lower levels of these fatty acids. Whereas, diet 2 and 3 (with 60 % of substitution) had medium levels of EPA, DHA and ARA, but diet 3 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 2. Diet 4 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 formulae:

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 1 : The types and % of oils in the experiemental diets

	FO	60 L	60 R	100 L
Fish oil	100	40	40	-
Rapeseed oil	-	10	24	17
Linseed oil	-	35	24	58
Palm oil	-	15	12	25

Table 2: Main fatty acids of the different experimental diets II (g/100 g fatty acid) (5mm) (Method Acid)

	Diet 1	Diet 2	Diet 3	Diet 4
% 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- 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 seringues from 6 fish per each tank (18 fish per diet) and transferred to an eppendorf tube coated lithium heparin as an anticoagulant. The blood was centrifuged immediately at 3000 rpm for 10 min 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) 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.

3- Blood leucocytes isolation

This experiment was done at the end of the second trial II only for three diet since fish fed 100 L were dead. A modification of the method developed by Izquierdo & Lall, 2002 for halibut leukocytes isolation was adapted to seabream. Seven ml of blood were collected in heparisined syringues from the caudal vein of 9 fish per diet and transferred to clean glass tube kept in ice. The blood was centrifuged at 500 × g for 10 min at 4 °C. Cells were diluted in 10 ml of HBSS, Ca-Mg free and centrifuged at 500 × g for 10 min at 4 °C. Cells were separated in to 2 samples, each sample was diluted in 6 ml of HBSS, Ca-Mg free; and layered carefully onto 6 ml of 46% Percoll and centrifuge at 450 × g for 40 min at 4 °C. The cells were collected from the white layer (intermediate) and diluted in 10 ml of HBSS, Ca-Mg free and centrifuged at 500 × g for 10 min at 4 °C. Cells were diluted in 6 ml HBSS, Ca-Mg free and layered carefully onto 6 ml of 46 % Percoll and centrifuged at 450 × g for 40 min at 4 °C. Cells were collected and diluted in 10 of HBSS, Ca-Mg free; and centrifuged at 500 × g for 10 min at 4 °C. Cells were diluted in 6 ml of HBSS, Ca-Mg free and layered carefully onto 6 ml 46 % and centrifuged at 450 × g for 40 min at 4 °C. The leucocytes (white intermediate layer) cells were collected and washed with 10 ml of HBSS, Ca-Mg free. The leucocytes obtained from 3 fish were pooled and were suspended in 4

ml of HBSS, 2 ml of chloroform were added and the sample is stored at – 80 °C prior to lipids extraction.

4- Lipids extraction and analysis

Extraction of total lipid from plasma samples, diets and leucocytes 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).

Individual phospholipid classes from leucocytes total lipids were isolated according to Bell et al. (1997) by loading 3-4 mg of total lipid onto a 2 cm origin of a 20X 20 cm* TLC plate and eluting with methyl acetate/isopropanol/chloroform/methanol/0.25% (w/v> aqueous KC1 (25:25:25: 10:9 by vol.). Individual phospholipids were identified by spraying the plate with 0.1% 2',7'-dichlorofluorescein in 97% methanol containing 0.05% BHT and the lipid classes visualised under UV light. Lipid classes were scraped from the silica and acid-catalysed transmethylation performed overnight at 50°C as described by Christie (1982).

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.

5- Extraction, separation and enzyme immunoassay of PGE isomers

5-1 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 °C) before analyzing by immunoassay.

5-2 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 196nm 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. Mesurement 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.

5-3 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 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 PGE2 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 the PGE2 tracer that is able to bind to the 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 contain 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: Diltuting 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 pastic film and incubated for 18 hours at 4°C wich 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.

6- Leptin immunoassay

6-1 -Principle of the assay

This assay uses a cuantitative sandwich enzyme immunoassay technique. A monoclonic antibody for leptine 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 ino 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.

6-2 Reagents

- Leptine microplates: 96- well. Mircoplate de polystyrene (12 columns of 8 well) with monoclínic antibody leptins.
- Leptin conjugate (Part 890574): 21 ml de antibody monoclonic against Leptin of rate with preservatif.
- Leptin estandard (Part 890575): 10 ng de recombinant leptin human in buefferd proteins with preservatif.
- Análisis diluente RD1-19 (Part 895467): 11ml de buffered protein base with preservatif.
- Calibrator diluente RD5P (5x) Concentrate (Part 895151): 21ml de solución 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.

6-3 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).

6-4 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 towels 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.

7- 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 a SPSS software (SPSS for windows 11.0).

RESULTS

1- Growth

All experimental diets were very well accepted by seabream, average feed intakes being only slightly, but not significantly, higher for fish fed 100 L diet at the end of the last part of feeding period (Fig. 5). Feed intakes were low at the beginning of the experiment (0.7-0.8 % bodyweight/fish/day) but increased over 1.2 % body weight/fish/day after the first 4 weeks of feeding. Specific growth rates ranged from 0.8 to 0.4 along the experiment (Fig. 6) being not significantly different for fish fed the different diets. Body weight was significantly lower in fish fed 100 L from intermediate sampling (Fig. 7), but those differences did not increased along the rest of the experiment. Average conversion indexes ranged from 1.3 at the beginning of the experiment to over 3 at the end, showing no significant differences among treatments (Table 3). No differences were neither found in eviscerated weight among fish fed the different experimental diets (Table 3). Liver weights or hepatosomatic indexes (HIS) were also similar among different fish (Table 3), although HSI was slightly higher in fish fed 100 L.

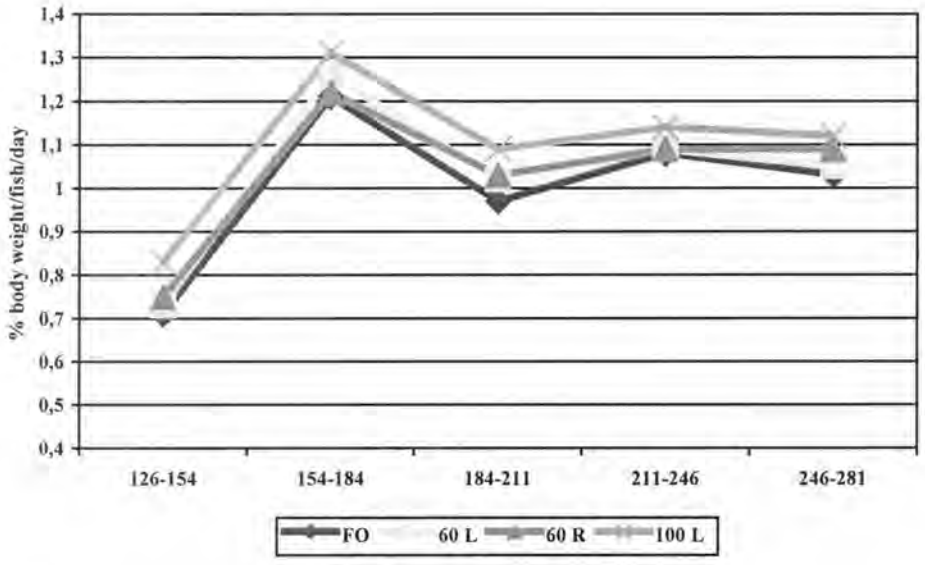

Fig. 5 : Feed intake in seabream along the last part of feeding period (5mm pellet)

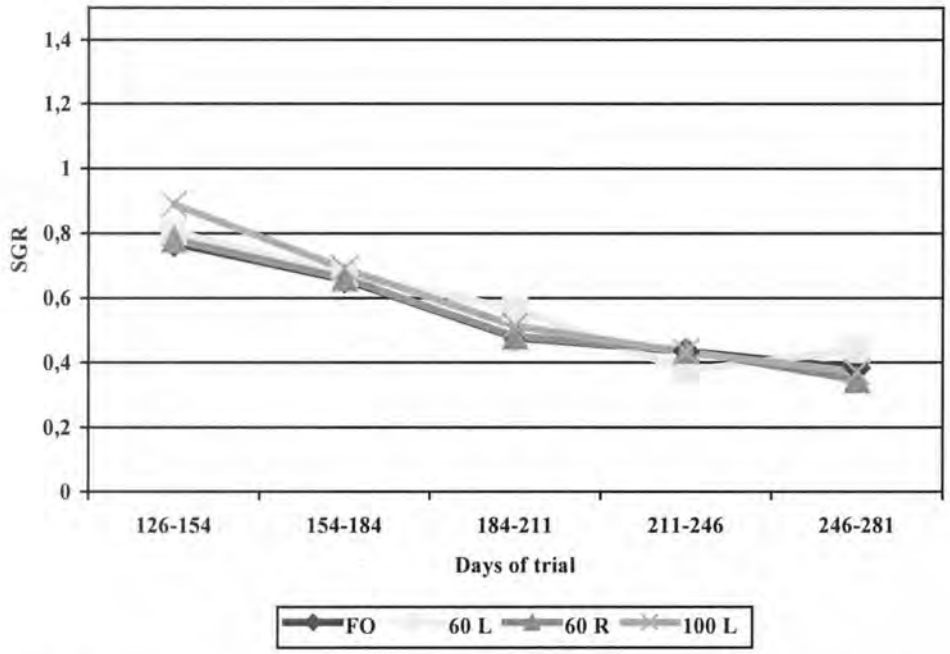

Fig. 6: Specific growth rates for seabream along the last part of the feeding period

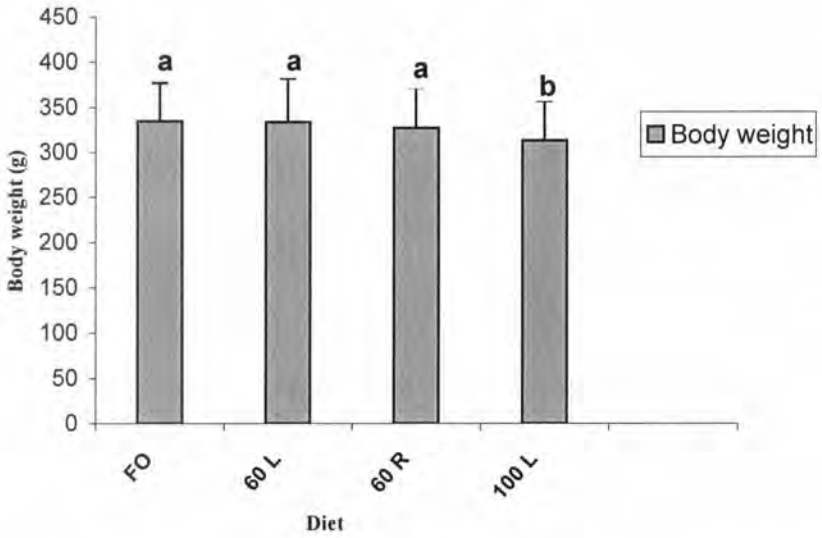

Fig. 7: Effect of experimental diets on fish body weight. Values are means ± SD from all fish in each of the three tank replicates. Bars with different superscript letters are significantly different ($P<0.05$)

Table 3: Liver weight. hepatosomatic index (HSI) and eviscerated weight of gilthead sea bream growth at intermediate (98 d) and final sampling (281d).

	FO	60 L	60 R	100 L
Eviscerated weight (%)				
98 d	92.29 ± 1.36	92.11 ± 1.26	91.94 ± 1.04	92.07 ± 0.89
281 d	94.24 ± 7.65	91.87 ± 4.40	93.20 ± 1.85	93.12 ± 1.97
Liver weight (g)				
98 d	1.16 ± 0.27	1.42 ± 0.31	1.34 ± 0.15	1.15 ± 0.23
281 d	3.59 ± 1.14	3.37 ± 0.55	3.46 ± 0.87	3.52 ± 0.92
HSI				
98 d	1.28 ± 0.26	1.47 ± 0.26	1.36 ± 0.16	1.33 ± 0.19
281 d	1.08 ± 0.30	1.02 ± 0.13	1.05 ± 0.18	1.11 ± 0.19

Values are means ± SD from three lots of 10 fish per tank. Values in the same row with different superscript letters are significantly different.

2- Fatty acids composition of plasma

The fatty acid composition of plasma total, polar and neutral lipids at the end of the second trial are summarized in Tables 4-6. The fatty acids composition of plasma total lipids are shown in Table 4. Regardless of diet, DHA was always the most abundant fatty acid in plasma total lipids, followed by 16:0 and 18:1n-9 (Table 4). Saturated fatty acids were similar among fish fed the four experimental treatments despite differences in diet, and the lowest content in 60 L fed fish. Total monoenoic fatty acids were significantly lower in fish fed FO compared to those fed 60 R, 60 L and 100 L, but in proportions much lower than in diets. Fish fed 60 R, 60 L and 100 L showed significantly higher levels of 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 compared to those fed fish oil. 18:3n-3 and 20:3n-3 were also significantly increased in fish fed vegetable oils compared to fish fed FO diet. DHA was significantly greater in FO-fed fish compared to the other three treatments, while total n-3 were significantly decreased with increasing dietary LA and GLA, with the highest value in fish fed FO diet followed by fish fed 60 R, 60 L and the lowest level in fish fed 100 L. N-3 HUFA were decreased but not significantly with increasing dietary LA and GLA, while n-6 were significantly increased with the highest level in fish fed 100 L, followed by those fed 60 R, 60 L and fish oil. EPA, DHA, ARA and DHGLA composition in plasma total lipids

are presented in figures 8 and 9. DHA was significantly reduced in fish fed vegetable oils, but were still higher than in diet. EPA levels were significantly different in each dietary treatment and proportional to dietary contents with the highest value in fish fed FO followed by 60 R, 60 L and, lastly, 100 L fed fish. ARA was significantly decreased in fish fed vegetable oils compared to those fed FO, while DHGLA significantly increased in fish fed vegetable oils with the highest content in fish fed 100 L. The n-3/n-6 PUFA ratio in fish fed FO was significantly higher than in fish fed 60 L, 60 R, and fish fed 100 L presented the lowest value.

The fatty acid composition of polar lipids from plasma broadly reflected those of total lipids and are shown in table 5. DHA was the most abundant fatty acid in fish fed FO, 60 R and 60 L, followed by 16:0 and 18:1n-9. However in fish fed 100 L, 18:1n-9 and 16:0 were the most abundant fatty acids in plasma followed by DHA. Total saturated fatty acids were lower in fish fed 60 L but not significantly different. Total monoenoic fatty acids were also lower in FO fed fish comparing to the other treatments but not significantly. 18:2n-6, 20:2n-6, 20:3n-6 and total n-6 were all increased with increasing dietary LA; 18:3n-3, was also significantly increased with increasing it's input in the diet. The elongase product of 18:3n-3, 20:3n-3, was also higher in fish fed 60 R, 60 L and 100 L diets than in control diet fed fish, despite being lowest in the diet. 20:4n-3 was also higher, but not significantly, in fish fed vegetable oil. The EPA, DHA, DHGLA, ARA, ARA/EPA and ARA/DHGLA in plasma polar lipids are shown in figures 10-13. DHGLA was significantly different for each dietary treatment with the highest value in fish fed diet with the highest level of LA (100 L), followed by diet 60 R and 60 L, and the lowest in FO fed fish. ARA was significantly decreased in fish fed 60 L, 60 R and 100 L compared with FO fed fish. EPA content showed a significant reduction in fish fed 60 L, 60 R and, lastly, 100 L compared with fish fed FO. ARA/EPA, ratio of eicosanoid precursors was relatively similar in the four treatments and around 0.14 value. ARA/DHGLA was significantly greater in fish fed FO (10.56) and decreased with increasing dietary LA and GLA, with the lowest value in fish fed 100 L

(2.21). DHA and total n-3 PUFA all decreased, but not significantly, with increasing dietary LA and GLA. Consequently, the n-3/n-6 PUFA ratio significantly decreased.

The fatty acids composition of neutral lipids is shown in Table 6. The fatty acids composition of neutral lipids was different comparing with the polar lipids, since this lipid class was more rich in LA and GLA. LA was significantly increased in fish fed vegetable oils with the highest value in fish fed diet 60 L, followed by 60 R and 100 L and, lastly, the FO fed fish. 18:3n-3 knew also an increase in fish fed vegetable oils. 20:2n-6 was higher, but not significantly, in fish fed 60 L, 60 R and 100 L compared with fish fed FO. No significant differences were observed in 20:3n-6, 20:4n-6, 20:3n-3 and 20:4n-3 concentrations among the four treatments. However, EPA and DHA were significantly higher in fish fed FO. EPA/DHA was decreased with feeding vegetable oils. By contrast AA/EPA was slightly but not significantly increased.

The study of the relations between some plasma fatty acid composition and dietary fatty acid contents are represented in table 7. Oleic acid was specially incorporated to plasma neutral lipid compared with polar lipid. The ratio of incorporation decreased with increasing dietary content in this fatty acid, being the highest incorporation ratio in fish fed fish oil (1.41) and the lowest in fish fed 100 L (0.75). The ratio of incorporation of palmitic acid was not different among the treatments, being around 1 and 1.46, with a slightly higher incorporation into polar than into neutral lipids. By contrast, arachidonic acid (ARA) was highly incorporated to polar lipid with high ratio in plasma phospholipids, superior to 2 and reaching extreme value (around 11) in fish fed 100 L, and low ratio in neutral plasma lipid from fish fed FO (0.79). Approximately, the same results were found for DHA, rising between 1 in plasma neutral lipids from fish fed FO to 11 in plasma polar lipids of fish fed 100 L. This indicate the role of these two HUFA in fish phospholipids. EPA plasma / diet was lower than that for ARA and DHA, but this fatty acid was more incorporated into polar than into neutral lipids being gradually higher as its level was reduced in the diets.

Table 4: Plasma total lipids composition of fatty acids (% of total fatty acids)

Fatty acids	FO	60 L	60 R	100 L
12:0	0.05	0.10 ± 0.04	0.86 ± 0.69	1.31 ± 1.20
14:0	1.60 ± 0.14	1.29 ± 0.26	1.03 ± 0.07	0.91 ± 0.44
15:0	0.26 ± 0.04	0.16 ± 0.05	0.13 ± 0.01	0.13 ± 0.07
16:0	21.47 ± 1.24	20.50 ± 4.90	19.06 ± 1.92	20.35 ± 1.98
16:1n-7	2.85 ± 0.15	2.21 ± 0.01	1.19 ± 0.15	0.83 ± 0.09
16:1n-5	0.11± 0.00	0.08 ± 0.00	0.06 ± 0.02	0.04 ± 0.01
16:2n-3	0.26 ± 0.02	0.15 ± 0.01	0.11 ± 0.01	0.04 ± 0.04
16:2n-4	1.25 ± 0.11	0.81 ± 0.01	0.62 ± 0.04	0.30 ± 0.11
17:0	0.32 ± 0.04	0.21 ± 0.03	0.16 ± 0.01	0.07 ± 0.05
17:1	0.30 ± 0.03	0.05 ± 0.03	0.16 0.01	0.12 ± 0.06
16:4n-3	0.14 ± 0.00	0.06 ± 0.04	0.04 ± 0.01	0.03 ± 0.00
16:4n-1	0.10 ± 0.04	0.04	0.05	0.04 ± 0.04
18:0	4.83 ± 0.07	5.86 ± 2.20	5.91 ± 0.45	6.90 ± 1.21
18:1n-9 + 18:1n-7	11.47 ± 0.07	19.53 ± 2.40	20.77 ± 1.08	20.90 ± 0.72
18:1n-5	0.13 ± 0.00	0.67 ± 0.74	0.12 ± 0.01	0.10 ± 0.03
18:2n-9	0.14 ± 0.01	0.14 ± 0.01	0.15 ± 0.02	0.14 ± 0.01
18:2n-6	3.10 ± 0.16^{c}	7.89 ± 1.37^{b}	9.56 ± 1.48^{b}	13.36 ± 1.44^{a}
18:3n-9	0.12 ± 0.00	0.06 ± 0.03	0.04 ± 0.01	0.06 ± 0.01
18:3n-6	0.11 ± 0.00	0.09 ± 0.03	0.06 ± 0.00	0.12 ± 0.01
18:4n-6	0.09 ± 0.00	0.08 ± 0.04	0.12 ± 0.03	0.03 ± 0.01
18:3n-3	0.62 ± 0.05^{c}	5.90 ± 0.71^{ab}	4.27 ± 0.44^{b}	7.27 ± 2.28^{a}
18:4n-3	0.41 ± 0.08	0.24 ± 0.09	0.19 ± 0.00	0.41 ± 0.04
18:4n-1	0.06 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.02
20:0	0.08 ± 0.00	0.10 ± 0.04	0.15 ± 0.05	0.16 ± 0.07
20:1n-9	0.80 ± 0.10	1.03 ± 0.22	1.00 ± 0.10	0.92 ± 0.19
20:1n-7	0.09	0.07 ± 0.00	0.08 ± 0.02	0.05 ± 0.02
20:1n-5	-	-	-	0.07 ± 0.06
20:2n-9	0.07 ± 0.00	0.04 ± 0.01	0.13 ± 0.02	0.07 ± 0.05
20:2n-6	0.16 ± 0.03^{b}	0.29 ± 0.08^{ab}	0.22 ± 0.14^{ab}	0.44 ± 0.16^{a}
20:3n-9	0.03 ± 0.01	0.02 ± 0.00	0.36 ± 0.09	0.10 ± 0.02
20:3n-6	0.10 ± 0.03^{b}	0.15 ± 0.02^{b}	0.23 ± 0.04^{ab}	0.40 ± 0.17^{a}
20:4n-6	1.33 ± 0.11^{a}	0.84 ± 0.15^{b}	0.81 ± 0.02^{b}	0.70 ± 0.03^{b}
20:3n-3	0.10 ± 0.03^{b}	0.39 ± 0.08^{a}	0.32 ± 0.05^{a}	0.12 ± 0.00^{b}
20:4n-3	0.69 ± 0.03	0.46 ± 0.14	0.45 ± 0.07	0.56 ± 0.16
20:5n-3	10.39 ± 0.73^{a}	6.20 ± 2.15^{b}	6.07 ± 0.31^{b}	3.56 ± 0.62^{c}
22:0	0.07 ± 0.01	0.10 ± 0.05	0.09 ± 0.06	0.09 ± 0.02
22:1n-11	0.52 ± 0.09	0.54 ± 0.07	0.43 ± 0.01	0.41 ± 0.05
22:1n-9	0.21 ± 0.03	0.26 ± 0.03	0.27 ± 0.02	0.25 ± 0.02
22:1n-7	0.09 ± 0.06	0.07 ± 0.05	0.04 ± 0.02	0.02 ± 0.01
22:4n-6	1.99 ± 0.16	0.04 ± 0.01	0.05 ± 0.03	0.05 ± 0.03
22:5n-6	0.41 0.01	0.28 ± 0.05	0.31 ± 0.02	0.18 ± 0.02
22:4n-3	0.05 ± 0.01	0.04 ± 0.00	0.06 ± 0.03	0.04 ± 0.00
22:5n-3	2.53 ± 0.10	1.72 ± 0.57	1.90 ± 0.07	1.20 ± 0.10
22:6n-3	30.51 ± 0.11^{a}	21.26 ± 8.51^{b}	22.46 ± 1.36^{ab}	17.15 ± 2.03^{b}
Total saturates	28.57 ± 1.40	28.31 ± 7.57	27.49 ± 1.30	29.97 ± 4.79
Total monoenes	15.36 ± 0.34^{b}	24.51 ± 1.96^{a}	24.21 ± 1.20^{a}	23.81 ± 0.72^{a}
n-3	45.44 ± 0.71^{a}	36.42 ± 10.71^{ab}	35.93 ± 1.34^{ab}	30.36 ± 3.78^{b}
n-6	7.29 ± 0.07^{d}	9.66 ± 1.30^{c}	11.39 ± 1.68^{b}	15.46 ± 1.30^{a}
n-9	11.90 ± 0.07^{d}	19.83 ± 2.36^{c}	22.81 ± 1.10^{a}	21.40 ± 0.80^{b}
n-3 HUFA	40.90 ± 0.62	30.07 ± 11.30	31.30 ± 1.60	22.59 ± 1.68
EPA/DHA	0.34 ± 0.03^{a}	0.30 ± 0.02^{a}	0.27^{ab}	0.21 ± 0.06^{b}
ARA/EPA	$0,13^{b}$	0.14 ± 0.03^{b}	0.13 ± 0.01^{b}	0.20 ± 0.04^{a}
DHGLA/ARA	0.08 ± 0.02^{b}	0.19 ± 0.07^{b}	0.28 ± 0.12^{b}	0.57 ± 0.06^{a}

Table 5 : Plasma polar lipids composition on fatty acids (% of total fatty acids):

Fatty acids	FO	60 L	60 R	100 L
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
15:0	0.39 ± 0.09	0.23 ± 0.10	0.30 ± 0.08	0.15 ± 0.04
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
16:1n-5	0.15 ± 0.04	0.13 ± 0.00	0.04 ± 0.02	0.08 ± 0.02
16:2n-3	0.22 ± 0.00	0.17 ± 0.01	0.05 ± 0.03	0.10 ± 0.01
16:2n-4	0.57 ± 0.01	0.60 ± 0.29	0.38 ± 0.02	0.32 ± 0.05
17:0	0.35 ± 0.01	0.20 ± 0.03	0.05 ± 0.03	0.04 ± 0.04
17:1	0.29 ± 0.08	0.08 ± 0.02	0.21 ± 0.04	0.14 ± 0.02
16:4n-3	0.12 ± 0.00	0.09 ± 0.03	0.05 ± 0.03	0.04 ± 0.04
16:4n-1	0.27 ± 0.01	0.26 ± 0.08	0.32 ± 0.02	0.26 ± 0.06
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
18:1n-5	0.14 ± 0.01	0.16 ± 0.06	0.06 ±0.03	0.08 ± 0.07
18:2n-9	0.12 ± 0.01	0.11 ± 0.11	0.05 ± 0.02	0.12 ± 0.03
18:2n-6	6.04 ± 2.64^{b}	8.47 ± 2.78^{ab}	8.07 ± 1.89^{ab}	11.75 ± 1.63^{a}
18:3n-9	0.07 ± 0.01	0.06 ± 0.02	0.01 ± 0.01	0.03 ± 0.03
18:3n-6	0.09 ± 0.00	0.07 ± 0.02	0.05 ± 0.02	0.10 ± 0.03
18:4n-6	0.10 ± 0.02	0.08 ± 0.05	0.03 ± 0.01	0.03 ± 0.02
18:3n-3	0.59 ± 0.23^{c}	3.05 ± 0.95^{ab}	2.19 ± 0.06^{bc}	4.98 ± 1.54^{a}
18:4n-3	0.10 ± 0.00	0.11 ± 0.08	0.04 ± 0.05	0.06 ± 0.06
18:4n-1	0.14 ± 0.00	0.04 ± 0.06	0.07 ± 0.11	0.12 ± 0.02
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
20:1n-7	0.08 ± 0.00	0.08 ± 0.01	0.06 ± 0.01	0.08 ± 0.03
20:1n-5	-	0.12 ± 0.01	-	-
20:2n-9	0.02 ± 0.00	0.06 ± 0.05	0.04 ± 0.06	0.10 ± 0.03
20:2n-6	0.16 ± 0.01	0.32 ± 0.01	0.36 ± 0.06	0.46 ± 0.09
20:3n-9	0.01 ± 0.01	0.01 ± 0.01	-	0.02 ± 0.01
20:3n-6	0.12 ± 0.02^{b}	0.26 ± 0.07^{ab}	0.31 ± 0.12^{a}	0.31 ± 0.06^{a}
20:4n-6	1.28 ± 0.15^{a}	1.02 ± 0.15^{ab}	1.05 ± 0.32^{ab}	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^{ab}	6.87 ± 0.82^{b}	4.52 ± 1.04^{b}
22:0	0.17 ± 0.02	0.13 ± 0.02	0.17 ± 0.08	0.11 ± 0.00
22:1n-11	0.32 ± 0.04	0.29 ± 0.14	0.31 ± 0.01	0.39 ± 0.20
22:1n-9	0.13 ± 0.00	0.15 ± 0.06	0.06 ± 0.08	0.17 ± 0.04
22:1n-7	0.15 ± 0.05	0.09 ± 0.07	0.03 ± 0.04	0.04 ± 0.04
22:4n-6	0.20 ± 0.01	0.12 ± 0.06	0.05 ± 0.07	0.07 ± 0.02
22:5n-6	0.47 ± 0.02	0.64 ± 0.28	0.51 ± 0.30	0.29 ± 0.04
22:4n-3	0.11 ± 0.02	0.09 ± 0.03	0.02 ± 0.03	0.04 ± 0.03
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	34.11 ± 0.06	28.56 ± 5.07	33.62 ± 3.37	31.92 ± 2.06
Total monoenes	15.24 ± 2.09	19.22 ± 5.93	17.12 ± 1.19	18.58 ± 0.76
n-3	41.01 ± 4.47	40.09 ± 3.47	37.97 ± 6.08	34.87 ± 1.21
n-6	8.44 ± 2.41	10.98 ± 2.23	10.42 ± 1.26	13.67 ± 1.75
n-9	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^{ab}	3.64 ± 1.03^{ab}	2.58 ± 0.40^{b}
EPA/DHA	0.31 ± 0.01	0.29 ± 0.01	0.26 ± 0.02	0.20 ± 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}

All Values are means ± SD from three fish. Values in the same row with different superscript letters are significantly different.

Table 6 : Plasma neutral lipids composition on fatty acids (% of total fatty acids)

Fatty acids	FO	60 L	60 R	100 L
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
15:0	0.58 ± 0.10	0.41 ± 0.25	0.32 ± 0.09	0.22 ± 0.05
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
16:1n-5	0.21 ± 0.05	0.18 ± 0.07	0.14 ± 0.03	0.12 ± 0.03
16:2n-3	0.17 ± 0.02	0.20 ± 0.04	0.16 ± 0.03	0.16 ± 0.05
16:2n-4	0.87 ± 0.73	1.03 ± 0.05	0.63 ± 0.42	0.74 ± 0.24
17:0	0.35 ± 0.01	0.23 ± 0.07	0.18 ± 0.02	0.16 ± 0.05
17:1	0.68 ± 0.05	0.46 ± 0.17	0.33 ± 0.09	0.26 ± 0.10
16:4n-3	0.26 ± 0.04	0.13 ± 0.00	0.13 ± 0.02	0.70 ± 0.08
16:4n-1	0.81 ± 0.11	0.53 ± 0.09	0.70 ± 0.13	0.73 ± 0.00
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
18:1n-5	0.29 ± 0.10	0.26 ± 0.12	0.19 ± 0.05	0.17 ± 0.04
18:2n-9	0.13 ± 0.04	0.17 ± 0.02	0.15 ± 0.03	0.16 ± 0.01
18:2n-6	6.34 ± 2.35^{b}	13.37 ± 3.18^{a}	11.09 ± 3.26^{ab}	10.61 ± 0.52^{ab}
18:3n-9	0.14 ± 0.02	0.12 ± 0.07	0.08 ± 0.03	0.06 ± 0.00
18:3n-6	0.14 ± 0.06	0.07 ± 0.00	0.16 ± 0.03	0.23 ± 0.5
18:4n-6	0.24 ± 0.07	0.17 ± 0.07	0.10 ± 0.03	0.14 ± 0.03
18:3n-3	1.06 ± 0.09^{c}	7.09 ± 0.44^{ab}	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
18:4n-1	0.06 ± 0.03	0.28 ± 0.06	0.38 ± 0.05	0.38 ± 0.02
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
20:1n-7	0.19 ± 0.00	0.12 ± 0.00	0.12 ± 0.01	0.04 ± 0.03
20:1n-5	0.02 ± 0.01	0.02 ± 0.03	0.13 ± 0.09	0.09 ± 0.04
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-9	0.02 ± 0.02	0.14 ± 0.06	0.03 ± 0.00	0.08 ± 0.00
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}
22:0	0.30 ± 0.00	0.19 ± 0.00	0.21 ± 0.04	0.16 ± 0.07
22:1n-11	1.12 ± 0.04	0.89 ± 0.13	0.83 ± 0.04	0.70 ± 0.05
22:1n-9	0.53 ± 0.11	0.50 ± 0.10	0.56 ± 0.08	0.53 ± 0.02
22:1n-7	0.15 ± 0.07	0.14 ± 0.10	0.08 ± 0.03	0.05 ± 0.00
22:4n-6	0.31 ± 0.06	0.14 ± 0.01	0.09 ± 0.07	0.16 ± 0.03
22:5n-6	0.37 ± 0.08	0.21 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
22:4n-3	0.21 ± 0.06	0.14 ± 0.00	0.12 ± 0.07	0.23 ± 0.03
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	38.08 ± 6.91	29.45 ± 0.70	29.68 ± 1.68	29.84 ± 1.42
Total monoenes	25.28 ± 3.47	29.36 ± 0.69	33.22 ± 0.62	29.81 ± 1.96
n-3	26.32 ± 6.13	24.05 ± 1.40	22.52 ± 3.01	24.92 ± 1.54
n-6	8.19 ± 1.90^{b}	14.68 ± 3.05^{a}	12.43 ± 3.14^{ab}	12.93 ± 1.30^{ab}
n-9	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}
EPA/DHA	0.42 ± 0.00	0.31 ± 0.04	0.27 ± 0.02	0.25 ± 0.11
ARA/EPA	0.08	0.09	0.12	0.18

Table 7 : The ration between fatty acid % in plasma lipid classes and dietary content on the same fatty acid (% Fatty acid in plasma / % fatty acid in diet)

Fatty acid in plasma lipid classes		FO	60 L	60 R	100 L
Oleic Acid (OA)	Total	0.98	0.79	0.70	0.63
	Polar	0.89	0.66	0.48	0.48
	Neutral	1.41	0.93	0.91	0.75
Palmitic acid	Total	1.11	1.37	1.19	1.28
	Polar	1.19	1.25	1.46	1.38
	Neutral	1.16	1.13	1.14	1.18
Arachidonic acid (ARA)	Total	2.02	2.47	2.89	11.67
	Polar	1.94	3.00	3.75	10.83
	Neutral	0.79	0.88	1.21	8.17
EPA	Total	0.87	1.02	1.26	3.36
	Polar	0.76	1.23	1.41	4.26
	Neutral	0.52	0.54	0.59	2.53
DHA	Total	2.16	2.89	7.00	8.17
	Polar	2.02	3.57	8.11	10.93
	Neutral	1.05	1.45	3.28	4.83

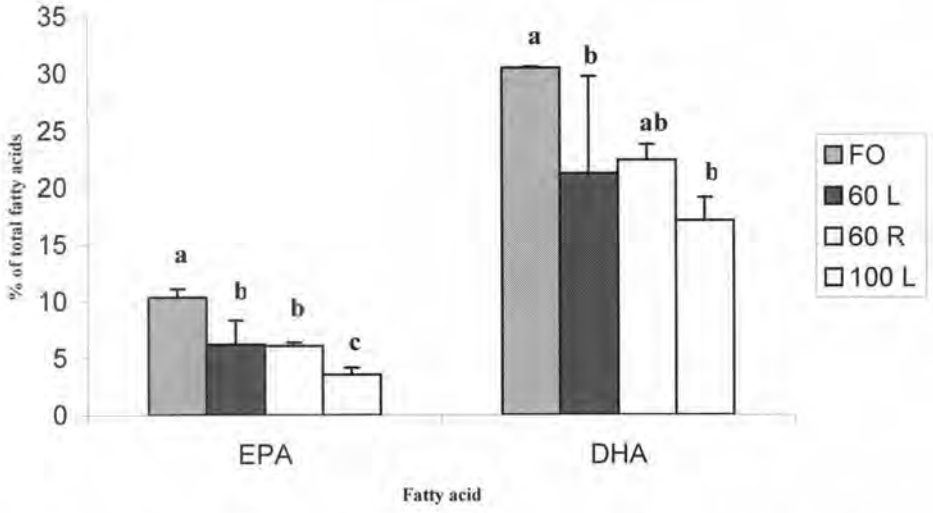

Fig. 8: Levels of EPA and DHA in plasma total lipids from sea bream fed experimental diets. Values are ± SD for 3 pools of fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P< 0.05$).

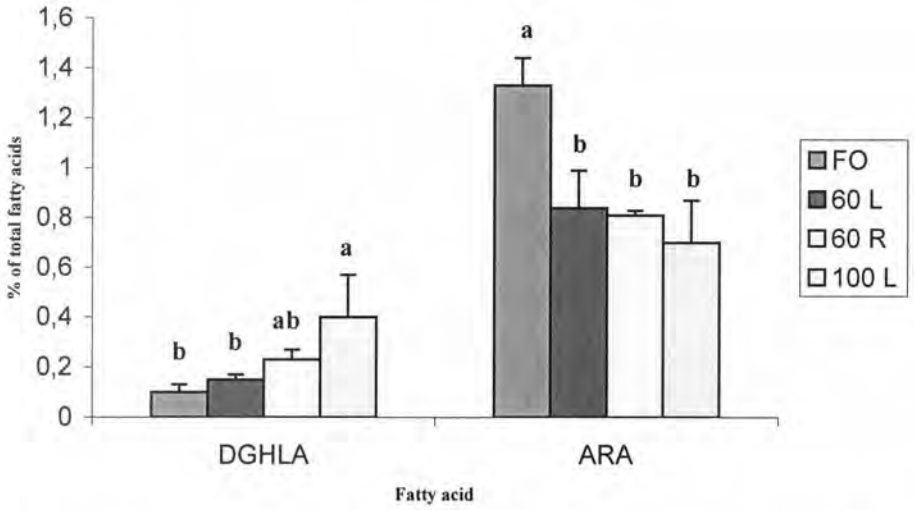

Fig. 9: Levels of DHGLA and ARA in plasma total lipids from sea bream fed experimental diets. Values are ± SD for 3 pools of fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P<$ 0.05).

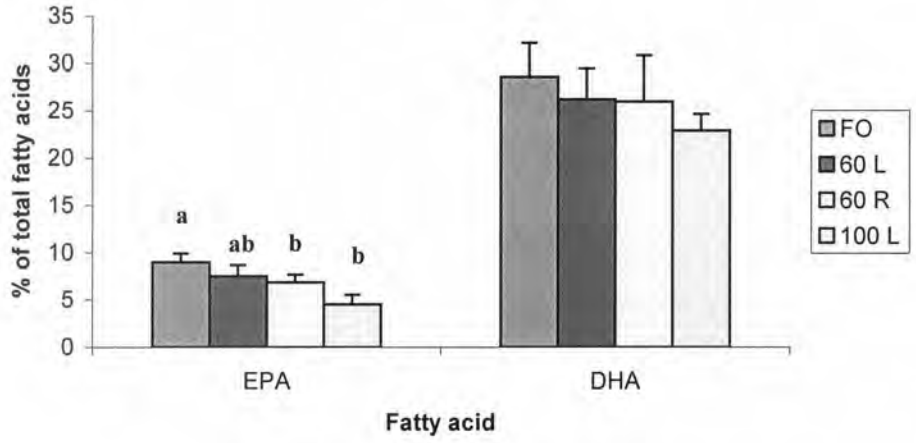

Fig. 10: Levels of EPA and DHA in plasma polar lipids from sea bream fed experimental diets. Values are ± SD for 3 polls of fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P<$ 0.05).

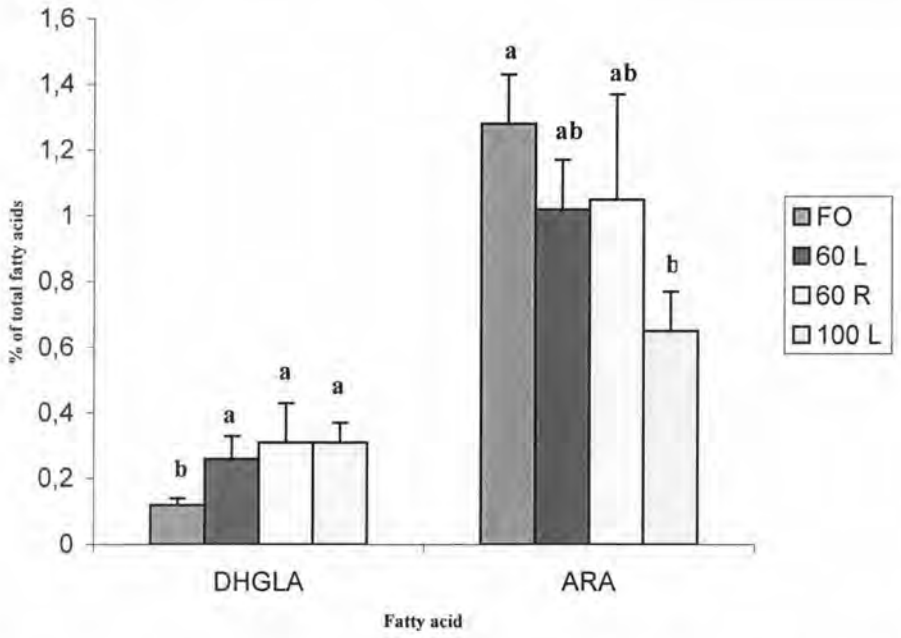

Fig. 11: Levels of DHGLA and ARA in plasma polar lipids from sea bream fed the 4 experimental diets.Values are means ± SD for 3 pools of fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P<0.05$).

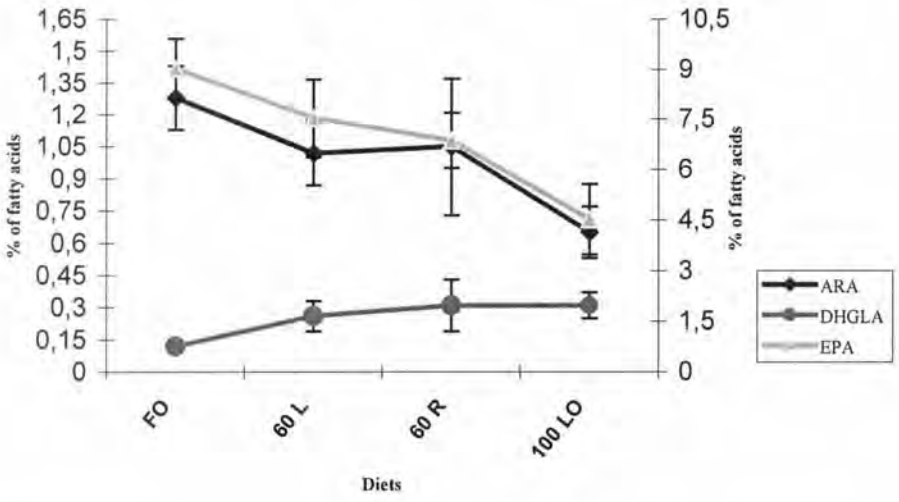

Fig. 12: Levels of eicosanoids precursors: ARA, DHGLA and EPA in plasma polar lipids from sea bream fed experimental diets. Values are means ± SD for 3 pools of fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P<0.05$).

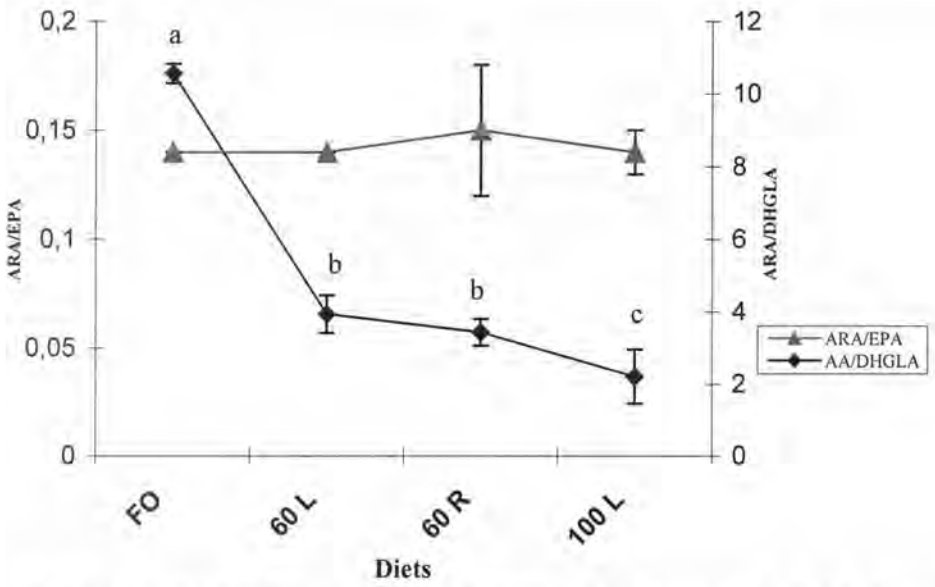

Fig. 13: Levels of ARA/EPA and ARA/DHGLA in plasma polar lipids from sea bream fed experimental diets. Values are means ± SD for 3 pools of fish per treatment. Values for each fatty acid having a different column letter are significantly different ($P<0.05$).

The fatty acid composition of PI from leucocytes is shown in table 8. Oleic acid was the predominant fatty acid in this lipid class and was more higher (up to 3.5 fold) in fish fed 60 L followed by fish fed 60 R (up to 3 fold) than FO-fed fish. PI from leucocytes contained more saturates, monoenes and n-6 compared with total and polar plasma lipids. By contrast, this phospholipid class contained lower levels of n-3 and n-3 HUFA giving a very low n-3/n-6 ratio. Fish fed fish oil incorporated significantly more 12:0, 14:0, 15:0, 17:0 and 20:0. Fish fed fish oil had more DHA, EPA and ARA compared to those fed vegetable oils, as well as 18:3n-6 and 20:3n-9. Oleic acid was markedly higher in PI from leucocytes of fish fed vegetable oils compared to FO-fed fish. AA/EPA was fairly constant among the treatments and higher than in polar and total plasma lipids. EPA/DHA was also higher than in plasma lipid. EPA/DHA contents were lower in comparison with plasma polar lipid ones, and was constant among dietary treatments.

Table 8: Fatty acids composition of leucocytes Phosphatidil Inositlol (PI) of sea bream fed experimental diets

Fatty acid	FO	60 L	60 R
12:0	7.61 ± 0.94	2.69± 0.65	2.26 ± 1.31
14:0	8.18 ± 1.05	2.65 ± 0.56	2.68 ± 0.21
14:1	1.61 ± 0.11	1.80 ± 0.22	1.69 ±0.16
15:0	1.14 ± 0.17	0.28 ± 0.10	0.46 ± 0.08
16:0iso	5.53± 1.35	1.52 ± 0.33	1.70 ± 0.18
16:0	10.28± 2.72^{b}	18.80 ± 0.10^{a}	17.77 ± 1.33^{a}
16: 1n-7	1.66 ± 0.20	3.55 ± 0.08	2.17 ± 1.15
16: 1n-5	1.24 ± 0.20	0.36 ± 0.10	1.28 ± 1.66
16: 2n-3	0.30 ± 0.25	n.d.	0.07 ± 0.12
17:0	1.37 ± 1.02	0.13 ± 0.11	0.07 ± 0.12
16: 2n-4	0.29 ± 0.22	0.15 ± 0.13	0.09 ± 0.16
16: 3n-4	0.39 ± 0.55	0.22 ± 0.20	0.10 ± 0.17
16: 4n-3	n.d.	0.08 ± 0.13	n.d.
16: 4n1	3.73 ± 1.99	0.82 ± 0.17	0.86 ± 0.08
18:0	7.28 ± 0.85	5.01± 0.73	7.03 ± 0.66
18: 1n-9	7.84 ± 2.47^{c}	31.13 ± 1.64^{a}	25.93 ± 1.85^{b}
18: 1n-7	0.52± 0.74	1.96 ± 0.14	1.76 ± 0.18
18: 1n-5	0.19± 0.26	0.16 ± 0.15	0.06 ± 0.10
18: 2n-9	0.23 ± 0.33	0.08 ± 0.14	0.31 ± 0.31
18: 2n-7	0.60	0.87 ± 0.33	0.64 ± 0.38
18: 2n-6	7.34 ± 2.84^{b}	7.09 ± 1.16^{b}	9.36 ± 2.59^{a}
18: 3n-6	6.81 ± 1.74^{a}	2.51± 1.19^{b}	2.45 ± 0.94^{b}
18: 4n-6	n.d.	n.d.	0.06 ± 0.11
18: 3n-1	0.48 ± 0.68	n.d.	0.08±0.13
18: 3n-3	0.74 ± 1.05^{a}	5.44 ± 5.98^{a}	5.95 ± 4.33^{a}
18: 4n-3	1.54± 0.84	0.59 ± 0.27	0.59 ± 0.20
18: 4n-1	0.71± 1.01	0.64 ± 0.40	0.68 ± 0.22
20:0	2.50 ± 2.24	0.47 ± 0.09	0.58 ± 0.06
20:1 n-9 + 20:1n7	1.53 ± 0	1.57 ± 0.09	2.00 ± 0.55
20: 2n-9	0.29 ± 0.41	0.60 ± 0.60	n.d.
20: 2n-6	1.10 ± 1.55	0.30 ± 0.06	0.09± 0.15
20: 3n-9	3.49 ± 1.63^{a}	1.05 ± 0.44^{b}	1.05 ± 0.34^{b}
20: 4n-6	1.15 ± 0.41^{a}	0.36 ± 0.16^{b}	0.71± 0.08^{b}
20: 3n-3	n.d.	0.08 ± 0.13	n.d.
20: 4n-3	n.d.	0.14 ± 0.12	0.06 ± 0.11
20: 5n-3	4.90 ± 1.91^{a}	2.42 ± 0.20^{b}	2.90 ± 0.36^{b}
22:0	n.d.	n.d.	0.07 ± 0.11
22: 1n-11+n-9	1.47 ± 0.42	0.78 ± 0.07	1.39 ± 0.58
22: 1n-9	0.93 ± 1.33	n.d.	0.09 ± 0.16
22: 1n-7	n.d.	0.08 ± 0.14	n.d.
22: 3n-6	n.d.	n.d.	0.05 ± 0.09
22: 5n-3	0.28 ± 0.40	0.45 ± 0.12	0.47 ± 0.02
22: 0	n.d.	n.d.	0.05 ± 0.09
22: 6n-3	4.19 ± 1.77	3.17 ± 0.82	4.40 ± 0.83
Saturados	40.32 ± 8.85	31.55 ± 1.86	32.73 ± 1.19
Monoenoicos	18.22 ± 3.95	41.40 ± 1.95	36.46 ± 1.95
n-3	13.38 ± 2.08	12.37 ± 5.95	14.45 ± 3.53
n-6	17.58 ± 3.85^{a}	10.25 ± 1.88^{b}	12.75 ± 3.77^{b}
n-9	15.28 ± 1.55^{b}	34.43 ± 1.47^{a}	29.45 ± 1.00^{a}
n-3HUFA	10.59 ± 1.17^{a}	6.26 ± 1.20^{b}	7.84 ± 1.16^{b}
AA/EPA	0.24 ± 0.01	0.15 ± 0.07	0.25 ± 0.05
EPA/DHA	1.19 ± 0.85	0.76 ± 0.16	0.67± 0.09
n-3/n-6	0.77± 0.05^{b}	1.32 ± 0.91^{a}	1.24 ± 0.55^{ab}

3- Plasma prostaglandins

The concentrations of PGE_2 and isolated PGE_3 in plasma are shown in Table 9. The concentration of PGE_2 was no significantly different between the four treatments with the lowest value in fish fed FO (33.72 pg/ml) and the highest in fish fed 60 R (44.74 pg/ml) (Figure 14).

However, the concentration of PGE_3 was significantly decreased with reducing EPA input in diet. Fish fed FO had significantly higher levels compared to fish fed 60 L and 60 R. Fish fed 100 L presented significantly the lowest plasma PGE_3 level compared to the other treatments (Figure 15).

Table 9: Concentration of PGE_2 and PGE_3 in plasma from sea bream fed experimental diets

Prostaglandins	FO	60 L	60 R	100 L
PGE_2 (pg/ml)	33.72 ± 6.52	41.87 ± 7.18	44.74 ± 16.47	35.15 ± 4.58
PGE_3 (pg/ml)	49.03 ± 0.49^{a}	41.70 ± 3.44^{ab}	40.77 ± 6^{ab}	33.89 ± 2.55^{b}
PGE_3/PGE_2	1.45 ± 0.33	1.00 ± 0.22	0.91 ± 0.46	0.96 ± 0.12

Values are means ± SD from three fish. Values in the same row with different superscript letters are significantly different . The specificity of the antibody was 100 % for PGE_2 . 43 % for PGE_3 and only 18.7 % for PGE_1.

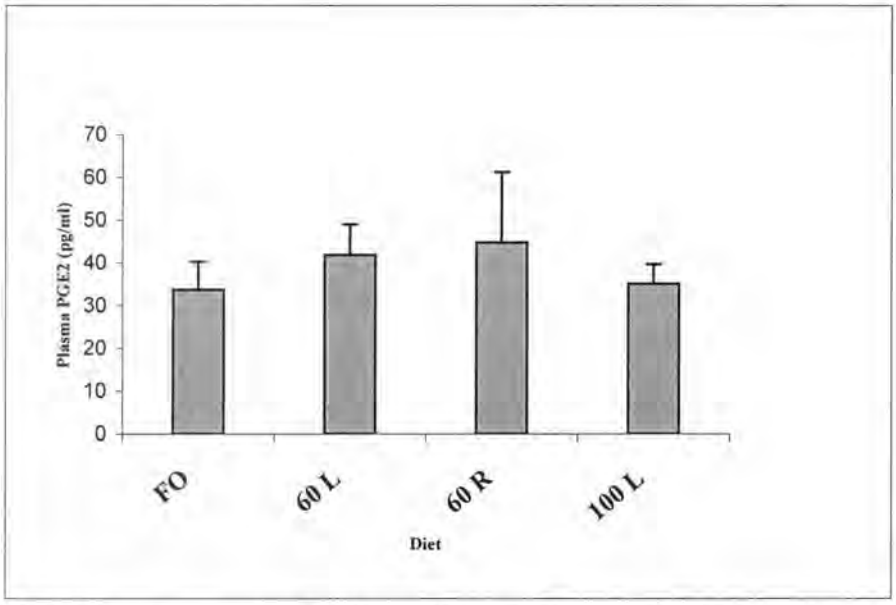

Fig. 14: PGE_2 concentration in plasma from sea bream fed the experimental diets. Values are means ± SD from three fish per treatment.

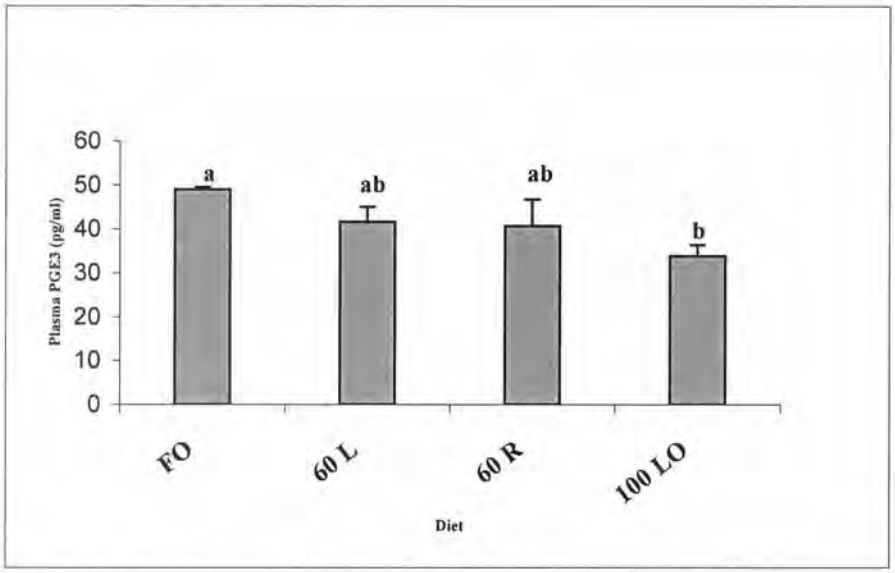

Fig. 15: PGE_3 plasma concentration from sea bream fed the experimental diets. Columns assigned a different letter are significantly different ($P<0.05$).

The study of correlation between PGE_3 and it's precursor (EPA) in plasma, is shown in Figure 16. A high correlation (0.98) was found between plasma PGE_3 and EPA concentration with a positive relation ($Y=3.26X + 18.61$). High correlation (0.96) was also found between plasma PGE_3 and EPA concentration in PI leucocytes ($Y= 3.30 X + 32.59$) (Fig. 17).

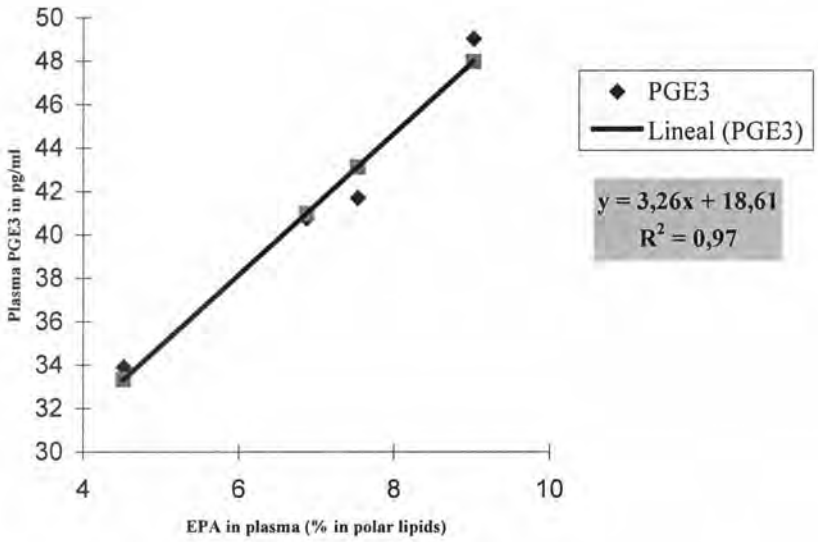

Fig.16: The relation between PGE_3 (pg/ml) and EPA (% in polar lipids) concentration in plasma from sea bream fed the experimental diets

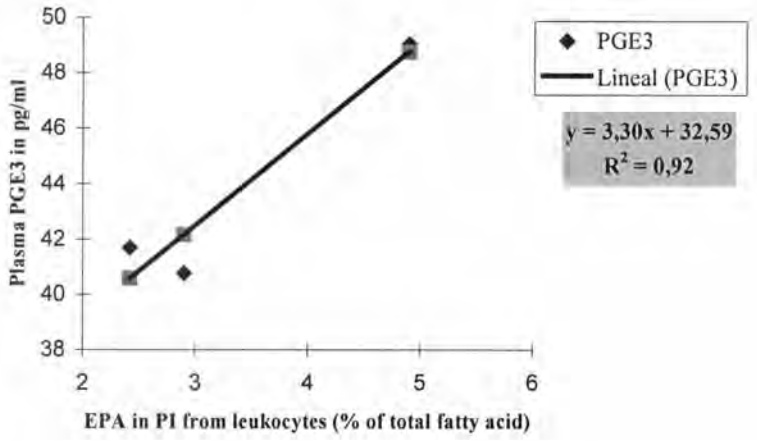

Fig. 17: The relation between PGE_3 (pg/ml) and EPA (% in) concentration in PI from leukocytes of blood sea bream fed the experimental diets

4- Plasma leptin

The production of the hormone leptin in plasma is shown in Figure 18. No significant differences were seen among the four diets.

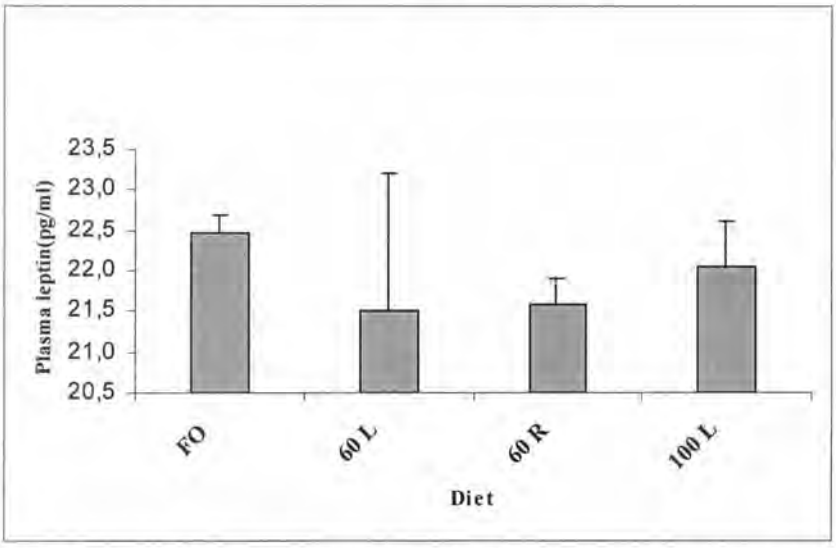

Fig. 18: The levels of leptin concentration in plasma from sea bream fed the four experimental diets.

To find out the effect of plasma leptin concentration on fish metabolism, we trace the correlation between these results with the some other parameters. We found that there was a correlation only with conversion index (feed intake/weight gain) and liver weight (Figure 19 and 20). High correlation (0.96) was found between plasma leptin level and conversion index (C.I) (Figure 19) with a negative relation (Y= -0.26 X + 8.74).

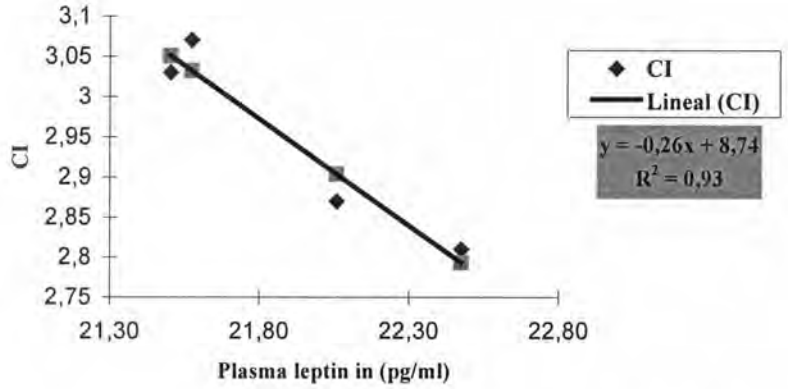

Fig. 19: Relation between plasma leptin and conversion index in sea bream fed the four experimental diets

There was also a high correlation (0.94) between plasma leptin levels and liver weight expressed in positive relation (Y= 0.19 X – 0.74) (Figure 20).

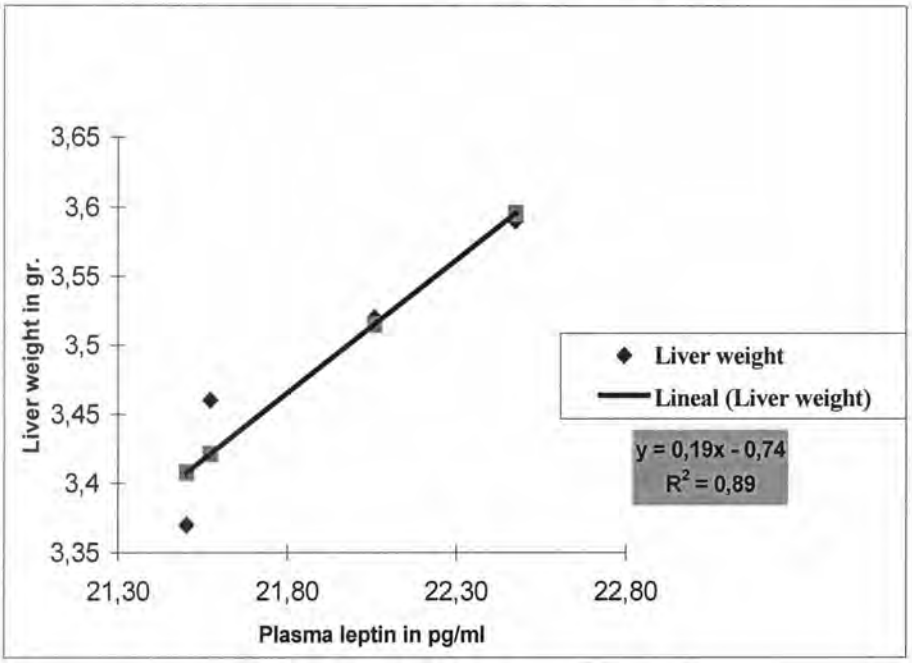

Fig. 20: Relation between liver weight and plasma leptin in sea bream fed the experimental diets.

No significant correlation was found between the plasma leptin concentration and the other parameters.

DISCUSSION

As shown in previous studies (Izquierdo et al., 2003; Izquierdo et al., submitted), substitution of 60 % dietary fish oil by vegetable oils did not affect growth and survival of gilthead sea bream. However, complete substitution (100 LO) of fish oil, and hence reduction of ARA, EPA and DHA dietary levels, was not able to cover the EFA requirements of this species in young sea bream as denote by the lower body weight reached in the first part of the trial. This is in agreement with the lower growth rates found by Izquierdo et al. (submitted) when sea bream were fed a 80 % fish oil substituted diet and the requirements of over 1.6 to 1.9 n-3 HUFA suggested for these species (Ibeas et al., 1996; Montero et al., 1996).

No significant differences were found in feed intake among fish fed the different experimental diets and this was in agreement with the results obtained for the leptin analysis. Thus, inclusion of vegetable oils did not significantly affected basal plasma leptin concentration, as it was also found in feed intake and conversion index (CI), suggesting also the importance of leptin in controlling feed intake. Leptin has been described in mammals to act as a "lipostat" sensing the body's adiposity, and regulating appetite and metabolism to maintain constant fat stores (Ahima and Flier, 2000). Circulating levels of leptin are sensitive to caloric intake (Ahima et al., 2000) and show profound decreases during caloric restriction imposed by fasting (Maffei et al., 1995; Havel, 2001). Both peripheral and central administration of leptin decrease food intake and body weight in birds and mammals (Ahima et al., 2000) and inhibits hypothalamic appetite-suppressing (anorexigenic) pathways in mammals (Meister, 2000). Few studies have been conducted to elucidate leptin activities in fish. Leptin administration in goldfish (Volkoff et al., 2003) causes a reduction in food intake and increases fat metabolism in sunfish (*Lepomis cyanellus*) (Londraville and Duvall, 2002). But, studies on the long-term effects of leptin (Londraville and Duvall, 2002) indicate that in fish as in other cold-blooded vertebrates with slower metabolism, leptin may predominantly act as a satiety factor rather than long-term regulator of energy homeostasis (Volkoff et al., 2003). In higher vertebrates, leptin is a protein secreted primarily by the adipose

tissue but also by a variety of tissues such as stomach, muscle, and placenta (Ahima et al., 2000). Adipose tissue is mainly represented in fish such as seabream in the perivisceral fat, including preipancreatic one. Seabream pancreatic tissue is imbibed in the hepatic tissue, liver being also considered as one hepatopancreas. Hence, correlation between fish liver weight and plasma leptin content may be a consequence of a higher leptin synthesis in such tissue.

Inclusion of vegetable oils in diets for salmon significantly reduced plasma leptin levels (Bell et al., unpublished data). Besides its role in regulation of energy balance by decreasing food intake and increasing energy expenditure, leptin have been claimed to play an important role in immune function and reproduction (Van Dijk, 2001). Thus, leptin seems to affect LH secretion in sea bass (Peyon et al., 2001) and LH/FSH in rainbow trout (Weil et al., 2003). More studies are needed to understand their effect both in reproduction and, particularly, on fish health. In this experiment, even there were no significant differences in basal plasma leptin levels among the four treatments, it should be of grand importance to measure the postprandial effects to see if there is an effect of alternative lipid oils sources.

There were no significant differences in plasma saturated fatty acids contents of fish fed blends of vegetable oils despite the dietary input differences on these fatty acids. Total saturates were more incorporated into polar than into neutral lipid fraction, as expected by the important structural role of these fatty acid (especially in phosphatidilcholine) (Mourente and Tocher, 1993). Monoenoic acid, mainly oleic acid, reflect the dietary input which was proved also in sea bass leucocytes (Farndale et al., 1999), cod (Waagbo et al., 1995) and recently in sea bream intestinal cells (Caballero, 2002). This fatty acid was high in fish fed vegetable oils compared to fish fed control diet, it was especially incorporated in neutral lipid which can be explained by it's energetic role (Ibeas et al., 1996). Deposition of LA and LNA acid in both polar (PL) and neutral lipids (NL) was related to dietary fatty acid level. Furthermore, the type of deposition in these two lipid classes was different as indicated by the ratio of plasma to dietary level. The plasma concentration in these fatty acids was

significantly increased in fish fed vegetable oils compared to those fed fish oil as a single added lipid source with a preferential incorporation into NL in which the ratio ranged from 0.78 to 1.13 for LA, while the ratio for LNA was only between 0.40 and 0.65. It appears that there was a preferential incorporation of LA compared with LNA; similar results have been found in previous studies (Izquierdo et al., 2003; Izquierdo et al., submitted) and in other species such as cod (Waagbo et al., 1995). The high content of either LNA or monoenes (mainly oleic acid) in the cell membrane could be responsible for imbalances among fatty acids and influence membrane physical properties and phagocytic activity (Montero et al., 2003).

All fish fed blends of vegetable oils had significantly increased levels of 20:2n-6 and 20:3n-3, which indicate the activation of the elongase enzyme in this species. There was also a significant increase in 20:3n-6 and 20:4n-3, products of elongation and Δ^6–desaturase, in fish fed vegetable oils compared to fish oil fed fish. The increase of 20:4n-3 and 20:3n-6 might be a further indication of a Δ^6 – desaturase activity in fish fed vegetable oils. A Δ^6 –desaturase like gene has been recently described to be present in gilthead sea bream (Seiliez et al., 2003). In freshwater fish, feeding vegetable oils contain LA but also high level of LNA (mainly linseed oil) which is competitor in desaturation and elongation of C_{18} PUFA, 20:3n-6 (DHGLA) may be a predominant product of 18:2n-6 in salmonids (Bell et al., 1991, 2002; Tocher et al., 2000). In salmon fed Rapseed, linseed and palm which do not contain ARA despite containing 18:2n-6, 20:3n-6 will accumulate while ARA will be reduced in the fish (Bell et al., 1991, 1993).

Furthermore, Bell et al. (1998) found an increase (up to 10 fold) of DHGLA, in total lipids of carcass, liver, gills, muscle and brain of turbot fed diets containing borage oil, rich in LA and γ-linolenic (GLA) acid. The highest levels of DHGLA were found in the PI fraction which is known to preferentially incorporated high levels of C_{20} HUFA (Sargent et al., 1995). In the present experiment, DHGLA was increased in fish fed vegetable oils with maximum ratios in fish fed 100% blend vegetable oils (up to 4 fold in plasma total lipids and up to 2.5 fold in plasma polar lipids). However, the level of ARA and EPA

(the product of Δ^5- desaturase from 20:3n-6 and 20:4n-3 respectively) have decreased in the plasma of fish fed vegetable oils comparing with fish fed the control diet. This can be explained by the low activity of Δ^5-desaturase in marine fish (Sargent et al., 1995, Henderson and Tocher 1987; Tocher, 1993). At the same time, the lack of Δ^5-desaturase in this fish specie enabled DHGLA to accumulate in plasma membrane while the concentration of ARA remained low, which agree with the results found in turbot by Bell et al.(1995).

DHA was the major PUFA in all lipid classes. The concentration of DHA in fish plasma was higher than their dietary input. This fatty acid was especially esterified in PL with high ratio comparing to NL. Hence, the ratio DHA in plasma PL/ DHA in diet rise from 2.02 (in fish fed fish oil) to 10.93 (in fish fed 100 % vegetable oils); this can be explained by the preference of plasma cells phospholipids to assimilate this fatty acid, through its high affinity with fatty acid binding protein (FABP) (Sire and Vernier., 1981) and with the enzyme 1-lisofosfatidilacilCoA transferase (Gurr and Harwood, 1991). Similar results were found in cod (Waagbo et al., 1995), European sea bass (Farndale et al., 1999) and sea bream (Caballero, 2002; Montero et al., 2003). Previous other studies have also demonstrated that DHA was preferentially retained under dietary essential fatty acids deficiency (Izquierdo et al., 1996, 2001; Montero et al., 2001), showing the importance of this fatty acid as a main structural component of fish membranes (Watanabe, 1993; Sargent et al., 1995).

The plasma content in EPA followed broadly the dietary input, this fatty acid was especially incorporated in plasma PL and its concentration was significantly decreased in fish fed blends of vegetable oils, particularly with 100% substitution levels. This fatty acid was especially incorporated into PL class with ratio of incorporation increased with increasing dietary LA and LNA, rising between 0.76 in fish fed control diet and the highest ratio (4.26) in fish fed 100% vegetable oils (with the lowest dietary EPA content). This may suggest a selective incorporation of this fatty acid in plasma membrane which agree with the results found sea bass leucocytes (Farndale et al., 1999) and sea bream

(Caballero, 2002). Together with DHA, EPA has been recorded to play important structural function in membranes (Sargent et al., 1995).

ARA concentration in fish plasma was markedly decreased in plasma of fish fed vegetable oils with the lowest concentration found in fish fed 100 % blend vegetable oil. Deposition of ARA in both polar and neutral lipids was related to dietary fatty acid levels. However, the type of deposition in the two lipid classes was different as indicated by the ratio of tissue to dietary level. In NL, this ratio was small and did not change much from high to low dietary concentrations among the three first diets, but in fish fed 100 % vegetable oil this ratio markedly increased. By contrast, a stronger deposition was noted for low dietary concentrations in the case of PL, with ratio rising between 1.94 in fish fed control diet to 10.83 in fish fed 100% blend of vegetable oils. This finding agrees well with results found by Farndale et al., (1999), Montero et al., (2003) and Fountoulaki et al., (2003) indicating the importance of this fatty acid for the proper cell function. The relative deposition of ARA in PL indicates that this fatty acid is under a specific control (Fountoulaki et al., 2003). Waagbo et al., (1995) have also found that in cod head kidney, the ratio ARA in macrophages/ARA in diet varied between 5 and 14. Other studies demonstrated that this fatty acid was retained in case of fatty acid deficiency (Izquierdo et al., 1996) and was incorporated and retained in phosphatidyl inositol of membrane from sea bream (Mourente & Tocher, 1993) and turbot (Linares and Henderson, 1991), it is also considered as the main PUFA in this phospholipid class (Bell and Sargent., 2002). Hence, evidences suggest that PI accumulate selectively C_{20} PUFA suggesting that this phospholipid class may be a source of precursors for the synthesis of eicosanoids (Bell, 1995). PI of blood leucocytes was very rich in oleic acid in fish fed diet 60 L and 60 R, despite the different levels in the diet, suggesting the competition of this fatty acid with palmitic acid in its frequent 1 *sn*-position of this phospholipid class, explaining the low content in the latter fatty acid. Comparing the fatty acid composition of PI and total polar lipids one, it appears that PI is less rich in PUFA and the PUFA represented only around 30

% of the total content of this fatty acid class, this might be explained by the competition of other fatty acids to esterification in *sn-2* of PI.

It is well established that the deposition of fatty acids in tissue lipids is strongly affected by dietary fatty acids (Bell et al., 1994; Sargent et al., 1995). In this study, n-3 HUFA were much higher in plasma of fish fed FO and decreased with increasing dietary LA and LNA input, but it was proportionally much higher than in diet. Nevertheless, plasma n-6 content was significantly different among treatments according to dietary input, with the lower level in fish fed FO diet and the highest in plasma of fish fed 100% vegetable oils. DHA, EPA, ARA and DHGLA were more abundant in polar than in neutral lipids, which confirm the role of polar lipids especially PI in accumulating C_{20} fatty acids (Bell et al., 1994). The existence of a competition between EPA and ARA during phospholipid esterification has been suggested to occur in fish (Bell and Dick, 1990). In this study, the fatty acids composition of PI from leucocytes, have shown a difference compared with plasma phospholipids contents. Even despite the diets 60 L and 60 R were very rich LA and LNA, those fatty were not very abundant in PI, whereas oleic acid was very high. By contrast with plasma phospholipids content, PI have not shown higher levels on DHGLA, but 20:3n-9 was strongly incorporated. N-3/n-6 was also significantly higher in fish fed vegetables oils than in those fed control diet.

There was high content in saturate and monoenes in fish fed vegetables oils, which may be explained by their importance as a source of energy and their estherification in the sn-1 position of this phospholipid class. ARA/EPA was constant among treatments but was higher than in phospholipids content, which confirm the specificity of PI in accumulating ARA compared to the other phospholipids classes. EPA/DHA was also higher than in plasma polar lipids.

ARA is known to turn over rapidly in response to external signals for prostaglandins synthesis (Sargent et al., 1989). There was also slightly higher levels of saturates and higher levels of polyunsaturates in plasma PL compared to NL in accordance with previous studies with freshwater fish (Henderson and Tocher, 1987) and Mediterranean species (Ibeas et al., 1996). There was also

increased levels of monoenes from control to 100% vegetable oils fed fish. Thus, the substitution of dietary FO by blend of vegetable oils in this experiment have demonstrated a profound modifications in plasma fatty acid composition.

Long-chain PUFA 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. Feeding vegetable oil could cause significant reductions in nonspecific immune parameters including haematocrit, total white blood cell and red blood cell counts, and macropahage respiratory burst in salmon (Good et al., 2001), and influence phagocytic activity of head kidney macrophages from sea bream (Montero et al., 2003). Fish offer a particularly useful model system for studying the production and interaction of eicosanoids derived from ARA and EPA because their tissues are naturally abundant in n-3 PUFA (Henderson and Tocher, 1987). Feeding dietary lipids with varying amounts of n-3 and n-6 PUFA, derived from fish and vegetable oils, have been demonstrated to affect the fatty acid composition, and in particular the incorporation of C_{20} eicosanoid precursors, in gill and kidney of salmon (Bell et al., 1996a). The dietary treatments employed in the present study have resulted in profound alterations of ARA/DHGLA ratio, but not in ARA/EPA one. ARA, EPA and DHGLA are all precursors for eicosanoid production (Bell et al., 1994), changes in the ratios of these HUFA have important consequences for the quantity and spectrum of eicosanoids produced by the turbot (Bell et al 1998). The lowest ARA/DHGLA ratio was noted in phospholipids from fish fed 100% vegetable oils (2.21) followed by those fed 60 L and 60 R with only 60% of FO substitution (3.94 and 3.44 respectively) and the highest ARA/DHGLA ratio in phospholipids of fish fed FO (10.56), thus, there was a significant decrease (up to 5 fold) in this ratio due the increase in DHGLA concentration in fish fed vegetable oils compared to fish fed control diet.

The basal concentrations of eicosanoids in plasma from sea bream fed dietary treatments were also affected by feeding vegetable oils. The methods used to measure PGE_2 and PGE_3 concentrations in this study were different,

which may make difficult the comparison of their concentrations. PGE_2 was determined by direct quantification with EIA after purification. However, PGE_3 was firstly separated with HPLC, collected, purified and, lastly, quantified with EIA. The slightly similar amount of ARA-derived eicosanoids (PGE_2), regardless the diet fed, could be a reflection of the constant eicosanoids precursor (ARA/EPA) in plasma phospholipids (around 0.14) among fish fed the different dietary treatments. Comparing with previous studies, feeding increasing dietary ARA level lead to significant increase in PGE_2 concentration in heart, brain and kidney in turbot (Bell et al., 1995). *In vitro* culture of turbot (*Scophtalmus maximus*) astrocytes have demonstrated that ARA is the preferred substrate for prostaglandin production, even when cultures are supplemented with other precursors fatty acids, and despite the excess of DHGLA or EPA in cellular lipid, demonstrating that PGE_2 was the major produced prostaglandin (Bell et al., 1994). Thus, in this study, despite a significant reduction in plasma ARA in vegetable oils fed fish, PGE_2 concentration remained constant which might be explained by the parallel reduction in EPA concentration leading to a constant ARA/EPA (eicosanoids precursors). Furthermore, the production of PGE_3 was significantly different among fish fed the four experimental diets and strongly correlated with plasma polar lipid (0.98) and PI from leucocytes concentrations on EPA. Fish fed FO, with high content in EPA, showed the highest PGE_3 concentration (49.03 pg/ml of plasma), whereas fed 100% vegetable oils showed the lowest (33.89 pg/ml of plasma). However, in contrast with previous studies "in vitro" conducted in juvenile turbot (*Scophtalmus maximus*) and salmon (*Salmo salar* L.) (Bell et al., 1994; Henderson et al., 1996), PGE_2 was not the major prostaglandin produced in fish plasma, since in fish fed FO, PGE_3 was higher than PGE_2 with a ratio PGE_3/PGE_2 reaching 1.45. Among the different fish species studied to date, differences in the relative concentrations of these substances have been found. The production of these compounds have been also reported to be different among tissues of the same species. Thus, PGE_2 production was higher in kidney macrophages than in blood leukocytes in turbot

(Tafalla et al., 1999). Further studies of prostanoid production in different tissues and species are required to clarify this effect.

Feeding vegetable oils lead to a decrease in plasma EPA which in turn give caused a reduction in plasma PGE_3 concentration. Phospholipid fatty acid composition is determinant of the physical properties of cell membranes which can influence the activities of membrane-associated proteins and enzymes (Spector and Yorek, 1985) and thereby influence the catalytic activity of the phospholipase (Bell et al., 1996). The production of plasma PGE_2 was not significantly different among the four treatments. Previous study, in rain bow trout, demonstrated that blood erythrocytes are not capable of eicosanoid synthesis (Pettit, Rowley and Barrow, 1989), thus plasma eicosanoids reflect principally leucocytes products. Despite the plasma PGE_2 levels were not significantly different, the lower production in fish fed only fish oil fed fish may be explained by the higher content of EPA in the PI of this fish, since this fatty acid is a potent competitor of AA. Besides the slight increase of PGE_2 between 60 L to 60 R agrees well with the slight increase in PI content on AA and the lower EPA levels in comparison with fish fed fish oil. In the present study, we found that substitution of fish oil with vegetable oils in diets for sea bream can affect profoundly plasma PUFA composition which in turn affect 2- and 3- series prostaglandins production.

In our experiment, it is notable in total and in polar lipid fatty acid composition that the amount of DHGLA was increased up to 4-fold in fish given the highest dietary LA (diet with 100 % vegetable oils); this fatty acid (as precursor of PGE_1) is known to inhibit the production of ARA-derived lipoxygenase metabolites (Miller et al., 1990) by increasing the synthesis of camp (Horrobin 1980). Supplementation with both DHGLA and EPA significantly reduced ARA-derived prostaglandins production in salmon (Bell et al., 1994) which indicate a competitive inhibition by those fatty acids at the cyclooxygenase active site (Bell et al., 1994). Also, phospholipase activity, which is the key of release of eicosanoid precursors fatty acids, is affected by the dietary fatty acids. In salmon fed diet a 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). Feeding fish with borage oil (rich in 18:2n-6 and 18:3n-6 precursors of DHGLA) decreased significantly PGE_2 concentration (Bell et al., 1995). 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 human (Horrobin, 1992). In this study we did not analysed plasma PGE_1 concentration, therefore we can not elucidate the effect of this slight increase in plasma DHGLA on PGE_1 production. It is notable in total and polar lipid fatty acid composition that the amount of DHGLA was increased in fish given the highest dietary LA (diet with 100 % LO), evidences exist suggesting that increase in concentration of DHGLA may increase PGE_1 production and consequently reducing PGE_2 one. This fatty acid is known to inhibit also the production of ARA-derived lipoxygenase metabolites (Miller et al., 1990). Thus, it might be an increase in plasma PGE_1 in fish feed vegetable oils compared with those fed FO.

PGE_2 is a mediator of inflammatory activity (Kinsella et al 1990), and is fundamental in the control of pathophysiological processes prevalent in numerous inflammatory conditions occurring in human populations (Horrobin, 1992). But high production of these compounds by leucocytes may be responsible for the severity of lesion (Bell et al., 1993). LTB_4 (derived from the same precursor) is a particularly powerful chemoatractic agent for neutrophils (Strasser et al., 1985), active in stimulating cellular uptake of calcium in human (Hunt and Rowley, 1986) and is able to increase lymphocytes proliferation (Secombes et al.,1994). While LTB_5 (derived from EPA) has similar activity but it is around 30 times less potent (Lee et al., 1984). There is good evidence that eicosanoids promote inflammatory processes and they are vital for the activation, proliferation and differentiation of B and T lymphocytes during the immune response (Yamaoka et al., 1989). The reduction in production of prostaglandins, or alteration in the spectrum and efficacy of prostaglandins produced, may be sufficient to alter immune cell composition and function (Kinsella et al., 1990), and cause increased inflammatory activity.

In summary, the present study have clearly demonstrated that inclusion of higher levels of vegetable oils in diets for sea bream may profoundly affect the fatty acid composition of their plasma and leukocytes, especially HUFA and consequently the production of eicosanoids in these cells. Thus, in sea bream, which seems to have a low Δ^5 than Δ^6 desaturase activity, there is a potential to provide membrane phospholipids with a high variability in the ratio of eicosanoids precursors, DHGLA/ARA/EPA. In light of these previous reports, using diets with higher percentages of substitution with vegetable oils for sea bream may affect their immunology system and health by changing eicosanoids production and affecting their resistance to diseases (Blazer, 1992; Thompson et al., 1996; Montero et al., 2003). This is an area where more research, in different immune system organs, is required to investigate the possible immunosuppressive effect of dietary vegetable oils in sea bream and its interaction with eicosanoids production.

CONSLUSIONS

Conclusions :

1- We confirm that vegetable Oils can be used in diets for seabream at percentage of substitution up to 60 % without affecting its growth, but extreme substitution may reduce the growth.

2- A method to effectively isolate circulating seabream leukocytes was developed for this species.

3- Membrane phospholipids in plasma and leukocytes of seabream may be profoundly altered by giving diets with higher content on vegetable oils.

4- There was a selective retention and incorporation of DHA and AA in plasma phospholipids which confirm their importance in plasma cell functions.

5- Confirmation of the presence of active elongase and some Δ^6 desaturase activity in seabream when fed vegetable oils.

6- There was a selective incorporation of C_{20} in PI from leukocytes.

7- The altered plasma phospholipid levels on AA and EPA are reflected in changes in prostaglandins production which could influence numerous physiologic functions affecting fish health.

8- Plasma leptin concentration reflect feed intake, and was not significantly affected by feeding vegetable oils.

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