UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

Instituto Canario de Ciencias Marinas
ICCM

PhD Thesis

IMPORTANCE OF THE PROPORTIONS OF DIETARY POLYUNSATURATED FATTY ACIDS AND ANTIOXIDANTS IN LARVAL DEVELOPMENT OF MARINE FISH

EYAD ATALAH

Grupo de Investigación en Acuicultura

Las Palmas de Gran Canaria, Spain
2008

DEPARTAMENTO DE BIOLOGIA
Universidad de Las Palmas de Gran Canaria

Anexo I

D. JUAN LUIS GÓMEZ PINCHETTI SECRETARIO DEL DEPARTAMENTO DE BIOLOGÍA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

CERTIFICA,

Que el Consejo de Doctores del Departamento en su sesión de fecha hoy tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada "IMPORTANCE OF THE PROPORTIONS OF DIETARY POLYUNSATURATED FATTY ACIDS AND ANTIOXIDANTS IN LARVAL DEVELOPMENT OF MARINE FISH" presentada por el doctorando D. EYAD ATALAH, dirigida por la Doctora Dª Mª SOLEDAD IZQUIERDO LÓPEZ y codirigida por la Doctora Dª CARMEN Mª HERNÁNDEZ CRUZ.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del Reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a dieciocho de noviembre de dos mil ocho.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
DPTO. DE BIOLOGIA

CAMPUS UNIVERSITARIO DE TAFIRA - 35017 Las Palmas - Islas Canarias - España - Tlf.: 34 - 928 45 29 11 - Fax: 34 - 928 45 29 22

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: **Biología & Instituto Universitario de Sanidad animales (IUSA)**

Programa de Doctorado: **Acuicultura: producción controlada de animales acuáticos**

Titulo de la Tesis

Importance of the proportions of dietary polyunsaturated fatty acids and antioxidants in larval development of marine fish

Tesis Doctoral presentada por **D. Eyad Atalah**

Dirigida por la **Catedrática. Marisol Izquierdo López**

Codirigida por **Prof. Carmen María Hernández Cruz**

La Directora **La Codirectora** **El Doctorando**

Las palmas de gran canaria, a 5 de Noviembre de 2008

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

Importance of the proportions of dietary polyunsaturated fatty acids and antioxidants in larval development of marine fish

EYAD ATALAH
Grupo de Investigación en Acuicultura
Instituto Canario de Ciencias Marinas
Universidad de Las Palmas de Gran Canaria

Being a thesis submitted for the degree of

Doctor of Philosophy

In the University of Las Palmas de Gran Canaria, 2008

Directors:

Prof. Marisol Izquierdo & Prof. Carmen María Hernández Cruz

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AA : Ascorbic acid

ARA : Arachidonic acid

DHA : Docosahexaenoic acid

EFA : Essential fatty acids

EPA : Eicosapentaenoic acid

HUFA : Highly unsaturated fatty acids with 20 or more carbon atoms and 3 or more double bonds

n-3 HUFA : Highly unsaturated fatty acids with 20 or more carbon atoms and 3 or more double bonds of the linolenic family

PE : Phosphatidylethanolamine

PI : Phosphatidylinositol

PL : Phospholipid

PUFA : Polyunsaturated fatty acids

Vit : Vitamin

α-T : α–Tocopherol

Acknowledgements

First of all I would like to express my gratitude to the Agencia Española de Cooperación Internacional for giving me the opportunity to pursue my future within the marine scientific field. I greatly thank my supervisor, Prof. Marisol Izquierdo, for the scientific, moral and professional support, sincerely without her help, this thesis would have never seen the light.

I would like to thank Prof. Carmen Hernández Cruz for the help and support provided as a co-supervisor and Prof. Juan Manuel Afonso for helping me in doing the statistical studies. A special thought to my close friend Rachid who was beside me along the entire long way. I am grateful to Prof. Daniel Montero and the Canary Islands Government through the project (Efectos de los ácidos grasos esenciales en el desarrollo de las respuestas endocrina e inmune temprana en larvas de lubina Dicentrarchus labrax 2004). I would like to thank Gercende for helping in contacting with French companies and Javier Roo, for his comments and advices which were very important for me.

I am grateful to the Instituto Canario de Ciencias Marinas, Grupo de Investigación en Acuicultura from the Universidad de Las Palmas de Gran Canaria and all the canarian people, with whom I lived very nice days of my life. Presently and at all moments, I would like to deeply thank my friends Tibi and Eneko for their help with the larvae culture, that was very nice, and finally my friends Orestes, Eduardo, Mentor, Mahamed, Reda and Yorya who were supporting me during the hard days and all Colleagues in the Grupo de Investigación en Acuicultura.

To my family

A mi familia

CHAPTER 1

GENERAL INTRODUCTION

1.1 - Importance of fish lipid nutrition for aquaculture development

Aquaculture is one of the most rapid developing animal production sectors in the world, growing at a rate higher than 5% per year in the last decade (Izquierdo, 2005). In aquaculture, the culture success of any marine fish species is limited by the quantity and quality of mass production of its fry (Izquierdo and Fernandez-Palacios, 1997). Hence, to meet the increasing demands of seed production, it is necessary improve the nutritional quality of its larvae, which still constitutes a major constraint for the development of marine fish species culture (Watanabe *et al*., 1983; Yúfera and Pascual, 1984; Sargent *et al*. 1997; Izquierdo *et al*., 2000).

While the Mediterranean production of marine fish culture has been increasing in several species, the gilthead sea bream *Sparus aurata* is still the most cultivated species (Izquierdo, 2005) with its annual fry production surpassing 120 million/yr. Demand for good quality fry has been increasing at a rate of 10% annually, but the success in mass production of juveniles is greatly affected by effective first feeding regimes and the high nutritional quality of starter diets (Kolkovski *et al*., 1993; Sargent *et al*., 1997; Izquierdo *et al*., 2000). In general, gilthead sea bream and European sea bass (*Dicentrarchus labrax*) are the most important marine finfish species reared in Mediterranean basin, which have characterized the development of marine aquaculture in this region over the last three decades (FAO, 1999, Manual on Hatchery Production of Sea bass and Gilthead Sea bream). Moreover, production of both species will still require a larger expansion (Basurco and Abellán, 1999).

However, while the production of these species is well controlled, the knowledge of their nutritional requirements in comparison with other species such as salmonids and carps is still incomplete (NRC, 1993). Particularly, larval rearing success is mostly affected by first feeding regimes and the nutritional quality of starter diets (Izquierdo *et al*., 2000).

Thus, to ensure a better growth and a high survival rate, a reliable diet that meet the nutritional requirements of larvae, both qualitatively and quantitatively is essential (Kolkovski *et al*., 1993; Sargent *et al*., 1997). Moreover, even before larval development, fish eggs should contain all nutrients to fulfil the requirements for the adequated development of the embryo (Izquierdo and Fernández-Palacios, 1997).

In the last years, more attention has been paid to study the importance of dietary lipids in marine fish larvae (Izquierdo *et al*., 2003), since they are essential for fish growth and development (Watanabe 1982; Sargent *et al*. 1999a). As a reflect of this importance, sea bream and sea bass diets have become highly energetical (25% lipid) in comparison with a decade or so ago (12 % lipid) (Izquierdo *et al*., 2003). Dietary lipids provide a rich source of energy and phospholipids which are vital as structure of biomembranes. Dietary lipids also serve as carriers for absorption of other nutrients, including the fat-soluble vitamins A, D, E, and K, and natural or synthetic pigments. Lipids are components of hormones and precursors for synthesis of various functional metabolites, such as prostaglandins and other eicosonoids. Moreover, dietary lipids are recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe *et al*., 1983), since they are the source of essential materials for the normal formation of cell and tissue membranes and organ development (Izquierdo *et al*., 1989b, 2003; Pousão *et al*., 2003). However, dietary lipid utilization by the larvae may be directly or indirectly affected by several morphological and physiological changes that occur during larval development. In recent years there has been an increasing interest in all these aspects of lipid nutrition in fish larvae, due to the importance of dietary lipid utilization for optimal larval growth and survival (Izquierdo *et al*., 2000).

1.2 - Essential fatty acids

Essential fatty acids (EFA) are key components of lipids, which cannot be biosynthesized in inadequate amounts by fish. Watanabe (1982) suggested that marine fish species have a limited ability to convert 18C fatty acids into longer polyunsaturated fatty acids as in most vertebrate. Probably all vertebrate have a requirement for n-3 and n-6 fatty acids in the diet (Teles, 2000), with qualitative requirements depending on the capacity of the species to synthetise EFA from precursors by the action of elongase and desaturase enzymes. In particular, probably all fish have a dietary requirement for three long chain polyunsaturated fatty acids (PUFA): docosahexaenoic acid (DHA, 22:6n-3),

eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (ARA, 20:4n-6), since they play very important functions in marine fish species (Izquierdo, 2005), but are poorly synthetised by these species. Whereas freshwater fish seem to posses Δ6 and Δ5 desaturase and elongase activities to produce ARA, EPA and DHA if their precursors linoleic (18:2n-6) and linolenic (18:3n-3) acids are present in the diet, such enzymatic activity is either lacking or very limited in marine fish and as a consequence, those long chain fatty acids have to be included in the diet and are considered essential (Sargent *et al*., 1995; Izquierdo, 1996). Few years ago, Δ6 desaturase-like genes were isolated from zebrafish (*Danio rerio*) (Hastings *et al*. 2001). Moreover, this type of gene was also found even in a marine fish species such as gilthead sea bream (Seiliez *et al*., 2003), although its expression is largely inhibited. More recently (Izquierdo *et al*., 2008), it has been shown that dietary lipids are able to regulate Δ6-desaturase expression in gilthead sea bream, although the ability of this fish to synthesise DHA was not enough to fulfill sea bream requirements for this fatty acid.

EFA play important physiological roles in fish as precursors of biologically active eicosanoids (Bell *et al*., 1986). For that reason, for a long time its study has been addressed by many research groups and the requirements for both DHA and EPA, included in the so called n-3 HUFA (highly unsaturated fatty acids with 20 or more carbon atom and 3 or more double bonds), in different fish species at different stages of fish development have been determined (Watanabe *et al*., 1978a,b, 1982; Izquierdo, 1988, 1996, 2005; Wilson, 1991; Wantanabe, 1993; Mourente *et al*., 1993; Rodríguez *et al*., 1993; Rodríguez, 1994; Salhi *et al*., 1994; Watanabe and Kiron, 1994; Sargent *et al*., 1999a; Izquierdo *et al*., 2000). The necessity of these fatty acids was pointed out by the high mortality and poor growth of larvae after 10-15 days of feeding an EFA-lacking diet (Izquierdo *et al*., 1989b; Sargent *et al*., 1997; Izquierdo, 2005) and the reduction in egg quality after 14 days of feeding broodstock with such a deficient diet (Harel *et al*., 1994; Izquierdo *et al*., 2001a). Reduced growth was observed in marine fish larvae fed EFA deficient rotifer, *Artemia* or microdiet in turbot (*Scophthalmus maximus*) (Gatesoupe and Le Milinaire, 1985), red sea bream (*Pagrus major*) (Izquierdo *et al*., 1989a, 1989b) and gilthead sea bream (Rodríguez *et al*., 1993, 1994; Salhi *et al*., 1994). Inadequate dietary n-3 HUFA resulted in reduction in survival rates in red sea bream (Izquierdo *et al*., 1989a, 1989b), gilthead sea bream (Rodríguez *et al*,, 1993, 1994; Salhi *et al*., 1994) and halibut (*Hippoglossus hippoglossus*) (Holmefjord and

Olsen, 1991). Poor pigmentation was as well related to low n-3 HUFA and especially very low DHA contents in polar lipids of turbot larvae (Rainuzzo *et al*., 1994).

Inadequate dietary EFA levels also resulted in poor feeding and swimming activities (Izquierdo, 1996) and altered fish larvae behaviour (Benítez *et al*., 2007). Moreover, EFA deficient feeds delayed the appearance of response to visual stimulus, in agreement with the reduction in DHA content in eyes and brains of these larvae and suggesting a delay in the functional development of brain and vision (Benítez *et al*., 2007). In agreement with this results, reduction in DHA and EPA content, reduced gilthead sea bream larvae eye diameter (Izquierdo *et al*., 2000; Roo *et al*., submitted). This fact, along with the higher density of cone photoreceptors, implies a significant improvement in larval visual potential (Roo *et al*., submitted). Lower swimming and feeding activities in EFA-deficient larvae (Izquierdo *et al*., 1989; Rodríguez *et al*., 1993; 1994) is frequently recognized by the larvae floating on the water surface denoting also alterations in the functioning of swim bladder (Koven, 1991).

Thus, n-3 HUFA have been recognised as a major limiting factor defining the nutritional value of larval diets for marine species, being indispensable through early stages of life (Watanabe and Kiron, 1994; Izquierdo, 1996; Sargent *et al*., 1999a).

Environmental factors affect both qualitatively and quantitatively the requirement of EFA, including temperature (Olsen and Skjervold, 1995), salinity (Borlongan and Benitez, 1992; Dantagnan *et al*., 2007) and light (Ota and Yamada, 1971).

Assuming that larvae grow faster than juveniles or adults, larval necessity of n-3 HUFA is higher than juvenile in the early stages of development (Izquierdo *et al*., 1989a). For instance, the elevation of dietary n-3 HUFA up to 1% improves growth and feed efficiency in gilthead sea bream whereas further increase of this levels resulted in the detriment of these parameters, hence 1% n-3 HUFA being the recommended requirement for juvenile gilthead sea bream (Ibeas *et al*., 1996). Besides, Rodríguez *et al*. (1998) showed that the elevation de n-3 HUFA from 0.9 to 1.5 % (d.w.) in rotifer *Brachionus plicatilis* improves growth in first feeding gilthead sea bream larvae, whithout further improvements with higher dietary levels of these fatty acids.

1.3 - Specific importance of DHA, EPA and ARA

DHA is necessary for fish reproduction, growth and survival (Izquierdo, 2005). Its incorporation into cell membrane regulates membrane integrity and function (Izquierdo, 2005) and this fatty acid is an important component of phosphoglycerides, particularly phosphatidyl ethanolamine and phosphatidyl choline in fish larvae. It is specifically retained in starved or low-EFA fed fish, due to the lower cell oxidation rates than other fatty acids (Madsen *et al*., 1999). DHA is more essential as EFA than EPA in marine fish larvae (Watanabe *et al*., 1989; Watanabe, 1993), promoting better larval growth and survival (Izquierdo, 1996). The minimum dietary DHA requirement recommended for sea bream larvae is 0.8% (Rodríguez *et al*., 1998; Salhi *et al*., 1999) and for juveniles 0.6% (Montero *et al*., 1996). Besides, no negative effects of high dietary levels of these fatty acids (5% in dry basis) have been determined for gilthead sea bream (Liu *et al*., 2002). Fish fed DHA as the major n-3 HUFA showed better growth and higher survival after activity test than larvae fed EPA as the major n-3 HUFA (Rodríguez *et al*., 1997).

EPA is another important EFA for marine fish larval growth (Watanabe *et al*., 1989), being a main precursor of prostaglandins in marine fish (Ganga *et al*., 2005), it enhances a non-specific lipase activity in sea bream larvae (Izquierdo *et al*., 2000) and DHA incorporation into larval PL (Izquierdo *et al*., 2000, 2001b). EPA is considered as a main component of polar lipids and its presence also contributes to regulate membrane integrity and function (Izquierdo, 2005).

Since it is known that ARA is an important precursor of eicosanoids, attention has been also paid to this fatty acid in marine fish aquaculture (Henderson and Sargent, 1985; Henderson *et al*., 1985; Bell *et al*., 1994; Sargent *et al*., 1994). Eicosanoids constitute a group of highly active compounds, once known as local hormones, which, among other molecules, include prostaglandins, thromboxanes, and leukotrienes, involved in the regulation of many several physiological processes. Besides, it is considered that the competitive interactions between EPA and ARA are important for the adequated balance in eicosanoids synthesis (Izquierdo, 1996). A number of studies have reported that dietary ARA improves fish growth (Castell *et al*., 1994; Bessonart *et al*., 1999), especially when offered together with suitable levels of other essential fatty acids such as EPA and DHA (Bessonart *et al*., 1999). Koven *et al*. (2001) showed that dietary ARA fed to gilthead sea bream larvae from 3 to 19 days posthatching markedly

improved survival following the acute stress. Moreover, feeding ARA prior to handling was much more effective in improving survival compared to feeding this fatty acid following this stress event. In juvenile turbot, dietary deficiencies in ARA have resulted in high mortality and obvious pathology (Bell *et al*., 1985), whereas in European sea bass, broodstock showed an elevated dietary requirement for ARA (Sargent *et al*., 1999a). Larvae of gilthead sea bream show a preferential retention of ARA during starvation (Castell *et al*., 1994; Rodríguez, *et al*., 1994; Izquierdo, 1996), denoting as well the importance of this fatty acid.

1.4 - Importance of different ratios of DHA, EPA and ARA

Competition among these three fatty acids has been demonstrated at several physiological levels, suggesting that not only the specific requirement of each of these fatty acids but also the proportions among them must be considered when determining their requirements (Izquierdo *et al*., 2001b). For instance, digestive lipases have shown different affinities for EPA and DHA (Izquierdo *et al*., 2000). Thus, an optimal EPA/DHA ratio seems to be necessary to obtain the best growth (Izquierdo and Fernández-Palacios, 1997) and pigmentation (Reitan *et al*., 1994) in early larval life stages.

Rodríguez *et al*. (1997) demonstrated that with the same n-3 HUFA the decreasing of EPA/DHA rotifer content down to 1/1.3 improves growth in gilthead sea bream larvae, denoting that DHA is more important than EPA during early larval stages (Watanabe *et al*., 1989). Moreover, Rodríguez *et al*. (1997) found a negative significant relationship between larval EPA/DHA (total polar lipid) and larvae growth. In turbot larvae, Reitan *et al*. (1994) have shown that the optimal dietary EPA/DHA ratio is about 2, denoting that optimum ratios differ with species. In juveniles of gilthead seabream, best growth and lowest hepatosomatic index were obtained when the EPA/DHA ratio increased from 1/2 to 2/1, when the total n-3 HUFA was kept constant in the diet (Ibeas *et al*., 1997), suggesting that optimum ratios may also vary along the life cycle. However, most of the studies assayed a narrow range of this ratio and the effect of striking differences in the dietary content of these fatty acids has not been yet determined. Moreover, these studies frequently assayed only 2 or 3 ratios, but never a wide variety of them.

Due to the importance of ARA and its interrelations with the other essential fatty acids, more recently the research has been focused on the ratio among these three fatty acids (McEvoy *et al*., 1998; Estévez *et al*., 1999; Sargent *et al*., 1999a). The relation among dietary DHA, EPA and ARA has been proposed to be a critical factor for broodstock and larvae performance (Sargent *et al*., 1999a, b; Bell and Sargent, 2003) due to competition interaction among them. For instance, evidences of competition among these three fatty acids (ARA, EPA and DHA) have been shown for digestive enzymes (Iijima *et al*., 1998; Izquierdo *et al*., 2000). Hence, not only absolute dietary values for each of these essential fatty acids but also optimum dietary ratios among them must be define since both factors will affect at least to their incorporation into the tissue lipids and hence membrane fluidity and function, energy values obtain from their beta-oxidation, and the production of metabolically active compounds (Izquierdo *et al*., 2000; Izquierdo, 2005). Thus, several studies demonstrate the importance of considering the relative amounts of DHA, EPA, and ARA (McEvoy *et al*., 1998; Bessonart *et al*., 1999; Estévez *et al*., 1999; Sargent *et al*., 1999a; Copeman *et al*., 2002; Bell and Sargent, 2003; Koven *et al*., 2003). Estévez *et al*. (1999) in turbot larvae showed that increasing of dietary ARA affected the fatty acid composition of turbot larvae brain phosphoglycerides (phosphatidylinositol PI and phosphatidylethanolamine PE) more than increasing dietary EPA, while EPA: ARA ratios ≥1 in PI (fraction of the brain) were associated with normal pigmentation, moreover in the same study observed that with giving a sufficiency dietary DHA, the optimum dietary level of EPA is not a function of DHA but of dietary ARA and finally HUFA dietary imbalances during larval development might have serious effects upon brain formation, neuroanatomical differentiation, and neuroendocrine action.

Copeman *et al*. (2002) observed in yellowtail flounder (*Limanda ferruginea*) larvae that fish fed with rotifer enriched with high DHA (low EPA and ARA) was significantly larger and had higher survival compared with larvae fed with rotifer enriched with DHA and more level of EPA, ARA or only with olive oil which was significantly the smallest and had the lowest survival, also a relationship was observed between the DHA/EPA ratio in the diet and larval size (r^2 = 0.75, P= 0.005) and survival (r^2 = 0.86, P= 0.001), moreover the incidence of malpigmentation was higher in the larvae fed high DHA+ARA diet than in all other treatments and finally larvae fed the DHA-enriched diet had higher ARA incorporation than larvae fed DHA+ EPA-enriched

diet, which could indicate a competitive interaction between EPA and ARA for incorporation into phospholipids.

Since both ARA and EPA have been shown to be precursors of eicosanoids (Ganga *et al*., 2005), competing for the same eicosanoid synthesizing enzymes, the ratio between both fatty acids influences in eicosanoid production in different tissues (Sargent *et al*., 1995, 1999b; Ganga *et al*., 2005, 2006). McEvoy *et al*. (1998) demonstrated that the higher EPA/ARA dietary levels improved the pigmentation in presence of high DHA in Atlantic halibut larvae. For instance, Bessonart *et al*. (1999) showed that increase in dietary ARA levels up to 1% improves gilthead sea bream larvae growth (DHA/EPA/ARA ratio is 1.4/0.8/1). Sargent *et al*. (1999b) suggest a beneficial DHA/EPA/ARA ratio of about 10:5:1 for marine fish larvae while the optimum ratio would be species specific, and Castell *et al*. (2001) suggest the ratio 40:5:4 for haddock (*Melanogrammus aeglefinus*) larvae. Bessonart *et al*. (1999) studied the effect of different ARA level in gilthead sea bream larvae without different ratio of EPA and DHA and the ratio of DHA/EPA/ARA suggested was 1.4/0.8/1. Thus there is an important benefit to study the ARA with different ratios and amount of EPA and DHA. A part of the present study was conducted to investigate in more detail the effect of different dietary DHA/EPA/ARA ratio in wide range for gilthead seabream and seabass larvae fed microdiet.

1.5 - Importance of antioxidant vitamins in lipid nutrition

Polyunsaturated fatty acids are very prone to peroxidation. Lipid peroxidation, is recognized as being highly deleterious, resulting in damage to cellular biomembranes (Kanazawa, 1991, 1993) and subcellular membranes, such as those of mitochondria, causing several pathological conditions in fish (Kawatsu, 1969; Watanabe *et al*., 1970; Murai and Andrews, 1974; Sakai *et al*., 1989). PUFA oxidation produces compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons which are implicated in several pathological conditions in fish (Kawatsu, 1969; Watanabe *et al*., 1970; Murai and Andrews, 1974; Sakai *et al*., 1989).

Fish have an endogenous antioxidant defence system (Filho *et al*., 1993), namely radical scavenging enzymes, playing an important role in physiological antioxidant protection (Blazer, 1982) such as catalase and superoxide dismutase, (SOD) acting on hydrogen peroxide (H_2O_2) and superoxide (O_2^-), respectively, and glutathione

peroxidase (GPX), which scavanges H_2O_2 and lipid hydroperoxides (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1996). There is a variation in the activities of antioxidant defence enzyme in fish in different organs of freshwater and marine fish (Wdzieczak *et al*., 1982; Lemaire *et al*., 1993), depending upon feeding behaviour (Radi and Markovics, 1988), feeding period (Mourente *et al*., 2002), ecological conditions (Winston and Di Giulio, 1991) and dietary antioxidant content. The vitamin E and C have a great importance as exogenous component in the diet of aquaculture fish acting as antioxidant defence enzymes in reduction of lipid peroxidation products and free radicals scavengers (Machlin, 1984). Mourente *et al*. (2002) showed a reduction of peroxidation products in liver of gilthead sea bream fed oxidised oil fed diets supplement with vitamin E in comparison with those fed diets lacking this vitamin.

Due to the high peroxidation risk of PUFAs, high HUFA requirements in early larval stages leading to dietary high levels of this fatty acids, must imply adequated levels of antioxidant compounds including these vitamins, in order to promote maximum benefits from these nutrients and avoid lipid oxidation problems that can cause pathologies, disease and subsequent mortalities particularly in marine fish larvae.

1.6 - Vitamin E

Vitamin E is one of the lipid soluble vitamins which are absorbed from the digestive tract in association with fat molecules and can be stored in fat reserves within the body. Vitamin E is a structural component of cell membranes (Putnam and Comben, 1987), functions as a lipid soluble antioxidant (Sargent *et al*., 1997) and maintains flesh quality, immunity, normal resistance of red blood corpuscles to haemolysis, and permeability of capillaries and heart muscle (Halver, 2002). The importance of vitamin E has been subsequently proven as a chain-breaking antioxidant process which plays important roles in biological processes (Burton *et al*., 1982; Burton and Ingold, 1989; Sies and Murphy, 1991). Numerous studies have demonstrated that vitamin E is essential for different fish species (Watanabe *et al*., 1970; Murai and Andrews, 1974; González *et al*., 1995) and affect a wide range of parameters.

Several studies denote the predominant role of vitamin E in antioxidant defense in many fish species improving the stability of tissue lipids to oxidation in trout (*Oncorhynchus mykiss)* (Frigg *et al*., 1990), Atlantic salmon (*Salmo salar)* (Waagbo *et al*., 1993), turbot (Stéphan *et al*., 1995) and sea bass (Messager, *et al*., 1992).

Erythrocyte fragility is one of the important parameters, which is used to determine the vitamin E status of fish (Halver, 1995). Vitamin E also enhances the activity of a non-specific immune system in sea bream (Montero *et al*., 1998). Besides, dietary vitamin E and n-3 HUFA had a synergistic effect on the non-specific immune responses and disease resistance in Japanese flounder (*Paralichthys olivaceus)* (Wang *et al*., 2006). Under chronic and acute stress, juveinile gilthead seabream fed a vitamin E-deficient diet showed a reduction in growth and survival (Montero *et al*., 2001), as well as a lower stress resistance. Tocher *et al*. (2003) have also shown that vitamin E supplementation improves growth in sea bream fed oxidized oil diet, and reduced the lipid peroxidation products in sea bream and turbot. These authors suggested that the antioxidant system is influenced by the species, development stage and culture temperature. Finally, increasing dietary vitamin E improves egg viability and the percentage of abnormal eggs in sea bream (Fernandez-Palacios *et al*., 1998).

A dietary requirement of vitamin E has been demonstrated in a number of fish (NRC, 1993) which includes 120 mg/kg diet (Hamre and Lie, 1995a) for Atlantic salmon, 30 to 50 mg/kg diet for channel catfish (*Ictalurus punctatus)* (Murai and Andrews, 1974; Wilson *et al*., 1984), 200 to 300 mg/kg diet for common carp (*Cyprinus carpio)* (Watanabe *et al*., 1977), 1200 mg/kg for gilthead sea bream (Ortuño *et al*., 2000) and 99 mg/kg for mrigal fry (*Cirrhinus mrigala)* (Paul *et al*., 2004). The level of 250 mg α-tocopherol/kg diet is sufficient to meet the requirements for successful reproduction in gilthead sea bream (Fernández-Palacios *et al*., 1998).

In larvae, higher HUFA requirements must be also associated with higher dietary levels of vitamin E as it is suggested in carp (Watanabe *et al*., 1981; Schwarz *et al*., 1988) and Atlantic salmon (Hamre and Lie, 1995b). Larvae of gilthead sea bream have shown an increasing content of vitamin E from hatching up to day 10^{th}, whereas from there on and until day 20, a marked decrease of vitamin E was observed (Izquierdo and Fernández-Palacios, 1997). This reduction was parallel to the reduction in the larval PUFA contents along larval development (Izquierdo, 1988), suggesting a close relation among both nutrients (Woodall *et al*., 1964; Watanabe *et al*., 1970; Izquierdo and Fernández-Palacios, 1997).

In gilthead sea bream larvae, González *et al*. (1995) tested a range of 22.27-780 mg/kg a-tocopherol in microdiets and found that dietary vitamin E elevation up to 136 mg/kg improves growth and survival, whereas further elevation markedly reduced larval survival.

1.7 - Vitamin C

Vitamin C (AA: ascorbic acid) constitutes a very important vitamin in fish (Kitamura *et al*., 1965; Mulero *et al*., 1998; Kolkovski *et al*., 2000; Ruff *et al*., 2001; Ortuño *et al*., 2003;). Fish larvae are particularly sensitive to vitamin C deficiency (Dabrowski *et al*., 1996). Their rapid growth rate suggests that larvae have higher vitamin requirements than juveniles and adults (Dabrowski *et al.,* 1988; Dabrowski, 1990; Kolkovski *et al*., 2000). Addition of vitamin C to larval diets improved survival, growth performance, skeleton development, stress resistance and immune response (Al-Amoudi *et al.,* 1992; Roberts *et al.,* 1995; Merchie *et al*., 1996; Montero *et al.,* 1999; Kolkovski *et al*., 2000; Anbarasu and Chandran, 2001; Ortuno *et al.,* 2001; Ai *et al.,* 2004; Lin and Shiau, 2005a).

Due to the low stability of ascorbic acid, up to 50% of the supplemented vitamin may be leached in the water in the first 10 s after feeding and approximaly 50% is destroyed during extrusion and 30 % during steam pelleting (Lim and Lovell, 1978). Thus stable forms of vitamin C as well as higher supplementation levels are included in fish diets.

The requirement level of AA depends on fish species, size, stage of sexual maturity, growth rate (Boonyaratpalin, 1997), diet and experimental conditions, as well as body vitamin storage status such as the hepatic AA concentration (Fournier *et al.,* 2000). Ascorbic acid supplied by diet is crucial for fish larvae development, since most species are unable to synthesise this vitamin (Gouillou-Coustans *et al.,* 1998). AA requirements seem to decrease as fish size increases (Boonyaratpalin *et al.,* 1989b). Inadequate supply of dietary AA usually results in a number of deficiency symptoms such as spinal deformation, impaired collagen formation, internal haemorrhaging, retarded growth and depressed immunity (Halver *et al.,* 1969; Al-Amoudi *et al.,* 1992; Gouillou-Coustans *et al.,* 1998). Beside, fish fed vitamin-C-deficient diets showed poor appetite, poor growth, dark coloration, fin erosion, gill arch pathology, bleeding gills and loss of equilibrium (Dabrowski *et al.,* 1988; Boonyaratpalin, 1997). Complete mortality of fish parrot (*Oplegnathus fasciatus*) after seven weeks of feed in an AA-free diet has been found by Wang *et al.* (2003).

Studies about the requirements of AA for marine finfish are scarce (Boonyaratpalin *et al.,* 1992; Saroglia and Scarano, 1992; Teshima *et al.,* 1993) and based on data obtained for freshwater species as reported by the NRC (1993). Published

research suggests the requirement of AA to be 200 mg of AA/kg in european sea bass for normal growth and hepatic saturation (Saroglia and Scarano, 1992), while other authores (Fournier *et al*., 2000) suggested 121 mg/kg and up to 1600 mg/kg dw *Artemia* in African catfish (*Clarias gariepinus*) larvae to improve growth (Merchie *et al.,* 1997). For gilthead seabream juveniles (150g) increasing AA from 500 to 3000 mg/kg increased the activity of non-specific immune responses (Ortuño *et al.,* 1999) while in fry (9g) increase up to 200 mg/kg improve protein efficiency ratio (Henrique *et al.,* 1998), and in younger fish (0.5g) a free AA diet caused high mortalities (Alexis *et al.,* 1997). For juvenile Korean rockfish (*Sebastes schlegeli)* a 103 mg AA/kg diet seem to required for improve growth (Montero *et al*., 1998); and about 45 mg/kg for common carp larvae, whereas more than 270 mg/kg is needed to maximize body vitamin C concentration (Gouillou-Coustans *et al.,* 1998). Juvenile grouper (*Epinephelus malabaricus)* requires about 45.3 mg AA/kg diet for optimal growth, whereas 6 times of this amout was needed to enhance the non-specific immune responses and promote survival of the fish infected with bacteria (Lin and Shiau, 2005a). In Japanese seabass 53.5 mg/kg was estimated as optimal for growth performance (Ai *et al.,* 2004). In parrot fish (*Oplegnathus fasciatus*), 118 ±12 mg AA/kg was required for maximum growth (Wang *et al.,* 2003). 1000mg/kg for Asian seabass fingerlings (*Lates calcarifer)* cultivated in freshwater (Boonyaratpalin *et al.,* 1989a) and 500mg/kg when it was cultivated in seawater or more has satisfactory growth (Boonyaratpalin *et al.,* 1989b). For mrigal larvae (*Cirrhina mrigala*) -an Indian major carp- the optimum requirement was found to be 650–700 mg AA/kg, based on weight gain, mortality or behavioural and morphological criteria (Mahajan and Agrawal, 1980).

1.8 - Relation between vitamin E and C

Ascorbic acid plays a role synergistically with vitamin E in the maintenance of glutatione peroxidase and superoxide dismutase activities (Le Grusse and Watier, 1993). Vitamin C and vitamin E function as biological antioxidants to protect cellular macromolecules (DNA, protein, lipids) and other antioxidant molecules from uncontrolled oxidation by free radicals during normal metabolism or under the conditions of high oxidative risk such as infection, stress or pollution (Chen *et al*., 2004). The beneficial effect of combined dietary vitamin E and C on growth and related parameters in fish has been studied in few species (Chávez de Martínez, 1990; Roem *et*

al., 1990; Thorarinsson *et al*., 1994; Chien *et al*., 1999). Hence, due to their potential for interaction, dietary requirements for vitamins C and E have sometimes been considered together (Moreau *et al*., 1999; Lee and Dabrowski, 2003).

AA seems to play an important role in the metabolism of *α*-tocopherol (α-T). Several studies (Cort, 1974; Packer *et al*., 1979; Niki, 1987a, b) demonstrated the ability of AA to reduce α-tocopheroxyl radicals and thereby regenerate them to *α*-tocopherol ('recycling' of vitamin E). However, hepatic vitamin E concentration decreased with increasing dietary vitamin C in hybrid striped bass (Sealey and Gatlin, 2002). Besides, a constant level of vitamin C and high levels of vitamin E diet reduced hepatic vitamin A in rainbow trout (Furones *et al*., 1992). These contradictory results could be related to an excess level in one or both vitamins and denotes the importance to specifically determine their requirements taking into consideration the dietary levels of each of these nutrients. Besides, their requirements in marine fish larvae are largely unknown.

1.9 - Objectives

Considering the importance of the amount and the ratios of DHA, EPA and ARA and considering the vitamin E and C as the most important dietary antioxidant, the global object of the present study is determination the optimal ratio of DHA/EPA/ARA and the optimal vitamin E and C in marine fish larvae, utilized gilthead seabream and seabass and fed them microdiet. For obtain this general objective the study was conducted to determine several objectives:

1. To determine the effect on larval rearing of a wide range of dietary EPA/DHA ratios in order to better understand the importance of these fatty acids and their consequences on dietary fatty acid utilization.
2. To find out the nutritional value of dietary ARA in microdiets for marine fish larvae and in relation to dietary EPA/ARA levels, determining their effects on survival, growth and stress resistance.
3. To improve the current knowledge on the incorporation of dietary essential fatty acids to larval lipids in relation to their relative values, mainly EPA/DHA and EPA/ARA and their implications for fish larvae production.

4. To better understand if the high larval requirements for essential fatty acids would imply increased dietary vitamin E values, determining the effects of dietary vitamin E in gilthead sea bream and seabass larviculture.

5. To elucidate the protective or damage effects of high vitamin E doses, studying its effect on larval lipids fatty acid composition and their implications in growth and survival.

6. To determine if increased vitamin E contents in larval diets should be associated to high dietary vitamin C contents and the nutritional value of the later nutrient for fish larvae performance.

Table 1.1. Optimal EPA/DHA, DHA/EPA, DHA/EPA/ARA or ARA ratios for different fish species

Species	**Ingredient**	**Fish size**	**Tested parameter**	**Requirement diet**	**Author**
Sparus aurata	EPA/DHA	Juveniles	Growth and hepatosomatic index	2/1	Ibeas *et al*., 1997
Melanogrammus aeglefinus	DHA/EPA/ARA	Larvae	Growth	40:5:4	Castell *et al*., 2001
Limanda ferruginea	DHA/EPA	Larvae	Growth and survival	8:1	Copeman *et al*., 2002
Paralichthys oliuaceus	EPA/DHA	Larvae	Dorsal pigmentation	< 4	McEvoy *et al*., 1998
Scophthalmus maximus	EPA/DHA	Larvae	Growth	2	Reitan *et al*.,1994
Sparus aurata	EPA/DHA	Larvae	Growth	1/1.3	Rodríguez *et al*.,1997
Sparus aurata	EPA/DHA	Larval	Growth	1/1.5	Rodríguez, 1994
Marine fish	DHA/EPA/ARA	Larvae	Non specified	10:5:1	Sargent *et al*.,1999b
Paralichthys dentatus	ARA	Larvae	Growth, survival and stress tolerance	> 6%	Willey *et al*., 2001

Table 1.2. Optimal vitamin E for different fish species.

Species	**Ingredient**	**Fish size**	**Tested parameter**	**Requirement diet**	**Author**
Sebastes schlegeli	Vitamin E	Juvenile	Growth	45 mg/kg	Bai and Lee, 1998
Ictalurus punctatus	Vitamin E	-------	Growth	50 mg/kg	Murai and Andrews, 1974
Cirrhina mrigala	Vitamin E	Fry	Growth	99 mg/kg	Paul *et al*., 2004
Epinephelus malabaricus	vitaimin E	Juvenile	Growth	≥100 mg/kg	Lin and Shiau, 2005a
Salmo salar	Vitamin E	Larvae	Growth	120mg/kg	Hamre and Lie, 1995a
Labeo rohita	Vitamin E	Fry	Growth	131.91 mg/kg	Sau *et al*., 2004
Sparus aurata	Vitamin E	Larvae	Growth and survival	136 mg/kg	González *et al*., 1995
Cyprinus carpio	Vitamin E	----------	Growth	300 mg/kg	Watanabe *et al*., 1977
Latris lineata	Vitamin E	Larvae	Growth	437 mg/kg	Brown *et al*., 2005
Sparus aurata	Vitamin E	Juvenile	Growth and survival	1000 mg/kg	Tocher *et al*., 2002
Sparus aurata	Vitamin E	Fry	Non-specific immune system	1200 mg/kg	Ortuño *et al*., 2000
Sparus aurata	Vitamin E	Broodstcok	Spawning quality	250mg/kg	Izquierdo *et al.*, 2001a

Table 1.3. Optimal vitamin C for different fish species

Species	**Ingredient**	**Fish size**	**Tested parameter**	**Requirement diet**	**Author**
Cyprinus carpio	Vitamin C	Larvae	Growth	45 mg/kg	Gouillou-Coustans *et al.,* 1998
Epinephelus malabaricus	Vitamin C	Juvenile	Growth	45.3 mg AA/kg	Lin and Shiau, 2005a
Sebastes schlegeli	Vitamin C	Juvenile	Growth	103 mg AA/kg	Montero *et al*., 1998
Oplegnathus fasciatus	Vitamin C		Growth	118 mg AA/kg	Wang *et al.,* 2003
Dicentrarchus labrax	Vitamin C	----------	Growth	121 mg/kg	Fournier *et al*., 2000
Sparus aurata	Vitamin C	Fry	Protein efficiency ratio	200 mg/kg	Henrique *et al.,* 1998
Dicentrarchus labrax	Vitamin C	---------	Growth	200 mg	Saroglia and Scarano, 1992
Cyprinus carpio	Vitamin C	Larvae	body vitamin C content	270 mg/kg	Gouillou-Coustans *et al.,* 1998
Epinephelus malabaricus	Vitamin C	Juvenile	Non-specific immune responses and disease resistance	271.8 mg AA/kg	Lin and Shiau, 2005a
Lutes calcarifer	Vitamin C	Fingerlings	Growth	500mg/kg	Boonyaratpalin *et al.,* 1989b
Cirrhina mrigala	Vitamin C	Larvae	Growth, survival, behaviour and morphological criteria	650–700 mg AA/kg	Mahajan and Agrawal, 1980
Lutes calcarifer	Vitamin C	Fingerlings	Growth	1000 mg/kg	Boonyaratpalin *et al.,* 1989a
Clarias gariepinus	Vitamin C	Larvae	Growth	1600 mg/kg d.w. in Artemia	Merchie *et al.,* 1997
Scophtalmus Maximus	Vitamin C	Juvenile	Non-specific immunity	2000mg/kg	Robers *et al*., 1995
Sparus aurata	Vitamin C	Juveniles	Non-specific immunity	500 to 3000 mg/kg	Ortuño *et al.,* 1999
Sparus aurata	Vitamin C	Juvenile	Immune system	2900 mg/kg	Cuesta *et al.,* 2002

1.10 - References

Ai, Q.H., Mai, K.S., Li, H.T., Zhang, C.X., Zhang, L., Duan, Q.Y., Tan, B.P., Xu, W., Ma, H.M., Zhang, W.B. and Liufu, Z.G., 2004. Effects of dietary protein to energy ratios on growth and body composition of juvenile Japanese seabass, *Lateolabrax japonicus*. Aquaculture, 230, 509– 516.

Al-Amoudi, M.M., El-Nakkadi, A.M.N. and El-Nouman, B.M., 1992. Evaluation of optimum dietary requirement of vitamin C for the growth of *Oreochromis spilurus* fingerlings in water from the Red Sea. Aquaculture, 105, 165–173.

Alexis, M.N., Karanikolas, K.K. and Richards, R.H., 1997. Pathological findings owing to the lack of ascorbic acid in cultured gilthead bream (*Sparus aurata* L.). Aquaculture, 151, 209–218.

Anbarasu, K. and Chandran, M.R., 2001. Effects of ascorbic acid on the immune response of the catfish, *Mystus gulio* (Hamilton), to different bacterins of *Ameromonas hydrophila*. Fish Shellfish Immunol., 11, 347–355.

Bai, S.C. and Lee, K.J., 1998. Different levels of dietary DL-α-tocopheryl acetate affect the vitamin E status of juvenile Korean rockfish, *Sebastes schlegeli*. Aquaculture, 161, 405–414.

Basurco, B. and Abellán, E., 1999. Finfish species diversification in the context of Mediterranean marine fish farming development. In: Marine Finfish Diversification: Current Situation and Prospects in Mediterranean Aquaculture. Options Mediterranéennes (eds. E. Abellan and B. Basurco), FAO, serie B: Etudes et Recherches, no. 24, pp. 9–25.

Bell, J.G. and Sargent, J.R., 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture, 218, 491– 499.

Bell, J.G., Henderson, R.J., Pirie, B.J.S. and Sargent, J.R., 1985. Effect of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gill structure in the turbot *Scophthalmus maximus*. J. Fish Biol., 26, 181–191.

Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of supplementation with 20:3n-6, 20:4n-6 and 20:5n-3 on the production of the prostaglandins E and F of the 1, 2 and 3 series in turbot *Scophthalmus maximus* L. brain astroglial cells in primary culture. Biochim. Biophys. Acta., 1211, 335–342.

Bell, M.V., Henderson, R.J. and Sargent, J.R., 1986. The role of polyunsaturated fatty acids in Fish. Comp. Biochem. Physiol., 83B, 711-719.

Benítez-Santana, T., Masuda, R., Juárez Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M. and Izquierdo, M.S., 2007. Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. Aquaculture, 264, 408–417.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M., González, M.M. and Fernández-Palacios, H., 1999. Effect of dietary arachidonic acid levels on growth and survival of gilthead seabream (*Sparus aurata* L.) larvae. Aquaculture, 179, 265-275.

Blazer, V. S., 1982. The ffect of marginal deficiencies of ascorbic acid and α-tocopherol on the natural resistance and immune response of rainbow trout (*Salmo gairdneri*). Ph.D. dissertation. University of Rhode Island.

Boonyaratpalin, 1997. Nutrient requirements of marine food fish cultured in Southeast Asia. Aquaculture, 151, 283-313.

Boonyaratpalin, M., Unprasert, N., Kosutharak, P., Sothana, W. and Chumsungnem, S., 1989a. Effect of different levels of vitamin C added in diet on growth, feed efficiency and survival rate of seabass. Technical Paper No. 6. National Institute of Coastal Aquaculture, Department of Fisheries, Thailand, 21 pp. (in Thai).

Boonyaratpalin, M., Unprasert, N. and Buranapanidgit, J., 1989b. Optimal supplementary vitamin C level in seabass fingerling diet. In: M. Takeda and T. Watanabe (Editors), The Current Status of Fish Nutrition in Aquaculture. Tokyo University of Fisheries, Tokyo, Japan, pp. 149-157.

Boonyaratpalin, M., Boonyaratpalin, S. and Supamattaya, K., 1992. Ascorbyl-phosphate-Mg as a dietary vitamin C source for seabass (*Lares calcarifer*). Presented at the 3rd Asian Fisheries Forum, 26-30 October 1992, Singapore.

Borlongan, I.G. and Benitez, L.V., 1992. Lipid and fatty acid composition of milkfish (*Chanos chanos* Forksskal) growth in freach water and seawater. Aquaculture, 104, 79-89.

Brown, M.T., Dunstan, G.A., Nichols, P.D., Battaglene, S.C., Morehead, D.T., Anna L. and Overweter, A.L., 2005. Effects of a-tocopherol supplementation of rotifers on the growth of striped trumpeter *Latris lineata* larvae. Aquaculture, 246, 367–378.

Burton, G.W. and Ingold, K.U., 1989. Vitamin E as an in vitro and in vivo antioxidant. Ann. NY Acad. Sci., 570, 7 –22.

Burton, G.W., Joyce, A. and Ingold, K.U., 1982. First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. Lancet 2, 327.

Castell, J.D., Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). Aquaculture, 128, 315– 333.

Castell, J.D., Blair, T., Neil, S., Howes, K., Mercer, S., Reid, J., Young-Lai, W., Gullison, B., Dhert, P. and Sorgeloos, P., 2001. The effect of different HUFA enrichment emulsions on the nutritional value of rotifers (*Brachionus plicatilis*) to larval haddock (*Melanogrammus aeglefinus*). In: Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloos, P. (Eds.), Larvi 01 Fish and Shellfish Larviculture

Symposium European Aquaculture Society, Special Publication, vol. 30. Oostende, Belgium, pp. 111 –114.

Chávez De Martínez, M.C., 1990. Vitamin C requirement of the Mexican native cichlid *Ciclasoma urophtalmus* (Gunther). Aquaculture, 86, 409-416.

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K. and Lee, K.J., 2004. Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). Aquaculture, 242, 553–569.

Chien, L.T., Hwang, D.F. and Jeng, S.S., 1999. Effect of thermal stress on dietary requirement of vitamin C in Thornfish *Terapon jarbua*. Fish. Sci., 65, 731–735.

Copeman, L.A., Parrish C.C., Brown, J.A. and Harel, M., 2002. Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. Aquaculture, 210, 285–304.

Cort, W.M., 1974. Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid, and their mode of action. Journal of the American Oil Chemists Society, 51, 321–325.

Cuesta, A., Esteban, M.A., Meseguer, J., 2002. Natural cytotoxic activity in seabream (*Sparus aurata* L.) and its modulation by vitamin C. Fish Shellfish Immunol., 13, 97–109.

Dabrowski, K., 1990. Absorption of ascorbic acid and ascorbic sulfate and ascorbate metabolism in stomachless fish, common carp. J. Comp. Physiol., B 160, 549–561.

Dabrowski, K., Hinterleitner, C., Sturmbauer, C., El-Fiky, N. and Wieser, W., 1988. Do carp larvae require vitamin C. Aquaculture, 72, 295-306.

Dabrowski, K., Moreau, R., El-Saidy, D. and Ebeling, J., 1996. Ontogenetic sensitivity of channel catfish to ascorbic acid deficiency. J. Aquat. Anim. Health, 8, 22-27.

Dantagnan, H., Bórquez, A.S., Valdebenito, I.N., Salgado, I.A., Serrano, E.A. and Izquierdo M.S., 2007. Lipid and fatty acid composition during embryo and larval development of puye Galaxias maculatus Jenyns, 1842, obtained from estuarine, freshwaterand cultured populations. J. Fish Biol., 70, 770–781.

Estévez, A., McEvoy, L.A., Bell, J.G. and Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot larvae fed live-prey enriched in arachidonic (ARA) and eicosapentaenoic (EPA) acids. Aquaculture, 180, 321–343.

FAO, 1999, Manual on Hatchery Production of Sea bass and Gilthead Sea bream. pp 5.

Fernández-Palacios, H., Izquierdo, M.S., Gonzalez, M., Robaina, L. and Valencia, A., 1998. Combined effect of dietary a-tocopherol and n-3 HUFA on egg quality of gilthead seabream broodstock (*Sparus aurata*). Aquaculture, 161, 475–476.

Filho, D.W., Giulivi, C. and Boveris, A., 1993. Antioxidative defences in marine fish: Teleosts. Comp. Biochem. Physiol., 106, 409– 413.

Fournier, V., Gouillou-Coustans, M.F. and Kaushik, S.J., 2000. Hepatic ascorbic acid saturation is the most stringent response criterion for determining the vitamin C requirement of juvenile European sea bass (*Dicentrarchus labrax*). *Journal of Nutrition,* 130, 617–620.

Frigg, M., Prabucki, A.L. and Ruhdel, E.U., 1990. Effect of dietary vitamin E levels on oxidative stability of trout fillets. Aquaculture, 84, 145-158.

Furones, M.D., Alderman, D.J., Bucke, D., Fletcher, T.C., Knox, D. and White, A., 1992. Dietary vitamin E and the response of rainbow trout, *Oncorhynchus mykiss* (Walbaum), to infection with Yersinia ruckeri. J. Fish Biol., 41, 1037-1041.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S., 2005. Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 142, 410–418.

Ganga, R., Tort. L., Acerote, L., Montero, D. and Izquierdo, M.S., 2006. Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream (*Sparus aurata*). Journal of Endocrinology, 190, 39–45.

Gatesoupe, F.J. and Le Milinaire, C., 1985. Adaptation de la qualite limentaire des filtreurs-proies aux besoins nutritifs des larvae de poissons marins. Coll. Fr.-japon Oceanogr., Marseille, 16-21 Sept., 8, 51-63.

González, M.M., Izquierdo, M.S., Salhi, M., Hernández- Cruz, C.M. and Fernández-Palacios, H., 1995. Dietary vitamin E for *Sparus aurata* larvae. Europ. Aqua. Soc. Spec. Publ., 24, 239–242.

Gouillou-Coustans, M.F., Bergot, P. and Kaushik, S.J., 1998. Dietary ascorbic acid needs of common carp (*Cyprinus carpio*) larvae. Aquaculture, 161, 453–461.

Halliwell, B. and Gutteridge, J.M.C., 1996. Lipid peroxidation: a radical chain reaction. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Clarendon Press, Oxford, pp. 188– 266.

Halver, J.E., 1995. Vitamin requirement study techniques. J. Appl. Ichthyol., 11, 215–224.

Halver, J.E., 2002. The vitamins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego, CA, pp. 61–141.

Halver, J.E., Ashley, L.M. and Smith, R.E., 1969. Ascorbic acid requirements of coho salmon and rainbow trout. Trans. Am. Fish. Soc., 98, 762–772.

Hamre, K. and Lie, O., 1995a. Minimum requirement of vitamin E for Atlantic salmon (*Salmo salar* L) at first feeding. Aquac. Res., 26, 175–184.

Hamre, K. and Lie, O., 1995b. Alpha-tocopherol levels in different organs of Atlantic salmon (*Salmo salar* L.) effect of smoltification, dietary levels of n-3 polyunsaturated fatty acids and vitamin E. Comp. Biochem. Physiol., 111A, 547–554.

Harel, M., Tandler, A. and Kissil, G.W., 1994. The kinetics of nutrient incorporation into body tissues of gilthead seabream (*Sparus aurata*) females and the subsequent effects on egg composition and egg quality. Br. J. Nutr., 72, 45– 58.

Hastings, N., Agaba, M., Tocher, D.R., Leaver; M.J., Sargent, J.R. and Teale, A.J., 2001. A vertebrate fatty acid desaturase with delta5 and delta6 activity. Proceedings of the National Academy of Science, USA. 98, 14304-14309.

Henderson, R.J., Sargent, J.R., 1985. Fatty acid metabolism in fish. In: Cowey, C.B., Mackie, A.M., Bell, J.G. (Eds.), Nutrition and Feeding in Fish. Academic Press, London, pp. 349–364.

Henderson, R.J., Bell, M.V. and Sargent, J.R., 1985. The conversion of polyunsaturated fatty acids to prostaglandins by tissue homogenates of the turbot, *Scophthalmus maximus* (L.). J. Exp. Mar. Biol., Ecol. 85, 93–99.

Henrique, M.M.F., Gomes, E.F., Gouillou-Coustans, M.F., Oliva-Teles, A. and Davies, S.J., 1998. Influence of supplementation of practical diets with vitamin C on growth and response to hypoxic stress of seabream, *Sparus aurata*. Aquaculture, 161, 415–426.

Holmefjord, I. and Olsen, Y., 1991. An intensive approach to Atlantic halibut fry production. In: *Larví'9l-Fish & Crustacean Larviculture Symposium* (P. Lavens, P. Sorgeloos, E. Jaspers and F. Ollevier eds.). European Aquaculture Society, Special publication No. 15, Gent, Belgium. 1991, pp, 331-334.

Ibeas, C., Cejas, J., Gbmez, T., Jerez, S. and Lorenzo, A., 1996. Influence of dietary n-3 HUFA levels on juvenile gilthead seabream (*Spans aurata*) growth and tissue fatty acid composition. Aquaculture, 142, 221-235.

Ibeas C., Cejas J.R., Fores R., Badía P., Gómez T. and Lorenzo Hernández A., 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream *Sparus aurata* juveniles. Aquaculture, 150, 91-102.

Iijima, N., Tanaka, S. and Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem., 18, 59–69.

Izquierdo, M.S., 1988. Estudio de los requerimientos de ácidos grasos esenciales en larvas de peces marinos. Modificación de la composición lipídica de las presas. Thesis Doctoral, La Laguna University, Spain.

Izquierdo, M. S., 1996. Review article: essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr., 2, 183–191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Options Mediterr., 63, 91-102.

Izquierdo, M.S. and Fernández-Palacios, H., 1997. Nutritional requirements of marine fish larvae and broodstock. Cah. Options Mediterr., 22, 243–264.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989a. Optimal EFA levels in Artemia to meet the EFA requirements of red seabream *Pagrus major*.. In: Takeda, M., Watanabe, T. _Eds.., The Current Status of Fish Nutrition in Aquaculture. Japan Translation Centre, Tokyo, Japan, pp. 221–232.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989b. Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi, 55, 859–867.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem., 22, 97–107.

Izquierdo, M.S, Fernández-Palacios, H. and Tacon, A.G.J., 2001a. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture, 197, 25-42.

Izquierdo, M.S., Tandler, A., Salhı´, M. and Kolkkovski, S., 2001b. Influence of dietary polar lipids quantity and quality on ingestion and assimilation of labelled fatty acids by larval gilthead seabream. Aquacult. Nutr., 6, 153– 160.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L. and Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquacult. Nutr., 9, 397– 407.

Izquierdo, M.S., Robaina, L., Juárez-Carrilo, E., Oliva, V., Hernández-Cruz, C.M. and Afonso, J.M., 2008. Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae *Sparus aurata*. Fish Physiol. Biochem., 34, 117-127.

Kanazawa, K., 1991. Hepatotoxicity caused by dietary secondary products originating from lipid peroxidation. In: Friedman, M. (Ed.), Nutritional and Toxicology Consequences of Food Processing. Plenum, New York, pp. 237–253.

Kanazawa, K., 1993. Tissue injury induced by dietary products of lipid peroxidation. In: Corongiu, F. (Ed.), Free Radicals and Antioxidants in Nutrition. Richelieu Press, London, pp. 383– 399.

Kawatsu, H., 1969. Studies on the anemia of fish-III. An example of macrocytic anemia found in brook trout, *Salvelinus fontinalis*. Bulletin of the Freshwater Fish Research Laboratory, 19, 161–167.

Kitamura, S., Ohara, S. and Suwa, T., 1965. Studies on vitamin requirements of rainbow trout, *Salmo gairdneri* I. On ascorbic acid. Bull. Jpn. Soc. Sci. Fish, 31, 818-827.

Kolkovski S., Tandler A. and Kissil G. Wm., 1993. The effect of dietary enzymes with age on protein and lipid assimilation and deposition in *Sparus aurata* larvae. In: Fish Nutrition in Practice, Les Colloques, No. 61 (ed. INRA). INRA, Paris, France.

Kolkovski, S., Czesny, S., Yackey, C., Moreau, R., Cihla, F., Mahan, D. and Dabrowski, K., 2000. The effect of vitamins C and E in (n-3) highly unsaturated fatty acids-enriched Artemia nauplii on growth, survival, and stress resistance of fresh water walleye Stizostedion vitreum larvae. Aquac. Nutr., 6, 199–206.

Koven, W.M., 1991. The combined effect of dietary n-3 highly unsaturated fatty acids and age, on growth, survival and lipid composition in larval gilthead seabream (*Sparus aurata*, Perciformes, Teleostei). Ph.D. Thesis, Hebrew University, Israel, 130 pp.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A., 2001. The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture, 193, 107– 122.

Koven, W., Van Anholt, R., Lutzky, S., Ben Atia, I., Nixon, O., Ron, B. and Tandler, A., 2003. The effect of dietary arachidonic acid on growth, survival, and cortisol levels in different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or daily salinity change. Aquaculture, 228, 307–320.

Le Grusse, J. and Watier, B., 1993. Les vitamines. Données biochimiques, nutritionnelles et cliniques. Centre d'Etude et d'Information sur les Vitamines, Neuilly-Sur- Seine.

Lee, K.J. and Dabrowski, K., 2003. Interaction between vitamins C and E affects their tissue concentrations, growth, lipid oxidation, and deficiency symptoms in yellow perch (*Perca flavescens*). Br. J. Nutr., 89, 589–596.

Lemaire, P., Viarengo, A., Canesi, L. and Livingstone, D.R., 1993. Pro-oxidant and antioxidant processes in gas gland and other tissues of cod (Gadus morhua). J. Comp. Physiol., 163, 477– 486.

Lim, C. and Lovell, R. T., 1978. Pathology of vitamin C deficiency syndrome in channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 108, 1137–1146.

Lin, M-F. and Shiau, S-Y., 2005a. Dietary l-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, Epinephelus malabaricus. Aquaculture, 244, 215– 221.

Lin, Y-H. and Shiau, S-Y., 2005b. Dietary vitamin E requirement of grouper, *Epinephelus malabaricus*, at two lipid levels, and their effects on immune responses. Aquaculture, 248, 235– 244.

Liu, J., Caballero, M.J., El-Sayed, Ali.T., Izquierdo, M.S., Hernández Cruz, C.M., Valencia, A. and Fernández-Palacios, H., 2002. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream (*Sparus aurata*). Fish Science, 68, 1165–1172.

Machlin, L.J., 1984. Vitamin E. In: Machlin, L.J. (Ed.), Handbook of Vitamins. Marcel Dekker, New York, pp. 99– 145.

Madsen, L., Rustan-Arild, C., Vaagenes, H., Berge, K., Dyroy, E. and Berge, R.K., 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and

peroxisomal fatty acid oxidation in relation to substrate preference. Lipid, 34 (9), 951-963.

Mahajan, C. and Agrawal, N.K., 1980. Nutritional requirement of ascorbic acid by Indian major carp, *Cirrhina mrigala*, during early growth. Aquaculture, 19, 37–48.

McEvoy, L.A., Estévez, A., Bell, J.G., Shields, R.J., Gara, B. and Sargent, J.R., 1998. Influence of dietary levels of eicosapentaenoic and arachidonic acids on the pigmentation success of turbot (*Scophthalmus maximus* L.) and halibut (*Hippoglossus hippoglossus* L.). Bull. Aquacult. Assoc. Can., 4, 17– 20.

Merchie, G., Lavens, P., Storch, V., Ubel, U., De Nelis, H., Leenheer, A. and Sorgeloos, P., 1996. Influence of dietary vitamin C dosage on turbot *Scophthalmus maximus* and European sea bass *Dicentrarchus labrax* nursery stages. Comp. Biochem. Physiol., 114, 123-133.

Merchie, G., Lavens, P. and Sorgeloos, P., 1997. Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture, 155, 165–181.

Messager, J.L., Stephan, G., Quentel, C. and Baudin Laurencin, F., 1992. Effects of dietary oxidized fish oil and antioxidant deficiency on histopathology, haematology, tissue and plasma biochemistry of sea bass, *Dicentrarchus labrax*. Aquat. Living Resour., 5, 205–214.

Montero, D., Tort, L., Izquierdo, M.S., Socorro, J., Robaina, L., Vergara, J.M. and Fernández-Palacios, H., 1996. Effect of alphatocopherol and n - 3 HUFA deficient diets on blood cells, selected immune parameters and proximate body composition of gilthead seabream (*Sparus aurata*). In: Modulators of Immune Response. The Evolutionary Trail (Stolen, J.S., Fletcher, T.C., Bayne, C.J., Secombes, C.J., Zelikoff, J.L., Twerdock, L., & Anderson D.P., eds.) pp. 251–266. S.O.S. Publications, Fair Haven.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L. and Vergara, J. M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by

α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiology and Biochemistry, 18, 399–407.

Montero, D., Marrero, M., Izquierdo, M.S., Robaina, L., Vergara, J.M. and Tort, L., 1999. Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. Aquaculture, 171, 269– 278.

Montero, D., Tort, L., Robaina, L., Vergara, J.M. and Izquierdo, M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish and Shellfish Immunology, 11, 473–490.

Moreau, R., Dabrowski, K., Czesny, S. and Cihla, F., 1999. Vitamin C-vitamin E interaction in juvenile lake sturgeon (*Acipenser fulvescens* R.), a fish able to synthesize ascorbic acid. J. Appl. Ichthyol., 15, 250–257.

Mourente, G., Rodríguez, A., Tocher, D.R. and Sargent, J.R., 1993. Effects of dietary docosahexaenoic acid DHA; 22:6n-3.on lipid and fatty compositions and growth in gilthead sea bream *Sparus aurata* L.. larvae during first feeding. Aquaculture, 112, 79–98.

Mourente, G., Diaz-Salvago, E., Bell, J.G. and Tocher, D.R., 2002. Increased activities of hepatic anti-oxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. Aquaculture, 214, 343–361.

Mulero, V., Esteban, M. A. and Mesenguer, J., 1998. Effects of in vitro addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes. Veterinary Immunology and Immunopathology, 66, 185–199.

Murai, T. and Andrews, J.W., 1974. Interaction of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 104, 1416–1431.

Niki, E., 1987a. Antioxidants in relation to lipid peroxidation. Chemistry and Physics of Lipids, 44, 227–253.

Niki, E., 1987b. Interaction of ascorbate and α-tocopherol. Ann. N.Y. Acad. Sci., 498, 186–199.

NRC, 1993. Nutritional Requirements of Fish, pp. 114.National Academic Press, Washington DC.

Olsen, Y. and Skjervold, H., 1995. Variation in content of omega 3 fatty acids in farmed Atlantic salmon, with special emphasis on effect of non dietary factor. Aquac, Int., 3, 2-35.

Ortuño, J., Esteban, M.A. and Meseguer, J., 1999. Effects of high dietary intake of vitamin C on non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 9, 429– 443.

Ortuño, J., Esteban, A. and Meseguer, J., 2000. High dietary intake of α-tocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 10, 293–307.

Ortuño, J., Cuesta, A., Esteban, A. and Meseguer, J., 2001. Effect of oral administration of high vitamin C and E dosages on the gilthead seabream (*Sparus aurata* L.) innate immune system. Vet. Immunol. Immunopathol., 79, 167–180.

Ortuño, J., Esteban, M.A. and Meseguer, J., 2003. The effect of dietary intake of vitamins C and E on the stress response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 14, 145–156.

Ota. T. and Yamada, M., 1971. Lipid of masu salmon Onchorhynchus masou. I. Variations of the lipid content and fatty acid composition of juvenile masu salmon during the period of smolt-transformation, and the influence of light upon those variations. Bull. Fac. Fish. Hokkaido Univ., 22, 151-158.

Packer, J.E., Slater, T.F. and Wilson, R.L., 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature, 278, 737–738.

Paul, B.N., Sarkar, S.N. and Mohanty., 2004. Dietary vitamin E requirement of mrigal, *Cirrhinus mrigala* fry. Aquaculture, 242, 529-536.

Pousão-Ferreira, P., Santos, P., Carvalho, A.P., Morais, S. and Narciso, L., 2003. Effect of an experimental microparticulate diet on the growth, survival and fatty acid profile of gilthead seabream (*Sparus aurata* L.) larvae. Aquaculture International, 11, 491–504.

Putnam, M.E. and Comben, M., 1987. Vitamin E. Veterinary Record, 121, 541-545.

Radi, A.A.R. and Markovics, B., 1988. Effects of metal ions on the antioxidant enzyme activities, proteins contents and lipid peroxidation of carp tissues. Comp. Biochem. Physiol., 90C, 69– 72.

Rainuzzo, J.R., Reitan, K.I., Jorgensen, L. and Olsen, Y., 1994. Lipid composition in turbot fed live feed cultured by emulsions of different lipid classes. Comp. Biochem. Physiol., 107A, 699–710.

Reitan, K.I., Rainuzzo, J.R. and Olsen, Y., 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquaculture International, 2, 33-48.

Roberts, M.L., Davies, S.J. and Pulsford, A.L., 1995. The influence of ascorbic acid (vitamin C) on non-specific immunity in the turbot (*Scophthalmus maximus* L.). Fish Shellfish Immunol., 5, 27– 38.

Rodríguez, C., 1994. Estudio de los requerimientos de acidos grasos esenciales de la dorada europea *Sparus aurata* L. durante las dos primeras semanas de alimentacion. PhD Thesis, University of La Laguna, Spain.

Rodríguez, C., Pérez, J.A., Izquierdo, M.S., Mora, J., Lorenzo, A. and Fernández-Palacios, H., 1993. Essential fatty acid requirements of larval gilthead seabream, *Sparus aurata* L.. Aquacult. Fish. Manage., 24, 295–304.

Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S. and Cejas, J.R., 1994. n-3 HUFA requirement of larval gilthead seabream *Sparus aurata* when using high levels of eicosapentaenoic acid. Comp. Biochem. Physiol., 107A, 693–698.

Rodríguez, C., Pérez, J.A., Díaz, M., Izquierdo, M.S., Fernandez-Palacios, H. and Lorenzo, A., 1997. Influence of the EPA/DHA ratio in rotifers on gilthead seabream *Sparus aurata* larval development. Aquaculture, 150, 77–89.

Rodríguez, C., Pérez, J.A., Badia, P., Izquierdo, M.S., Fernández Palacios, H. and Hernández, A.L., 1998. The n-3 highly unsaturated fatty acids requirements of gilthead seabream (*Sparus aurata* L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture, 169, 9–23.

Roem, A.J., Kohler, C.C. and Stickney, R.R., 1990. Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. Aquaculture, 87, 155–164.

Ruff, N., P. Lavens, P., Huo, J.Z., Sorgeloos, P., Nelis, h.J. and DE Leenheer, A., 2001. Antioxidant effect of dietary tocopherol and ascorbic acid on growth and survival of *Litopenaeus vannamei* Postlarvae. Aquaculture International, 9, 115–126.

Sakai, T., Murata H., Endo, M., Yamauchi, K., Tabata, N. and Fukudome, M., 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola aquiqueradiata*. Agricultural and Biological Chemistry, 53, 1739–1740.

Salhi, M., Izquierdo, M.S., Hernández-Cruz, C.M., Gonzalez, M. and Fernández-Palacios, H., 1994. Effect of lipid and n-3 HUFA levels in microdiets on growth survival and fatty acid composition of larval gilthead seabream *Sparus aurata*. Aquaculture, 124, 275–282.

Salhi, M., Izquierdo, M.S., Hernández-Cruz, C.M., Bessonart, M. and Fernández-Palacios, H., 1999. Effect of different dietary polar lipid levels and different n-3 HUFA content in polar lipids on the gut and liver histological structure of seabream *Sparus aurata* larvae. Aquaculture, 179, 253–264.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1994. The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: Lahlou, B., Vitiello, P. (Eds.), Aquaculture: Fundamental and Applied Research. American Geophysical Union, pp. 103–113.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol., 11, 183– 198.

Sargent, J.R., McEvoy, L.A. and Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEnvoy, L., Tocher, D. and Estevez A., 1999a. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177,191-199.

Sargent, J.R., McEvoy, L.A., Estevez, A., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1999b. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture, 179, 217–229.

Saroglia, M. and Scarano, G., 1992. Experimental induction of ascorbic deficiency in sea bass in intensive aquaculture. Bull. Eur. Ass. Fish Pathol., 12, 97–99.

Sau, S.K., Paul, B.N., Mohanta, K.N. and Mohanty, S.N., 2004. Dietary vitamin E requirement, fish performance and carcass composition of rohu, *Labeo rohita* fry. Aquculture, 240, 359–368.

Schwarz, F.J., Kirchgessuner, M., Steinhart, H. and Runge, G., 1988. Influence of different fats with varying additions of α-tocopherol acetate on growth and body composition of carp (*Cyprinus carpio* L.). Aquaculture, 69, 57–67.

Sealey, W.M. and Gatlin III, D.M., 2002. Dietary vitamin C and vitamin E interact to influence growth and tissue composition of juvenile hybrid striped bass (*Morone chrysops XM. Saxatilis*) but have limited effects on immune responses. J. Nutr., 132, 748– 755.

Seiliez, I., Panserat, S., Corraze, G., Kaushik, S. and Bergot, P., 2003. Cloning and nutritional regulation of a Δ6-desaturase-like enzyme in marine teleost gilthead seabream (*Sparus aurata*). Comparative biochemistry and physiology, Part B: Biochemistry and Molecular Biology., 135, 449-460.

Sies, H. and Murphy, M., 1991. Role of tocopherols in the protection of biological systems against oxidative damage. J. Photochem. Photobiol., B8, 211– 224.

Stéphan, G., Guillaume, J. and Lamour, F., 1995. Lipid peroxidation in turbot (*Scophthalmus maximus*) tissue: effect of dietary vitamin E and dietary n-6 or n-3 polyunsaturated fatty acids. Aquaculture, 130, 251-268.

Teles, O.A., 2000. Recent advances in European sea bass and gilthead sea bream nutrition. Aquaculture International, 8, 477–492.

Teshima, S.I., Kanazawa, A., Koshio, S. and Itoh, S., 1993. L-Ascorbyl-2-phosphate-Mg as vitamin C source for the Japanese flounder *(Paralichthys oliuaceus*). In: S.J. Kaushik and P. Luquet (Editors), Fish Nutrition in Practice. Coll. Les. Colloq., No. 61. INRA, Paris, pp. 157-166.

Thorarinsson, R., Landolt, M.L., Elliott, D.G., Pascho, R.J. and Hardy, R.W., 1994. Effect of dietary vitamin E and selenium on growth, survival and the prevalence of *Renibacterium salmoninarum* infection in chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture, 121, 343–358.

Tocher D.R., Mourente G., Van der Eecken A., Evjemo J.O., Diaz E., Bell J.G., Geurden I., Lavens P. and Olsen Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquaculture Nutrition, 8, 195–207.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz E., Wille, M., Bell J.G. and Olsen, Y., 2003. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International, 11, 195–216.

Waagbo, R., Sandnes, K., Torrissen, O. J., Sandvin, A. and Lie, É., 1993. Chemical and sensory evaluation of fillets from Atlantic salmon (*Salmo salar*) fed three levels of n-3 polyunsaturated fatty acids at two levels of vitamin E. Food Chemistry, 46, 361-366.

Wang, X.J., Kim, K.W., Bai, S.C., Huh, M.D. and Cho, B.Y., 2003. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). Aquaculture, 215, 21– 36.

Wang Z., Mai K., Liufu Z., Ma H., Xu W., Ai Q., Zhang W., Tan B. and Wang X., 2006. Effect of high dietary intakes of vitamin E and n-3 HUFA on immune responses and resistance to Edwardsiella tarda challenge in Japanese flounder (*Paralichthys olivaceus*, Temminck and Schlegel) Aquaculture Research, 37, 681-692.

Watanabe, T., 1982. Lipid nutrition in fish. Comp. Biochem. Physiol., 73, 3-15.

Watanabe, T., 1993. Importance of docosahexanoic acid in marine larval fish. J. World Aquacult. Soc., 24, 152– 161.

Watanabe, T. and Kiron, Y., 1994. Prospects in larval fish dietetics. Aquaculture, 124, 223–251.

Watanabe, T., Takashima, F., Ogino, C. and Hibiya T., 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi, 36, 623–630.

Watanabe, T., Takeuchi, T., Matsui, M., Ogino, C. and Kawabata, T., 1977. Effect of a-tocopherol deficiency on carp: VII. The relationship between dietary levels of linoleate and a-tocopherol requirement. Bull. Jpn. Soc. Sci. Fish, 43, 935– 946.

Watanabe, T., Kitajima, C., Arakawa, T., Fukusho, K. and Fujita, S., 1978a. Nutritional quality of rotifer *Brachionus plicatilis* as a living feed from the viewpoint of essential fatty acids for fish. Nippon Suisan Gakkaishi, 44, 1109– 1114.

Watanabe, T., Arakawa, T., Kitajima, C., Fukusho, K. and Fujita, S., 1978b. Nutritional quality of living feed from the viewpoint of essential fatty acids for fish. Nippon Suisan Gakkaishi, 44, 1223– 1227.

Watanabe, T., Takeuchi, T. and Wada, M., 1981. Dietary lipid levels and a-tocopherol requirement of carp. Nippon Suisan Gakkaishi, 47, 1585-1590.

Watanabe, T., Kitajima, C. and Fujita, S., 1983. Nutritional value of live organisms used in Japan for mass propagation of fish: a review. Aquaculture, 34, 115–143.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C., 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. Nippon Suisan gakkaishi, 55 (9), 1635-1 640.

Wdzieczak, J., Zalesna, G., Wujec, E. and Peres, G., 1982. Comparative studies on superoxide dismutase, catalase and peroxidase levels in erythrocytes and livers of different freshwater and marine fish species. Comp. Biochem. Physiol., 73B, 361– 365.

Willey, S., Bengtson, D.A., Harel, M., 2001. Arachidonic acid requirements for larval summer flounder (*Paraalichthys dentatus*). In: Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloos, P. (Eds.), Larvi 01—Fish and Shellfish Larviculture

Symposium European Aquaculture Society, Special Publication, vol. 30. Oostende, Belgium, pp. 634–637.

Wilson, R.P. (ed.) 1991. Handbook of Nutrition Requirements of finfish. C.R.C. Press, Bocaraton, 196 pp.

Wilson, R.P., Bowser, P.R. and Poe, W.E., 1984. Dietary vitamin E requirement of fingerling channel catfish. J. Nutr., 114, 2053– 2058.

Winston, G.W. and Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol., 19, 137– 161.

Woodall, A.N., Ashley, L.M., Halver, J.E., Scott, H.S. and Van de Veen, J., 1964. Nutrition of salmonid fishes: XIII. The α-tocopherol requirement of chinook salmon. J. Nutr., 84, 125– 135.

Yúfera M. and Pascual E., 1984. La producción de organismos zooplanctónicos para la alimentación larvaria en acuicultura marina. Informes Técnicos Instituto de Investigación Pesquera, 119, 3–27.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and methods

2.1. Fish

2.1.1. Gilthead sea bream

Gilthead sea bream (*Sparus aurata*) larvae were obtained from natural spawning from broodstocks at the GIA (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain) facilities where all experiments were carried out. In sea bream trials, larvae were previously fed enriched Selco (DHA Protein Selco, INVE, Dendermonde, Belgium) rotifers (*Brachionus plicatilis*), until they were 18 days old. At day 18th larvae were randomly distributed into the experimental tanks and were fed one of the experimental diets tested in triplicates.

2.1.2. Sea bass

Sea bass (*Dicentrarchus labrax*) larvae were obtained from natural spawnings from France (Ecloserie Marine de Gravelines, Gravelines, France). The larvae were previously fed enriched Selco (DHA Protein Selco, INVE, Dendermonde, Belgium) rotifers (*Brachionus plicatilis*) followed by a commercial microdiet (Commercial microdiet, Skretting, Norway) until they reached 18 and 35 days old.

2.2. Experimental conditions

All experiments and laboratory analysis presented in the thesis were carried out in the GIA facilities (ICCM & IUSA), Canary Islands, Spain, between 2005 and 2007.

All tanks were supplied with marine water (about 37 ppm salinity) filtered by 50μm mesh. Light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). Temperature and oxygen were daily measured by using an Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, USA). Tanks were daily cleaned by hand between 18:00 and 20:00 pm with a hose by a siphon system.

All tanks (170 L light grey colour cylinder fibreglass tanks, Fig. 1) were supplied with filtered sea water previously stored in a 500 l tank for degasification. Tanks were subjected to an open circulation at different water flowing rates which were increasingly adjusted along the feeding trials. Water quality was daily tested and no deterioration was observed. Water was continuously aerated (125ml/min). Water temperature and dissolved oxygen were daily recorded at 15:00 (attaining 5-8 ppm) and saturation ranged between 60 and 80% in all experimental tanks. Photoperiod was kept at 12h light: 12h dark by fluorescent lights.

2.3. Diet and feeding

2.3.1. Diet formulation

Several isonitrogenous and isolipidic (16,8-20.56 y 70.01-71.1 protein/lipid respectively) experimental microdiets (Pellet size < 250 μm) having differing ratios of DHA, EPA, ARA, vitamin E or vitamin C were formulated in the different experiments using: EPA50 oil and DHA50 oil in a triglycerides form as an sources of EPA and DHA (CRODA, East Yorkshire, England, UK), DHA45, EPA45 and ARA44 (Polaris, Pleuven, France) as DHA, EPA and ARA sources and vitamins E (Sigma-Aldrich, Madrid, Spain) and C (ROVIMIX Stay-C-35, Roche, Paris, France). The desired lipid content was completed with a non essential fatty acid source, oleic acid (Merck, Darmstadt, Germany). The protein source used squid meal (Riber & Son, Bergen, Norway) which was defatted (3 consecutive times with a chloroform:meal ratio of 3:1) to allow a better control of the microdiet fatty acid profile. The defatted squid meal (2.6 % lipid content), DHA50, DHA45, EPA50, EPA45 and ARA44 fatty acids profiles are shown in Table 1.

2.3.2. Microdiets preparation

The microdiet was prepared in the followed manner: the squid powder was carefully mixed with the other hidrosoluble ingredients (attractants, minerals and hidrosoluble vitamins shown in Table 2, Sigma-Aldrich, Madrid, Spain) in a mortar. In a separated mixture, oils and fat-soluble vitamins were combined to obtain a homogeneous mix which was afterwards joint together with the powder mixture. Then, gelatin was dissolved in warm water and when its temperature was lower than 35ºC it was added to the rest of the previously mixed ingredients. The paste was compressed

pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain the desired particle size. Diets were analyzed for proximate and fatty acid composition of dry basis and each diet was tested in triplicate.

2.4. Larval feeding

Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00. In the sea bream trails, conducted with larvae which were younger than sea bass ones, rotifers that had been fed only baker's yeast for at least 7 days, to avoid any n-3 HUFA content, were added to the feeding tanks twice a day (at 12:00 and 16:00h) at a fixed ratio of 2 ind/ml at the beginning of the experiment and gradually reducing the density to 1 ind/ml at the end of the experiment.

To guarantee feed availability Daily Feed Supply was kept at 2 and 2.5 g/tank during the first and second week of feeding, respectively. After feeding, larvae were observed under the binocular microscope to determine feed acceptance. If apparent feed intake differences were observed along different experimental diets, diet acceptance was determined calculating the percentage of gut occupation by the microdiet by image analysis in pictures of 30 larvae/tank. For such study, 30 larvae per tank were taken, their abdominal cavity was observed in a stereoscope (Leica Wild M3Z, Optotek, California, USA) and the area of the gut occupied by digested matter was measured on the optic micrographs taken at a magnification of 25µm, using Image Pro Plus® (Media Cybernetics inc., Silver Springs, MD, USA) semiautomatic image analysis system.

2.5. Sampling

At the beginning, in the middle and at the end of each experiment, samples of alive larvae unfed during the night were taken to determine total length and whole body weight. At the end of the experiment, alive fed larvae were sampled for the activity test. Finally, to analyze biochemical composition, all the remaining larvae in each tank, after a starvation period of 12 h, were collected, washed with distilled water, sampled and kept at -80ºC in air free individually labelled plastic sampling bags until analysis.

2.6. Activity tests and survival

Before the end of the experiment an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and, subsequently allocating them in another tank supplied with clean seawater and aeration, to determine survival after 24 hours. In another stress test, 20 larvae/tank were translated in 15^{o}C sea water tanks, to determine survival after 24 hours. Final survival was calculated by individually counting all the alive larvae at the beginning and at the end of the experiment.

2.7. Growth evaluation

Growth was determined by measuring dry body weight and Total length of starved larvae. Whole body weight was determined by 4-3 replicates of 5-10 starved larvae washed with distilled water and dried in a glass slide at an oven at 110ºC until constant weight, for approximately 24 h, followed by 1 h periods. Total or standard length of 20-30 anesthetised larvae from each tank were measured in a Profile Projector (V-12A Nikon, Nikon Co., Tokyo, Japan).

Specific growth rate (SGR)

SGR was determined according to the equation:

SGR= {(ln final body weight) – (ln initial body weight)} X 100/t

Where t = days of the feeding period.

2.8. Biochemical Analysis

All samples of larvae and diets were taken out from –80ºC freezers and melt and homogenized inside the sampling bag to avoid water evaporation.

2.8.1. Moisture

Moisture content was determined by thermal drying to constant weight in an oven at 110 ºC, with a first 24 h drying period, followed by 1 h periods until weight was not reduced any further. Sample weight was recorded before drying and after each drying period, following the cooling in a desiccator to weight always at the same temperature. Moisture was expressed as a percentage of the weight according to Official Methods of Analysis (A.O.A.C., 1995), using the following equation:

Moisture content % = (100 (B - A) – (C – A))/ (B – A)

Where:

A= weight of empty flask

B= weight of wet sample + flask

C= weight of dry sample + flask

2.8.2. Protein

Protein analysis was done according to Kjeldahl Method (A.O.A.C., 1995), which measures the total nitrogen content in the sample, and converting this figure to a total crude protein value by multiplying by the empirical factor 6.25.

2.8.3. Total lipids

Lipids were extracted following the method of Folch *et al*. (1957). Weight samples of diets and whole body larvae (0.1-0.2g) were homogenised in 10 ml chloroform: methanol (2:1 v:v) mixture by ultra turrax during 5 min. Lipid was then separated by centrifugation during 5 min (2000 rpm), the lower chloroform phase containing the lipid was carefully removed and evaporated to obtain lipid weight.

2.8.4. Separation of polar lipids

Neutral and polar fractions of the larval total lipids were separated by adsorption chromatography on silica cartridges (Sep-pak; Waters S.A., Massachussets, USA) using 30 ml chloroform and 20 ml chloroform/methanol (49: 1, v/v) as solvent for neutral lipid, followed by a 30 ml methanol wash to obtain the polar fractions according to Juaneda and Rocquelin (1985).

2.8.5. Fatty acid methyl esters preparation and quantification

Total or polar lipids were dissolved in toluene and fatty acid methyl esters obtained by transmethylation with 1 % sulphuric acid in methanol (Christie, 1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16h at 50 ºC. Afterwards, fatty acid methyl esters were extracted with hexane: diethyl ether (1:1 v/v) and purified by adsorption chromatography on NH2 Sep-pack cartridges (Waters S.A., Massachussets, USA) as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused

silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC/min and then held at 215 ºC for 10 min, following the conditions described in Izquierdo *et al*. (1990). Fatty acid methyl esters were quantified by FID and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd Tokyo; Japan).

2.9.Statistical analysis

Mean and standard deviations were calculated for each parameter measured. All data were passing by normality test and transformation was done in none normality data. Differences among groups were determined using one-way ANOVA and means were compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA). For Analysis of two-ways for growth data (weight, length, SGR, biomass), normality and homogeneity of variance were checked, and following general linear model was used:

$$\mathbf{Y}_{ijk} = \mu + \mathbf{F}_i + \mathbf{H}_j + (\mathbf{FH})_{ij} + \varepsilon_{ijk}$$

Where $\mathbf{Y}_{ijk}$ is the mean value of the tank, μ is the mean population, $\mathbf{F}_i$ is the fixed effect of the first factor (vitamin for example), $\mathbf{H}_j$ the fixed effect of the second factor (PUFA for example), $(\mathbf{FH})_{ij}$ the interaction between fixed effects, and ε_{ij} is the residual error.

Table 2.1. Main fatty acids of the major dietary lipid sources used in these experiments

	DHA 50	EPA 50	DHA 45	EPA 45	ARA 44	Defatted squid meal
14:0	0.031	0.042	0.361	1.196	0.428	1.685
14:1n-5	0.202	0.245	n.d.	n.d.	n.d.	0.036
14:1n-7	0.003	0.011	n.d.	0.053	n.d.	0.026
15:0	0.004	0.009	0.042	0.159	0.133	n.d.
15:1n-5	0.005	n.d.	n.d.	n.d.	n.d.	0.036
16:0iso	0.008	0.016	0.047	0.030	n.d.	0.108
16:0	1.214	0.362	3.226	5.151	8.250	23.808
16:1n-9	0.000	n.d.	n.d.	n.d.	n.d.	0.02
16:1n-7	0.472	0.427	1.099	2.000	0.375	0.517
Me 16:0	0.010	0.011	n.d.	n.d.	n.d.	0.097
16:1n-5	0.020	n.d.	n.d.	0.048	n.d.	0.163
16:2n-6	n.d.	0.054	0.095	0.122	n.d.	0.06
16:2n-4	0.158	0.494	n.d.	0.041	n.d.	0.485
17:0	0.121	n.d.	0.218	0.407	0.228	0.056
16:3n-4	0.097	0.212	0.326	0.375	0.038	0.072
16:3n-1	0.060	0.145	0.254	0.455	n.d.	0.116
16:4n-3	n.d.	0.002	0.133	0.112	n.d.	0.381
16:4n-1	n.d.	0.326	0.251	0.380	n.d.	0.034
18:0	2.316	0.271	5.749	2.652	6.929	3.639
18:1n-9	4.921	1.812	10.061	6.417	13.652	1.735
18:1n-7	0.870	0.555	4.149	1.329	0.779	0.941
18:1n-5	0.043	n.d.	0.109	0.068	n.d.	0.238
18:2n-9	n.d.	0.445	0.292	0.045	0.063	n.d.
18:2n-6	0.588	2.159	1.213	0.853	9.069	0.241
18:2n-4	0.074	0.679	0.580	0.122	n.d.	n.d.
18:3n-6	0.342	0.734	0.447	0.279	n.d.	n.d.
18:3n-4	0.038	0.439	n.d.	n.d.	0.238	n.d.
18:3n-3	0.204	1.670	0.769	0.313	2.568	n.d.
18:3n-1	n.d.	0.317	3.506	0.873	n.d.	n.d.
18:4n-3	0.312	10.441	0.429	0.055	n.d.	0.066
18:4n-1	0.001	1.007	n.d.	0.021	1.014	0.002
20:0	0.727	0.417	1.175	0.447	n.d.	0.004
20:1n9+n7	3.985	0.395	3.361	2.650	0.724	2.804
20:1n-5	n.d.	n.d.	0.371	0.242	n.d.	n.d.
20:2n-9	0.071	0.410	0.721	0.060	n.d.	n.d.
20:2n-6	0.543	0.128	0.256	0.293	n.d.	0.197
20:3n-9 +7	0.216	0.720	0.522	0.072	0.454	n.d.
20:3n-6	n.d.	n.d.	0.033	0.078	4.041	n.d.
20:4n-6	2.456	3.494	2.094	1.531	50.354	0.493
20:3n-3	0.443	0.114	0.178	0.179	n.d.	0.344
20:4n-3	0.916	2.486	2.245	0.661	n.d.	0.062
20:5n-3	12.662	46.483	43.232	15.582	0.113	4.315
22:1n-11	2.298	0.313	0.355	2.369	n.d.	0.041
22:1n-9	1.208	0.737	0.352	0.501	n.d.	0.106
22:4n-6	2.843	0.490	0.257	1.590	n.d.	0.126
22:5n-6	0.190	0.000	0.088	0.278	n.d.	n.d.
22:5n-3	5.527	3.209	2.057	3.609	0.124	n.d.
22:6n-3	53.803	17.721	9.345	46.304	0.426	9.397

*n.d.=not detected.

Table 2.2. Mix of attractants, minerals and vitamins

Attractants		mg/100g MD
	Inosine-5-monophosphate	500
	Betaine	660
	L-Serine	170
	L-Tyrosine	170
	L-Phenylalanine	250
	DL-Alanine	500
	L-Sodium Aspartate	330
	L-Valine	250
	Glycine	170
	Total	3000.000
VITAMINS		
Hydro-soluble vitamins		mg/100g MD
	Cyanocobalamin	0.030
	Astaxanthin	5.000
	Folic Acid	5.440
	Pyridoxine-HCl	17.280
	Thiamine-HCl	21.770
	Riboflavin	72.530
	Calcium Pantothenate	101.590
	p-aminobenzoic acid	145.000
	Ascorbic polyphosphate(V.C)	180.000
	Nicotinic Acid	290.160
	myo-Inositol	1450.900
	Sub Total	2289.700
Lipo-soluble vitamins		mg/100g MD
	Retinol acetate	0.180
	Ergocalciferol	3.650
	Menadione	17.280
	α-Tocopherol acetate	150.000
	Sub total	171.110
	Choline chloride	2965.800
	Total	5426.610
Minerals		mg/100g MD
	NaCl	215.133
	MgSO4.7H2O	677.545
	NaH2PO4.H2O	381.453
	K2HPO4	758.949
	Ca(H2PO4).2H2O	671.610
	FeC6H5O7	146.884
	C3H5O3.1/2Ca	1617.210
	Al2(SO4)3.6H2O	0.693
	ZnSO4.7H2O	14.837
	CuSO4.5H2O	1.247
	MnSO4.H2O	2.998
	KI	0.742
	CoSO4.7H2O	10.706
	Total	4500.007

Fig. 2.1. Tanks used for larval rearing during feeding experiments.

Fig. 2.2. Larval microdiet formulated and prepared for the feeding experiments.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Juaneda, P. and Rocquelin, G., 1985. Rapid and convenient separation of phospholipids and non-phosphorous lipids from rat heart using silica cartridges. Lipids, 20, 40–41.

CHAPTER 3

EFFECT OF DIFFERENT DIETARY EPA/DHA RATIOS ON INCORPORATION OF ESSENTIAL FATTY ACIDS IN GILTHEAD SEA BREAM LARVAE *Sparus aurata* (Linnaeus, 1758)

E. Atalah*, C. M. Hernández Cruz, A. Valencia, E. Ganuza, T. Benítez-Santana, R. Ganga, O. Vega-Orellana, J. Roo and M.S. Izquierdo.

Abstract

Dietary unbalances between eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) markedly reduce larval growth and survival in several fish species. The present study was conducted to precise in more detail the effect of dietary EPA/DHA ratios for gilthead seabream larvae fed microdiets with a wide range of values of these fatty acids for 14 and 17 days of feeding.

Twelve isonitrogenous and isolipidic microdiets differing in their EPA (0.3-4%) and DHA (0.6-9%) content were formulated. Average survival rates were significantly correlated with dietary EPA contents, highest values being obtained in larvae fed diets 4/1 and 4/3 (25.67% and 27.59%). Besides, for a given dietary EPA level, survival improved when larvae were fed higher DHA. Subsequently, higher dietary n-3 HUFA levels were associated with higher larval survival. Larval resistance to handling stress was higher with higher dietary EPA/DHA and EPA/ARA. Fish growth was improved by the elevation of dietary n-3 HUFA up to 8% in the diet and dietary DHA levels up to 6%, increase of dietary EPA/DHA over 1 or EPA/ARA over 10 markedly reduced growth, best growth obtained with EPA/ARA values of 7-8. Highest total length was found in larvae fed diets containing a (EPA+DHA+ARA)*(DHA/EPA)/ARA ratio close to 50. The average larvae lipid content of the whole larval body and the fatty acid composition reflected the dietary content of EPA, DHA and n-3 HUFA. Elevation of EPA/DHA ratio over 2, markedly decreased DHA incorporation into larval lipids

denoting the negative and competitive effect of EPA. This reduction in DHA content in the larvae was associated with a lower survival, except when EPA levels were as high as 4%. In conclusion, larval survival was markedly associated to dietary EPA, DHA and n-3 HUFA levels, whereas growth was more affected by DHA contents. Besides, EPA/DHA and EPA/ARA dietary ratios should not be increased over 1 and 8, respectively, to avoid negative effects on growth.

Keywords: *Sparus aurata*; larval nutrition; Stress resistance; EPA, DHA

Abbreviations: ARA: arachidonic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid.

Introduction

Gilthead sea bream (*Sparus aurata*) constitutes the most important marine fish species for Mediterranean aquaculture (Izquierdo, 2005) with an annual fry production surpassing 120 million/yr. Demand for good quality fry has been increasing at a rate of 10% annually, but the success in mass production of juveniles is greatly affected by effective first feeding regimes and the high nutritional quality of starter diets (Kolkovski *et al.,* 1993; Sargent *et al.,* 1997; Izquierdo *et al.,* 2000). Dietary lipids are recognized as one of the most important nutritional factors that affect larval growth and survival (Watanabe *et al.,* 1983) since they are determinant sources of metabolic energy and essential components for the normal formation of cell and tissue membranes and organ development (Izquierdo, 1996, Izquierdo *et al.,* 2003). Besides, essential fatty acids (EFA) play important physiological roles in fish as precursors of biologically active eicosanoids (Bell *et al.,* 1986). Several studies reported that marine fish species require docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids for normal growth, development and survival (Izquierdo *et al.,* 1989a,b, 1992,; Mourente *et al.,* 1993; Rodríguez *et al.,* 1993; Watanabe, 1993; Watanabe and Kiron; 1994, Izquierdo, 1996; Sargent *et al.,* 1999).

Despite both fatty acids share a number of common functions in fish metabolism, in particular, DHA is very effective in regulation of membrane integrity and function, being an important component of phosphoglycerides, such as phosphatidyl ethanolamine and phosphatidyl choline in fish larvae. This fatty acid also accumulates in neural tissues and sensory organs (Takeuchi, 2001) and it is specifically retained in starved or low-EFA fed fish (Izquierdo, 2005), possibly due to the lower cell

oxidation rates than other fatty acids (Madsen *et al.,* 1999). DHA is also a precursor of lypoxigenases derived eicosanoids, regulating steroid hormones release (Ganga *et al.,* 2006) and other aspects of fish metabolism. Hence, some studies have shown that it has a greater potential as an essential fatty acid for marine fish larvae than EPA (Watanabe *et al.,* 1989; Watanabe, 1993). EPA is also particularly important for larval growth (Watanabe *et al.,* 1989) and being a main precursor of prostaglandins in marine fish (Ganga *et al.,* 2005), plays very important roles in regulation of fish metabolism. Its presence in live preys enhances non specific lipase activity in the larvae (Izquierdo *et al.,* 2000). Moderate dietary levels of this fatty acid also enhance DHA incorporation into larval PL (Izquierdo *et al.,* 2000, 2001).

However, dietary unbalances among these two fatty acids, which compete among them in several physiological processes (Izquierdo *et al.,* 2000) have been found to markedly reduce larval growth and survival in several fish species (Mourente *et al.,* 1993; Reitan *et al.,* 1994; Rodríguez *et al.,* 1994, 1997), and hence, it is important to determine for each one the adequate EPA/DHA ratio (Watanabe and Kiron, 1994; Mourente *et al.,* 1993; Reitan *et al.,* 1994). In gilthead seabream, Rodríguez *et al.* (1994) reported that for the same dietary total content of EPA and DHA, a lower EPA/DHA ratio improves larval growth. In juveniles of this species Ibeas *et al.* (1997) suggested the dietary EPA/DHA ratio to be 2/1 for growth improvement, whereas 1/1.5 was recommended for larval stages (Rodríguez, 1994). In most of the ratios studies in this and other species, results are contradictory, since they are based in feeding live preys whose content on EPA/DHA ratio is difficult to completely control due to the own prey metabolism. Besides, numbers of replicates limited the range of EPA/DHA ratios applied. Finally, optimum EPA/DHA ratios may be also dependant on the total amount of both fatty acids in the diet. The present study was conducted to precise in more detail the effect of dietary EPA/DHA ratios for gilthead seabream larvae fed microdiets with a wide range of values of these fatty acids.

Materials and methods

Gilthead Sea Bream (*Sparus aurata)* larvae were obtained from natural spawnings from broodstocks at the GIA facilities (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain), where the experiment was carried out. Twelve experimental microdiets were tested in duplicate and in two different trials.

Gilthead seabream larvae were previously fed enriched rotifers (Selco, DHA Protein Selco, INVE, Dendermonde, Belgium), until they reached 18 days old. In trial 1, larvae (standard length: 4.98 ± 0.899 mm, mean±SD) were randomly distributed into the experimental tanks at a density of 2380 larvae/tank and were fed one of the experimental diets tested in triplicates for 14 days, at a water temperature of 19.2 to 21ºC. In Trial 2, larvae (standard length 6.92 ± 0.471 mm, dry body weight 310 µg) were randomly distributed into the experimental tanks at a density of 2000 larvae/tank and were fed one of the experimental diets tested in triplicates for 17 days, at a water temperature of 23 to 24.5 ºC.

All tanks (170 L light grey colour cylinder fibreglass tanks, were supplied with filtered sea water (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L/min along the feeding trials. Water flowed in at the top of the tank and get out from the bottom by using a siphon system, water quality was daily tested and no deterioration was observed. Water was continuously aerated (125ml/min) attaining 5-8 ppm dissolved O_2 and saturation ranged between 60 and 80% in all tanks. Photoperiod by fluorescent lights was kept at 12h light: 12h dark.

Table 3.1. Lipid sources (% total ingredients) and crude lipid (% dry basis) and moisture (% wet basis) content of the experimental diets

Diet (EPA/DHA)	EPA50[1]	DHA50[1]	Oleic acid[2]	Crude lipids	Moisture
0.3/0.6	0.00	1.30	11.70	16.04	10.91
1/1	1.60	0.80	10.60	15.90	11.47
1/3	0.70	4.80	7.50	17.29	14.03
1/6	0.00	8.60	4.40	16.66	10.57
1.5/8.5	3.50	9.50	0.00	16.83	11.63
1.5/9	12.00	1.00	0.00	17.16	12.45
2/7.5	4.00	7.50	1.50	17.35	9.9
2.5/1	4.00	0.00	9.00	16.67	11.67
2.5/2	3.00	4.00	6.00	16.93	12.29
2.5/6	1.90	8.20	2.90	16.90	11.44
4/1	6.00	0.00	7.00	16.95	11.9
4/3	5.30	3.30	4.40	16.99	10.35

[1] CRODA, East Yorkshire, England, UK.
[2] Oleic acid vegetable, Merck, Darmstadt, Germany.

Twelve isonitrogenous and isolipidic (70.1/16.80 formulated protein/lipid) experimental microdiets (pellet size < 250 µm) differing in their EPA (0.3-4%) and DHA (0.6-9%) content (Table 3.1) were formulated using EPA50 and DHA50 (CRODA, East Yorkshire, England, UK) as sources of EPA and DHA, and completing the desired lipid content with a non essential fatty acid source, oleic acid (Oleic acid vegetable, Merck, Darmstadt, Germany). The protein source used (squid meal) was

Table 3.2. Main fatty acid composition of the experimental diets for gilthead seabream fed microdiets containing different EPA/DHA proportions (g/100 g dry weight)

EPA/DHA	.3/0.6	1/1	1/3	1/6	1.5/8.5	1.5/9	2/7.5	2.5/1	2.5/2	2.5/6	4/1	4/3
Lipid d.w.	16.1	15.9	17.29	16.7	16.8	17.2	17.4	16.7	16.9	16.9	17.0	17.0
14:0	0.32	0.28	0.25	0.17	0.06	0.06	0.10	0.27	0.22	0.13	0.22	0.16
14:1n-5	0.08	0.07	0.05	0.03	0.00	0.00	0.01	0.06	0.05	0.02	0.05	0.03
14:1n-7	0.03	0.03	0.02	0.01	0.00	0.00	0.00	0.02	0.02	0.01	0.02	0.01
15:0	0.05	0.04	0.04	0.03	0.02	0.02	0.02	0.04	0.04	0.02	0.04	0.03
15:1n-5	0.03	0.02	0.02	0.01	0.01	0.00	0.01	0.02	0.02	0.01	0.02	0.01
16:0	1.21	1.06	1.10	1.05	0.87	0.92	0.91	1.17	1.09	0.94	1.01	0.90
16:1n-9	0.05	0.04	0.04	0.02	0.07	0.00	0.01	0.01	0.03	0.02	0.03	0.02
16:1n-7	0.70	0.60	0.50	0.33	0.00	0.07	0.16	0.56	0.44	0.22	0.45	0.32
16:1n-5	0.03	0.02	0.02	0.02	0.00	0.00	0.01	0.02	0.02	0.01	0.02	0.01
16:2n-6	0.11	0.10	0.08	0.05	0.00	0.00	0.02	0.09	0.07	0.03	0.07	0.05
16:2n-4	0.04	0.04	0.04	0.04	0.03	0.03	0.03	0.04	0.04	0.03	0.04	0.03
16:3n-4	0.22	0.21	0.16	0.10	0.01	0.02	0.05	0.18	0.14	0.07	0.15	0.10
18:0	0.22	0.20	0.26	0.31	0.39	0.40	0.34	0.20	0.26	0.30	0.18	0.21
18:1n-9	10.15	8.96	7.69	4.58	0.92	0.96	2.16	7.77	6.33	3.28	6.38	4.51
18:1n-7	0.05	0.04	0.51	0.38	0.15	0.15	0.25	0.43	0.42	0.31	0.42	0.37
18:1n-5	0.00	0.00	0.03	0.02	0.00	0.01	0.01	0.07	0.02	0.02	0.03	0.02
18:2n-9	0.05	0.02	0.04	0.02	0.01	0.00	0.01	0.03	0.03	0.00	0.02	0.02
18:2n-6	1.34	1.29	1.27	1.21	1.12	1.21	1.11	1.31	1.31	1.18	1.27	1.22
18:3n-3	0.13	0.15	0.15	0.15	0.16	0.17	0.16	0.17	0.18	0.17	0.19	0.20
18:4n-3	0.00	0.10	0.04	0.02	0.04	0.04	0.11	0.22	0.22	0.14	0.36	0.35
18:3n-1	0.00	0.01	0.00	0.00	0.00	0.00	0.06	0.02	0.00	0.01	0.03	0.03
18:4n-1	0.00	0.00	0.00	0.05	0.00	0.08	0.00	0.00	0.03	0.05	0.00	0.00
20:1n-9	0.12	0.11	0.15	0.31	0.39	0.05	0.04	0.06	0.20	0.04	0.00	0.00
20:1n-7	0.00	0.01	0.01	0.02	0.04	0.47	0.37	0.01	0.03	0.30	0.06	0.13
20:3n-6	0.00	0.00	0.03	0.04	0.07	0.07	0.06	0.00	0.00	0.04	0.00	0.02
20:4n-6	0.04	0.08	0.12	0.18	0.29	0.29	0.27	0.14	0.21	0.25	0.22	0.27
20:3n-3	0.00	0.00	0.02	0.04	0.06	0.06	0.05	0.00	0.03	0.00	0.02	0.00
20:4n-3	0.00	0.05	0.05	0.06	0.11	0.11	0.12	0.11	0.12	0.12	0.17	0.18
20:5n-3	0.28	1.14	1.03	1.02	1.60	1.62	2.18	2.56	2.70	2.37	4.09	4.14
22:1n-11	0.00	0.02	0.07	0.16	0.28	0.28	0.22	0.00	0.08	0.17	0.02	0.08
22:1n-9	0.00	0.01	0.03	0.08	0.14	0.14	0.11	0.00	0.03	0.08	0.00	0.03
22:4n-6	0.03	0.02	0.00	0.20	0.34	0.34	0.28	0.02	0.11	0.21	0.02	0.10
22:5n-6	0.00	0.00	0.00	0.01	0.00	0.02	0.00	0.00	0.01	0.00	0.00	0.00
22:5n-3	0.06	0.07	0.23	0.39	0.67	0.66	0.56	0.09	0.26	0.44	0.13	0.27
22:6n-3	0.62	0.99	3.19	5.48	8.94	8.86	7.49	0.93	2.09	5.84	1.12	3.09
Saturated	1.8	1.58	1.65	1.56	1.34	1.4	1.37	1.68	1.61	1.39	1.45	1.3
Monoenoics	11.24	9.93	9.14	5.97	2	2.13	3.36	9.03	7.69	4.49	7.5	5.54
n-3	1.09	2.5	4.71	7.16	11.58	11.52	10.67	4.08	5.6	9.08	6.08	8.23
n-6	1.52	1.49	1.5	1.69	1.82	1.93	1.74	1.56	1.71	1.71	1.58	1.66
n-9	10.37	9.14	7.95	5.01	1.53	1.15	2.33	7.87	6.62	3.42	6.43	4.58
n-3HUFA	0.96	2.25	4.52	6.99	11.38	11.31	10.4	3.69	5.2	8.77	5.53	7.68
ARA/EPA	0.14	0.07	0.12	0.18	0.18	0.18	0.12	0.05	0.08	0.11	0.05	0.07
EPA/DHA	0.45	1.15	0.32	0.19	0.18	0.18	0.29	2.75	1.29	0.41	3.65	1.34
Ratio *	52.0	24	112.0	199.4	208.7	203.1	126.5	9.4	18.4	83.4	6.8	20.7

(EPA+DHA+ARA)(DHA/EPA)/ARA

defatted (3 consecutive times with a chloroform:meal ratio of 3:1) to allow a better control of the microdiet fatty acid profile. The microdiets were prepared by mixing squid powder and water soluble components, then the lipid and fat soluble vitamins and finally, warm water solved gelatin. The paste was pelleted and dried in oven at 38 ºC for 24 h. Pellets were ground and sieved to obtain particle size below 250 µm. Diets were analyzed for proximate and fatty acid composition of dry basis (Tables 3.1 and 3.2). Diets were manually supplied fourteen times per day each 45 min from 9:00-19:00. Daily feed supplied was 2 and 2.5 g/tank during the first and second week of feeding, respectively.

Before the end of the experiment an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and, subsequently allocating them in another tank supplied with clean seawater and aeration, to determine survival after 24 hours. Final survival was calculated by individually counting all the alive larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC until constant weight) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish/tank at the beginning, in the middle and at the end of the trial.

The specific growth rate (SGR) was determined according to the equation: SGR= {(ln final body weight) – (ln initial body weight)} X 100/t, where t = days of the feeding period. Besides, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition after 12 hours of starvation at the end of the trials. Moisture (A.O.A.C., 1995), crude protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al.* (1990) and identified by comparison to previously characterized standards and GLC-MS.

All data were treated using one-way ANOVA and means were compared by Duncan's test ($P< 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results

All the experimental microdiets were well accepted by larvae. Final average survival rates for both trials (Table 3.3) were significantly correlated ($r^2=0.55$) with

dietary EPA contents (Fig. 3.1), highest values being obtained in larvae fed diets 4/1 and 4/3 (25.67% and 27.59%, respectively). Besides, for a given dietary EPA level (1, 1.5, 2.5 and 4), survival showed a tendency to improve when larvae were fed higher DHA dietary levels (Table 3.3, Fig. 3.2). Subsequently, in general higher dietary n-3 HUFA levels were associated with higher larval survival. Larval resistance to handling stress was significantly affected by the diet, being higher with higher dietary EPA/DHA (r^2=0.38) and EPA/ARA (r^2=0.30).

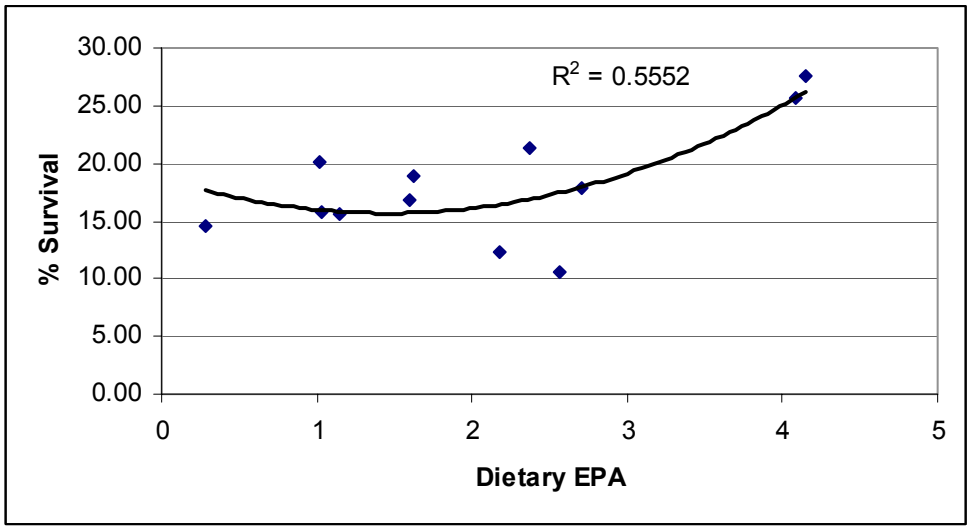

Figure 3.1. Effect of dietary EPA (% d.w.) on survival of gilthead seabream larvae fed different EPA/DHA ratio.

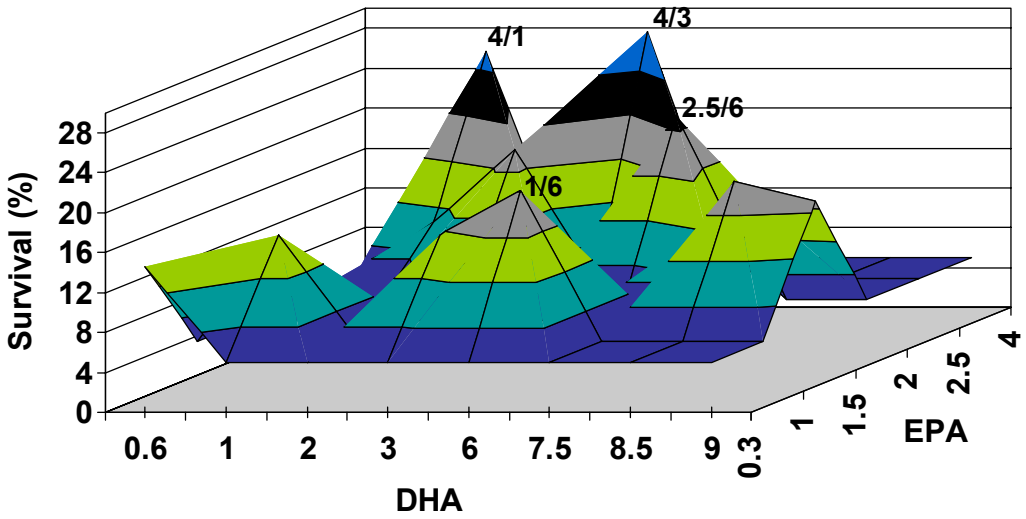

Figure 3.2. Effect of dietary EPA and DHA (% d.w.) on survival of gilthead seabream larvae fed different EPA/DHA ratio.

Table 3.3. Effect of dietary EPA and DHA levels on average survival and resistance to handling stress of larval gilthead seabream in both trials

EPA/DHA	Final survival	Survival after handling
0.3/0.6	14.60±8.8	67.50±17.7^{a}
1/1	15.68±10.7	52.50±10.6^{ab}
1/3	15.70±4.3	35.00±14.1^{ab}
1/6	20.15±5.7	45.00±7.1^{ab}
1.5/8.5	16.90±10.9	43.75±26.5^{ab}
1.5/9	18.88±10.4	45.00±28.3^{ab}
2/7.5	12.38±7.0	17.89±3.0^{b}
2.5/1	10.66±6.4	62.50±17.7^{ab}
2.5/2	17.89±4.4	45.28±14.5^{ab}
2.5/6	21.29±10.3	56.97±31.1^{ab}
4/1	25.67±21.7	72.50±24.7^{a}
4/3	27.59±9.1	48.95±12.7^{ab}

(Mean±SD; n=2; different letters in the same column denote significant differences)

Regarding fish growth, average final total length for both experiments (Table 3.4) was lowest for fish fed lower n-3 HUFA levels and improved when these fatty acids increased up to about 8% in the diet (Fig. 3.3). Total length was also markedly improved by the elevation of DHA dietary levels up to 6%, particularly in trial I (Table 3.4). Besides, in trial 1, larvae fed with dietary EPA/DHA values higher than 1 or EPA/ARA values higher than 10 showed lower final growth, whereas higher final total length was associated to EPA/ARA values of 7-8. Finally, in trial 1 highest total length was found in larvae fed diets containing a (EPA+DHA+ARA)*(DHA/EPA)/ARA ratio close to 50 (Fig. 3.4). No significant differences were found in final total length in trial 2.

Table 3.4. Effect of dietary EPA and DHA levels on final total length and body weight of gilthead sea bream in trials 1 and 2

	Final Total Length (mm)*	
Diet EPA/DHA	Trial 1 (mean±SD)	Trial 2 (mean±SD)
0.3/0.6	6.97±0.87^{a}	8.29±0.92^{ab}
1/1	6.15±0.68^{cd}	8.33±0.75^{ab}
1/3	6.73±1.01^{abc}	7.93±0.73^{b}
1/6	6.39±0.68^{abcd}	8.52±0.98^{ab}
1.5/8.5	6.51±0.93^{bcd}	8.27±0.83^{ab}
1.5/9	6.27±0.77^{abcd}	7.91±0.62^{b}
2/7.5	6.69±0.70^{abc}	8.21±0.89^{b}
2.5/1	5.91±0.78^{d}	8.39±0.81^{ab}
2.5/2	6.31±0.91^{bcd}	8.16±1.19^{b}
2.5/6	6.87±0.86^{ab}	8.18±0.86^{b}
4/1	6.51±1.25^{abcd}	8.46±1.02^{ab}
4/3	6.19±0.73^{cd}	8.86±1.1^{a}

*(Mean±SD; n=40; different letters in the same column denote significant differences)

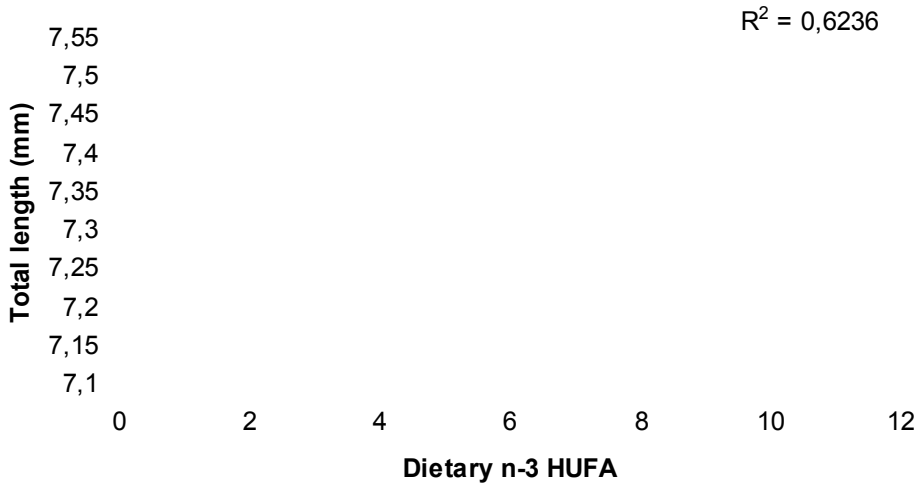

Figure 3.3. Effect of dietary n-3 HUFA (% d.w.) on average final total length of gilthead seabream larvae fed different EPA/DHA ratio.

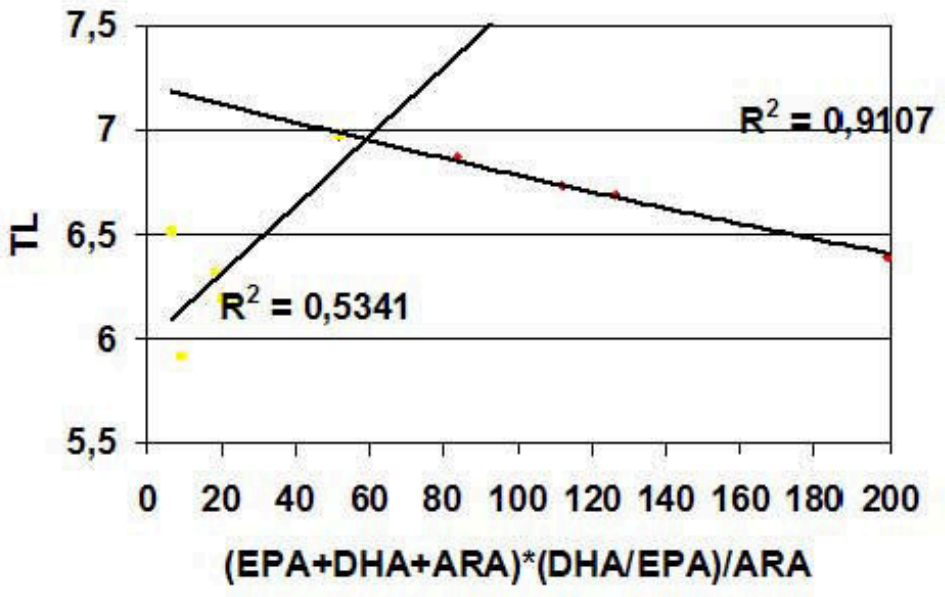

Figure 3.4. Effect of (EPA+DHA+ARA)*(DHA/EPA)/ARA on total length of gilthead seabream larvae fed several EPA/DHA ratio at the end of trial1.

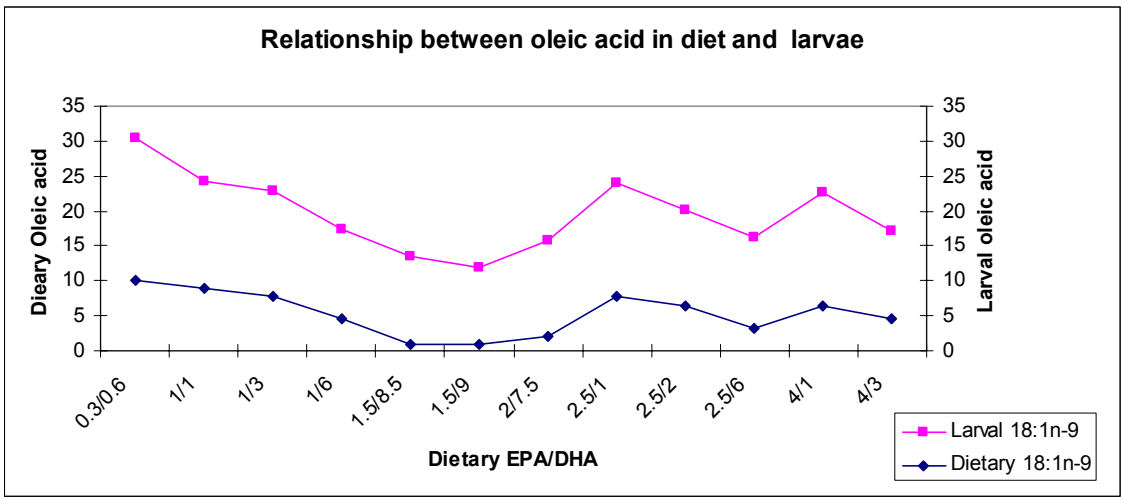

Figure. 3.5 Evolution of dietary oleic acid (% fatty acid) in diet and gilthead seabream larvae (% fatty acid) fed different EPA/DHA ratio.

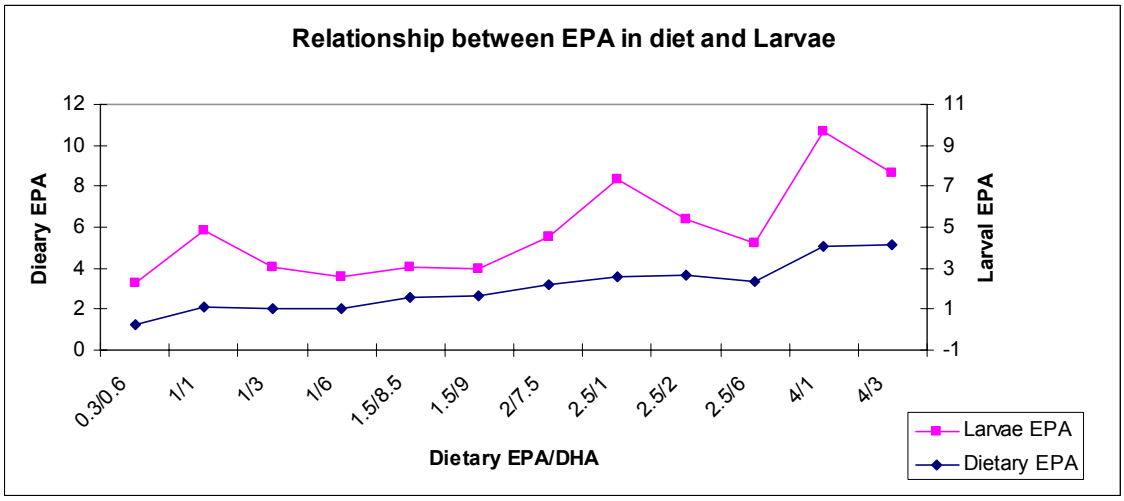

Figure 3.6. Evolution of EPA in diets and gilthead seabream larvae fed different EPA/DHA ratio.

The average larvae lipid content of the whole larval body and the fatty acid composition (Table 3.5) reflected the dietary content of EPA, DHA and n-3 HUFA. The elevation of dietary oleic acid from 0.92 to 10.15 (g/100g diet) induced an increase in the larval oleic acid content (Fig, 3.5) being significantly correlated (r^2 = 0.938). The larvae content of linoleic acid was not affected by dietary linoleic acid, but by the dietary n-6/ARA (r^2=0.92), suggesting a higher incorporation of linoleic acid into larval tissues when dietary ARA, together with EPA and DHA, were lower. However, dietary elevation of either EPA (Fig. 3.6, r^2= 0.82) or DHA markedly increased the incorporation of these fatty acids into larval tissues. On the contrary, elevation of EPA/DHA ratio over 2, markedly decreased DHA incorporation into larval lipds denoting the negative and competitive effect of EPA. This reduction in DHA content in

the larvae was associated with a lower survival, except when EPA levels were as high as 4%. While in the diet there were no 20:2N-9 and 18:3N-6 the larval fatty acid contain these fatty acid.

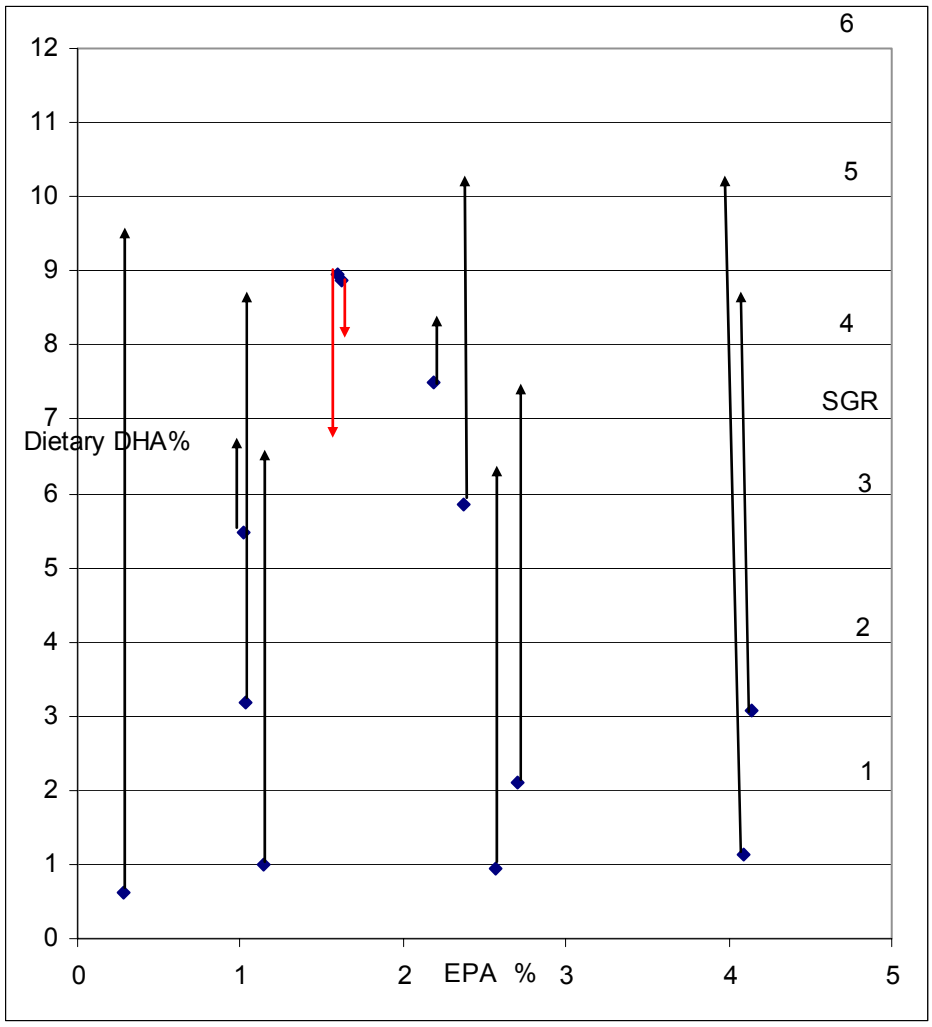

Figure 3.7. Graphical representation of EPA (x axis) and DHA (y axis) contents on diets fed to gilthead seabream and the larval growth (SGR, secondary y)

Discussion

Dietary lipids are recognized as one of the most important nutritional factors affecting larval growth and survival (watanabe *et al.* 1983). The fatty acids, especially DHA and EPA, are key components of lipids, for larval rearing. Due to the important effect that, not only quantitative amounts of EPA and DHA, but also its ratio plays in performance of marine fish larvae, several studies have been conducted in the past years in relation to the dietary ratios for these essential fatty acids (Izquierdo, 2005). Frequently these studies assay a small number of ratios in few experimental diets. In the

present experiment up to 12 different ratios of EPA/DHA with different amounts of these fatty acids were tested in gilthead seabream.

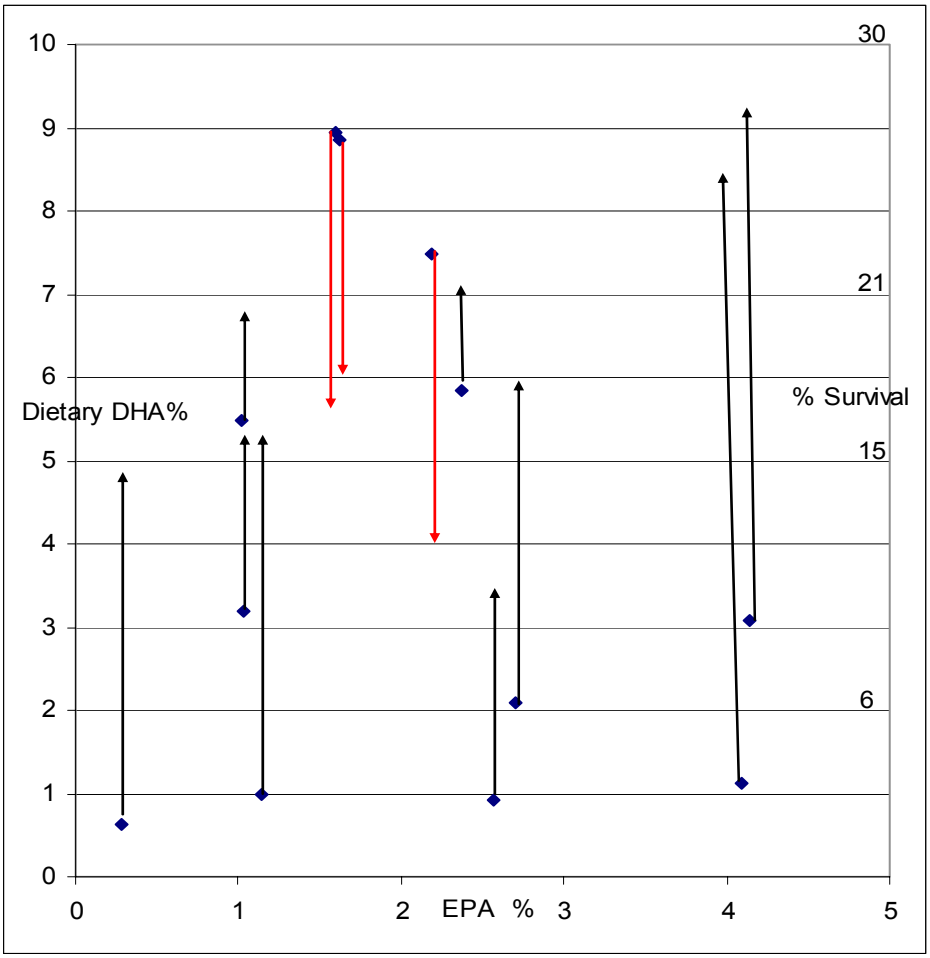

Figure 3.8. Graphical representation of EPA (x axis) and DHA (y axis) contents on diets fed to gilthead seabream and the larval survival (secondary y)

In this study when dietary DHA was between 2 and 3 % (1/3, 2.5/2 and 4/3 treatments) the elevation of EPA improved larval stress resistance to handling r^2= 0.95 and survival r^2= 0.85. Previous studies also suggested that when dietary DHA levels are high, elevation of dietary EPA is necessary to improve larval growth, survival, stress resistance to handling and temperature tests (Liu *et al.,* 2002). These could be related to its possible role as a regulator of cortisol production, and a main precursor of prostaglandins in marine fish (Ganga *et al.,* 2005), where ARA and EPA influences in eicosanoid production in different tissues (Ganga *et al.,* 2005). EPA has also been shown to be essential when DHA was absent from the diet (Watanabe *et al*., 1989) and

Table 3.5. Average final lipid and fatty acid composition of larval gilthead seabream from both trials

Diet EPA/DHA	0.3/0.6	1/1	1/3	1/6	1.5/8.5	1.5/9
Lipid w.b.	2.22±0.0	2.5±0.0	2.93±0.47	2.22±0.53	2.84±0.53	2.26±0.28
14:0	0.70	0.60	0.65±0.03	0.63±0.10	0.59±0.06	0.57±0.08
14:1n-5	0.02	0.01	0.01±0.01	n.d.	0.05±0.03	0.02±
14:1n-7	0.10	0.08	0.22±0.20	0.21±0.21	0.21±0.18	0.23±0.21
15:0	0.42	0.34	0.42±0.07	0.43±0.09	0.43±0.08	0.44±0.06
15:1n-5	0.16	0.13	0.19±0.10	0.17±0.09	0.15±0.08	0.16±0.09
16:0iso	0.44	0.38	0.34±0.04	0.31±0.04	0.35±0.09	0.37±0.13
16:0	21.62	17.12	15.31±2.55	15.89±2.53	15.78±2.73	16.10±4.20
16:1n-9	0.00	0.80	0.88±0.23	0.77±0.31	0.48±0.04	0.66±0.25
16:1n-7	1.37	1.74	3.87±3.18	3.19±2.57	3.07±2.20	2.89±2.30
Me16:0	0.18	0.17	0.13±0.01	0.11±0.02	0.07±0.04	0.07±0.04
16:1n-5	0.32	0.28	0.57±0.45	0.55±0.41	0.53±0.36	0.59±0.38
16:2n-6	0.52	0.41	0.30±0.08	0.29±0.02	0.18±0.04	0.21±0.03
16:2n-4	0.81	0.71	0.82±0.18	0.90±0.17	0.90±0.11	0.96±0.07
16:3n-4	1.06	0.86	1.00±0.32	0.86±0.31	0.42±0.60	0.72±0.22
16:3n-3	n.d.*	0.03	0.04±0.06	0.05±0.07	0.04±0.06	0.04±0.06
16:3n-1	n.d.	0.01	n.d.	0.01±0.01	n.d.	n.d.
16:4n-3	0.95	0.88	0.91±0.05	0.96±0.05	1.04±0.05	1.06±0.15
16:4n-1	0.88	0.80	0.54±0.30	0.46±0.20	0.50±0.10	0.50±0.03
18:0	9.23	8.23	8.74±0.90	9.28±0.63	9.53±0.10	9.80±0.62
18:1n-9	30.37	24.23	22.97±0.93	17.43±0.16	13.53±0.85	11.84±0.63
18:1n-7	4.74	4.01	4.82±1.57	4.17±1.50	3.51±1.59	3.47±1.40
18:1n-5	0.22	0.19	0.25±0.13	0.19±0.08	0.15±0.06	0.18±0.25
18:2n-9	0.15	0.09	1.10±1.44	0.78±0.96	0.69±0.81	0.68±0.77
18:2n-6	6.59	4.90	3.60±0.70	3.16±0.46	3.31±0.47	3.07±0.63
18:2n-4	n.d.	n.d.	0.27±0.38	0.15±0.21	0.05±0.07	0.11±0.14
18:3n-6	0.14	0.12	n.d.	0.09±0.13	0.05±0.07	0.26±0.03
18:3n-4	0.10	0.01	n.d.	n.d.	n.d.	n.d.
18:3n-3	n.d.	0.26	0.22±0.05	0.21±0.06	0.30±0.01	0.23±0.07
18:3n-1	n.d.	n.d.	0.28±0.40	n.d.	0.10±0.13	0.09±0.11
18:4n-3	n.d.	n.d.	n.d.	0.02±0.03	0.00±0.01	n.d.
18:4n-1	0.28	0.24	0.25±0.05	0.19±0.27	0.40±0.15	0.40±0.15
20:1n-9	n.d.	n.d.	n.d.	0.02±0.01	n.d.	n.d.
20:1n-7	0.67	0.55	0.83±0.22	0.89±0.18	1.05±0.11	1.00±0.12
20:2n-9	0.06	n.d.	0.24±0.34	0.22±0.31	0.23±0.32	0.33±0.21
20:2n-6	n.d.	n.d.	0.33±0.46	n.d.	0.03±0.04	0.19±0.27
20:3n-9	0.19	0.15	n.d.	0.12±0.17	0.17±0.11	0.17±0.11
20:3n-6	0.16	n.d.	n.d.	0.00±0.00	0.07±0.09	0.09±0.13
20:3n-3	n.d.	0.03	n.d.	0.06±0.08	0.00±	0.13±0.01
20:4n-6	2.52	2.52	0.92±0.65	2.42±0.44	2.66±0.59	2.75±0.69
20:4n-3	n.d.	0.17	0.11±0.15	0.05±0.07	0.04±0.06	0.09±0.13
20:5n3	3.27	5.81	4.07±0.05	3.60±0.21	4.02±0.32	3.98±0.12
22:1n-11	n.d.	0.01	0.81±0.97	0.56±0.47	0.58±0.34	0.34±0.48
22:1n-9	n.d.	n.d.	0.05±0.07	0.12±0.06	0.18±0.02	0.09±0.13
22:4n-6	0.80	0.56	0.88±0.13	1.13±0.09	1.31±0.09	1.34±0.07
22:5n-3	1.05	1.28	0.89±0.19	1.33±0.42	1.45±0.31	1.44±0.44
22:6n-3	9.92	21.29	22.15±7.96	28.03±5.21	31.80±3.89	32.33±2.30

Table 3.5. (Cont.) Average final lipid and fatty acid composition of larval gilthead seabream from both trials

Diet EPA/DHA	2/7.5	2.5/1	2.5/2	2.5/6	4/1	4/3
lipid w.b.	2.97±0.28	2.86±0.0	2.47±0.53	2.48±0.46	2.86±0.59	2.5±0.01
14:0	1.07±0.64	0.70	0.63	0.63±0.12	0.77	1.08±0.79
14:1n-5	0.06±0.07	0.00	0.01±0.01	0.01±0.01	0.01	n.d.
14:1n-7	0.22±0.20	0.45	0.25±0.25	0.23±0.21	0.12	0.21±0.20
15:0	0.49±0.01	0.45	0.42±0.06	0.43±0.07	0.40	0.43±0.09
15:1n-5	0.17±0.06	0.29	0.19±0.10	0.17±0.10	0.13	0.16±0.07
16:0iso	0.38±0.17	0.28	0.32±0.07	0.34±0.12	0.44	0.33±0.12
16:0	16.59±2.77	13.36	15.60±3.33	16.02±4.33	18.54	15.76±2.81
16:1n-9	0.26±0.36	1.34	0.88±0.26	0.48±0.68	0.80	0.78±0.10
16:1n-7	3.48±2.88	6.60	3.50±2.71	3.15±3.21	1.80	3.58±3.12
Me16:0	0.09±0.04	0.15	0.13	0.10±0.02	0.17	0.13±0.01
16:1n-5	0.50±0.39	0.98	0.63±0.50	0.58±0.44	0.32	0.51±0.31
16:2n-6	0.01±0.01	0.40	0.33	0.27±0.01	0.42	0.29±0.02
16:2n-4	0.88±0.13	0.91	0.88±0.18	0.87±0.15	0.83	0.83±0.01
16:3n-4	0.59±0.01	1.35	0.58±0.82	0.82±0.31	0.81	0.65±0.02
16:3n-3	0.05±0.06	0.09	0.05±0.07	0.05±0.07	n.d.	0.06±0.04
16:3n-1	n.d.	n.d.	n.d.	n.d.	n.d.	0.06±0.08
16:4n-3	1.06±0.20	0.84	0.92±0.02	0.97±0.02	0.93	0.99±0.27
16:4n-1	0.66±0.18	0.31	0.50±0.25	0.62±0.04	1.00	0.69±0.15
18:0	9.26±0.40	8.68	8.81±0.71	9.11±0.39	8.50	8.48±0.23
18:1n-9	15.89±2.87	24.08	20.15±1.03	16.14±0.37	22.76	17.17±0.14
18:1n-7	3.79±1.17	6.30	4.61±1.78	4.18±1.71	4.12	4.25±1.49
18:1n-5	0.18±0.24	0.56	0.25±0.14	0.25±0.36	0.19	0.28±0.20
18:2n-9	0.61±0.86	2.12	0.92±1.15	0.95±1.10	0.26	0.73±0.84
18:2n-6	3.35±0.77	3.85	3.30±0.65	2.94±0.52	3.96	3.28±0.04
18:2n-4	0.04±0.06	0.53	0.19±0.26	0.17±0.24	n.d.	0.06±0.08
18:3n-6	0.11±0.16	0.06	0.08±0.08	0.08±0.11	n.d.	0.09±0.09
18:3n-4	n.d.	n.d.	n.d.	0.02±0.03	0.12	0.06±0.09
18:3n-3	0.16±0.22	0.09	0.27±0.05	0.20±0.05	0.29	0.31±0.09
18:3n-1	0.07±0.10	0.32	0.11±0.16	0.10±0.14	n.d.	0.05±0.07
18:4n-3	0.16±0.20	0.10	0.11±0.15	0.12±0.04	0.32	0.36±0.14
18:4n-1	0.13±0.19	0.10	0.26±0.06	0.32±0.11	0.28	0.27±0.10
20:1n-9	0.07±0.10	0.09	0.13±0.01	0.00	n.d.	0.09±0.13
20:1n-7	0.63±0.89	1.04	0.93±0.29	0.92±0.22	0.54	0.84±0.23
20:2n-9	0.21±0.30	0.45	0.26±0.37	0.24±0.34	n.d.	0.24±0.23
20:2n-6	0.13±0.18	0.48	0.19±0.27	0.27±0.39	n.d.	0.14±0.20
20:3n-9	0.06±0.08	0.00	0.00±	0.18±0.02	n.d.	0.14±0.01
20:3n-6	0.00	0.39	0.15±0.21	0.17±0.10	0.15	0.12±0.17
20:3n-3	0.05±0.07	0.00	0.04±0.06	0.06±0.09	n.d.	0.04±0.06
20:4n-6	2.78±0.85	1.81	2.38±0.52	2.54±0.69	3.18	2.73±0.95
20:4n-3	0.00	0.29	0.00±0.00	0.21±0.07	0.34	0.36±0.17
20:5n-3	5.57±0.50	5.72	6.35±0.10	5.20±0.05	10.67	8.68±1.10
22:1n-11	0.35±0.50	1.58	0.60±0.66	0.60±0.85	0.11	0.46±0.54
22:1n-9	0.02±0.00	0.10	0.08±0.11	0.10±0.14	0.08	0.10±0.04
22:4n-6	0.54±0.76	0.56	0.78±0.00	1.04±0.11	0.42	0.70±0.04
22:5n-3	0.96±1.36	1.44	1.52±0.50	1.46±0.54	2.09	1.86±0.64
22:6n-3	28.36±3.55	10.77	21.69±5.38	26.68±6.41	14.16	21.56±6.13

*n.d. not detected.

it has important functions in larval lipid metabolism (Izquierdo, 1996), in the present study when dietary DHA is about 1% (1/1, 2.5/1 and 4/1treatment) the elevation of EPA improve larval stress resistance to handling r^2=1. Ganga *et al.* (2005) showed a high correlation between EPA (present in polar lipid) and prostaglandin PGE3 (r^2=0.97). Increase in dietary EPA and DHA has been found to increase stress resistance of juvenile gilthead sea bream by inhibition of the plasma cortisol levels induced by chronic, acute and repetitive stress (Montero *et al.,* 1998). In the present study the elevation of dietary EPA/ARA improve the resistance to stress r^2=0.93 in the presence of dietary DHA 1-3.2%.

It has been demonstrated that the gilthead seabream requires n-3 HUFA for healthy development (Izquierdo *et al*., 1989a,b, 1992). Not only a correlation between dietary EPA and larval whole body content EPA (r^2=0.83) but also a more correlated relationship between dietary linolenic acid 18:3n-3 and larval EPA (r^2= 0.88) was found. Besides the dietary DHA had a correlation with larval DHA (r^2= 0.83), but the dietary ratio linolenic acid/DHA showed a more significant correlation (r2= 0.88). These results suggested the presence of Δ5 and Δ6 desaturases and elongase enzymes and the dietary linolenic acid regulation of these enzymes. Recently a Δ6 desaturase like gene has been found in gilthead seabream (Seiliez *et al.* 2003). Izquierdo *et al.* (2008) confirmed the activity of that enzyme and also its regulation by dietary lipids. Feeding gilthead sea bream larvae with vegetable oil contain high level of dietary linolenic acid increased the fatty acids produced by Δ5 and Δ6 (Izquierdo *et al.,* 2008). Also in fresh water fish like zebra fish and tilapia, feeding vegetable oils containing 18:3n-3 increased Δ6 desaturase activity, resulting in increased levels of 18:4n-3, EPA and DHA (Tocher *et al.,* 2001). In the present study and in most treatments there were 18:3n-6 and 20:2n-9 in whole larval lipid, despite these fatty acids were not found in the diet, suggesting the activation of elongase and Δ5 and Δ6 desaturases. In this study the increasing of DHA in 1.5 and 2.5 EPA categories improved SGR values in agreement with Rodríguez *et al.,* (1997) who demonstrated in seabream larvae the better growth was obtained with the increasing of dietary DHA/EPA. In fish fed 2.5 EPA diets the elevation of DHA showed a relationship with SGR (r^2 =0.9948) and with n-3 HUFA (r^2 = 0.999). For first feeding seabream the elevation of n-3 HUFA from 0.9 to 1.5 % in rotifers improved growth (Rodríguez *et al.,* 1998).

The incorporations of EPA, DHA and n-3 HUFA were higher than the relative proportion in diets, but the dietary DHA affected the incorporation of DHA (r^2 =0.83) as well as the dietary EPA affected the incorporation EPA (r^2 =0.82).

Despite the dietary elevation of DHA for a given range of EPA markedly improved growth (Fig. 3.7) and survival (Fig. 3.8) of the larvae, elevation of DHA over 6% had a negative impact on larval performance reducing both parameters. For instance, for a given dietary EPA range of 1.14 to 1.2 elevation of DHA from 1 to 3.2% induced a similar or slightly improved survival (Fig. 3.8) and growth (Fig. 3.7) whereas the elevation of dietary DHA over 6 % did not induced any improvement in these parameters. Castell *et al.* (1994) have found a reduction in growth and survival in juvenile turbot when dietary DHA was increased up 6.2 % fatty acids. Also Sargent *et al.* (1999) have suggested that in marine fish larvae nutrition the concentration and ratio of EPA, DHA and ARA must be considered.

In summary, the results of this study have shown that larval survival was markedly associated to dietary EPA, DHA and n-3 HUFA levels, whereas growth was more affected by DHA contents. Besides, EPA/DHA and EPA/ARA dietary ratios should not be increased over 1 and 8, respectively, to avoid negative effects on growth.

Acknowledgements

This study was supported by a grant of the Spanish International Cooperation Agency (AECI) to the first author.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Bell, M.V., Henderson, R.J. and Sargent, J.R., 1986. The role of polyunsaturated fatty acids in Fish. Comp. Biochem. Physiol., 83B, 711-719.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S., 2005. Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 142, 410–418.

Ganga, R., Tort. L., Acerote, L., Montero, D. and Izquierdo, M.S., 2006. Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream (*Sparus aurata*). Journal of Endocrinology, 190, 39–45.

Ibeas C., Cejas J.R., Fores R., Badía P., Gómez T. and Lorenzo Hernández A., 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream *Sparus aurata* juveniles. Aquaculture, 150, 91-102.

Izquierdo, M. S., 1996. Review article: essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr., 2, 183–191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Options Mediterr., 63, 91-102.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989a. Optimal EFA levels in Artemia to meet the EFA requirements of red seabream *Pagrus major*.. In: Takeda, M., Watanabe, T. _Eds.., The Current Status of Fish Nutrition in Aquaculture. Japan Translation Centre, Tokyo, Japan, pp. 221–232.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989b. Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi, 55, 859–867.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red sea bream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The Current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Izquierdo, M.S., Arakawa, T., Takeuchi, T, Haroun, R. and Watanabe, T., 1992. Effect of n-3 HUFA levels in Attemia on growth of larval Japanese flounder (*Paralichthys olivaceous*). Aquaculture, 105, 73-82.

Izquierdo, M.S., Socorro, J., Arantzamendi, L., Hernández-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem. 22, 97– 107.

Izquierdo, M.S., Tandler, A., Salhı´, M. and Kolkkovski, S., 2001. Influence of dietary polar lipids quantity and quality on ingestion and assimilation of labelled fatty acids by larval gilthead seabream. Aquacult. Nutr., 6, 153– 160.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L. and Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquacult. Nutr., 9, 397– 407.

Izquierdo, M.S., Robaina, L., Juárez-Carrilo, E., Oliva, V., Hernández-Cruz, C.M. and Afonso, J.M., 2008. Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae *Sparus aurata*. Fish Physiol. Biochem., 34, 117-127.

Kolkovski S., Tandler A. and Kissil G. Wm., 1993. The effect of dietary enzymes with age on protein and lipid assimilation and deposition in *Sparus aurata* larvae. In: Fish Nutrition in Practice, Les Colloques, No. 61 (ed. INRA). INRA, Paris, France.

Liu, J., Caballero, M.J., El-Sayed, Ali.T., Izquierdo, M.S., Hernández Cruz, C.M., Valencia, A. and Fernández-Palacios, H., 2002. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream (*Sparus aurata*). Fish Science, 68, 1165–1172.

Madsen, L., Rustan-Arild, C., Vaagenes, H., Berge, K., Dyroy, E. and Berge, R.K., 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipid, 34 (9), 951-963Mourente, G. and Tocher, D.R., 1993. The effects of weaning on to a dry pellet diet on brain lipid and fatty acid compositions in post-larval gilthead sea bream *Sparus aurata* L. Comp. Biochem. Physiol., 104A, 605–611.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L. and Vergara, J. M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiology and Biochemistry, 18, 399–407Mourente, G., Rodríguez, A., Tocher, D.R. and Sargent, J.R., 1993. Effects of dietary docosahexaenoic acid DHA; 22:6n-3.on lipid and fatty compositions and growth in gilthead sea bream *Sparus aurata* larvae during first feeding. Aquaculture, 112, 79–98.

Reitan, K.I., Rainuzzo, J. and Olsen, Y., 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquacult. Int., 2, 23–48.

Rodríguez, C., 1994. Estudio de los requerimientos de acidos grasos esenciales de la dorada europea *Sparus aurata* L. durante las dos primeras semanas de alimentacion. PhD Thesis, University of La Laguna, Spain.

Rodríguez, C., Pérez, J.A., Izquierdo, M.S., Mora, J., Lorenzo, A. and Fernández-Palacios, H., 1993. Essential fatty acid requirements of larval gilthead seabream, *Sparus aurata* L.. Aquacult. Fish. Manage., 24, 295–304.

Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S. and Cejas, J.R., 1994. n-3 HUFA requirement of larval gilthead seabream *Sparus aurata* when using high levels of eicosapentaenoic acid. Comp. Biochem. Physiol., 107A, 693–698.

Rodríguez, C., Pérez, J.A., Díaz, M., Izquierdo, M.S., Fernandez-Palacios, H. and Lorenzo, A., 1997. Influence of the EPA/DHA ratio in rotifers on gilthead seabream *Sparus aurata* larval development. Aquaculture, 150, 77–89.

Rodríguez, C., Pérez, J.A., Badia, P., Izquierdo, M.S., Fernández Palacios, H. and Hernández, A.L., 1998. The n-3 highly unsaturated fatty acids requirements of gilthead seabream (*Sparus aurata* L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture, 169, 9–23.

Sargent, J.R., McEvoy, L.A. and Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEnvoy, L., Tocher, D. and Estevez A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177,191-199.

Seiliez, I., Panserat, S., Corraze, G., Kaushik, S. and Bergot, P., 2003. Cloning and nutritional regulation of a Δ6-desaturase-like enzyme in marine teleost gilthead seabream (*Sparus aurata*). Comparative biochemistry and physiology, Part B: Biochemistry and Molecular Biology., 135, 449-460.

Takeuchi, T., 2001, A review of feed development for early life stages of marine finfish in Japan. Aquaculture, 200, 203–222.

Tocher, D.R., Bell, J.G., MacGlaughlin, P., McGhee, F. and Dick, J.R., 2001. Hepatocyte fatty acid desaturation and polyunsaturated fatty acid composition of liver in salmonids: effects of dietary vegetable oil. Comp. Biochem. Physiol., B, 130, 257–270.

Watanabe, T., 1993. Importance of docosahexanoic acid in marine larval fish. J. World Aquacult. Soc., 24, 152– 161.

Watanabe, T. and Kiron, Y., 1994. Prospects in larval fish dietetics. Aquaculture, 124, 223–251.

Watanabe, T., Kitajima, C. and Fujita, S., 1983. Nutritional value of live organisms used in Japan for mass propagation of fish: a review. Aquaculture, 34, 115–143.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C., 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabreamN. ippon Suisan gakkaishi, 55(9), 1635-1 640.

CHAPTER 4

IMPORTANCE OF RELATIVE LEVELS OF DIETARY ARA AND EPA FOR CULTURE PERFORMANCE OF GILTHEAD SEABREAM (*Sparua aurata*) LARVAE

E. Atalah*, C. M. Hernández Cruz, E. Ganuza, T. Benítez-Santana, R. Ganga, O. Vega-Orellana, J. Roo, Fernández-Palacios, H. and M.S. Izquierdo

Abstract

The effect of different ARA dietary contents at several dietary EPA levels on growth, survival and biochemical composition of gilthead seabream larvae was studied to better define the importance of this fatty acid as a function of EPA. Larvae of 18 days were fed one of the eight isonitrogenous and isolipidic microdiets with 4 different EPA (0.3, 2, 3 and 4 %) and ARA amounts (0.1, 0.3, 0.6 and 1.2 %). Despite, dietary increase of either ARA or EPA alone did not significantly improved survival, the increase in both fatty acids significantly enhanced growth and survival, suggesting an optimum dietary ratio (EPA/DHA) close to 4:1. Dietary ARA was more efficiently incorporated into larval tissues than EPA. Increased dietary EPA or ARA contents respectively reduced the incorporation of ARA or EPA into larval lipids, denoting their competition as substrates for different enzymes. The possible negative effect of further elevation of dietary ARA and its competition with EPA for phospholipids synthesis deserves further studies in marine fish larvae.

Keywords: *Saprus aurata*; larval nutrition; arachidonic acid; EPA; DHA.

Abbreviations: ARA: arachidonic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, d.w.: dry weight basis, PC: phosphatidylcoline; DAH: days after hatching

Introduction

The importance of dietary essential fatty acids (EFA) for marine fish larvae has been largely recognized (Watanabe and Kiron, 1994; Sargent *et al*., 1999b). Particularly, both docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are required for growth, normal larval development, survival and stress resistance (Izquierdo *et al*., 1989a,b, 1996; Mourente *et al*., 1993; Rodríguez *et al*., 1993; Watanabe, 1993; Rodríguez, 1994; Salhi *et al*., 1994). More recent studies have been also focusing the value of arachidonic acid (ARA, 20:4n-6) as an EFA for marine fish (Bessonart *et al*., 1999; Sargent *et al*., 1999b; Copeman *et al*., 2002; Bell and Sargent, 2003; Ganga *et al*., 2006). Dietary ARA improves growth and survival (Castell *et al*., 1994) in juvenile turbot (*Psetta maxima*), when it is supplied (0.78%) as an EFA source, better than when it is combined with DHA. In gilthead seabream (*Sparus aurata*) larvae, increased dietary ARA up to 1.8 (% DW) improved growth and survival when both DHA and EPA were provided in the diet (Bessonart *et al*., 1999). Moreover, ARA has been found to be necessary to improve handling stress resistance of marine fish larvae (Koven *et al.*, 2001, 2003), since it regulates cortisol production by interrenal ACTH stimulated cells (Ganga *et al*., 2006). This fatty acid is a main component of phosphatidyl inositol, and whereas *in vitro*, ARA is a preferred substrate for most cycloxigenases, being a main precursor for prostaglandin synthesis (Bell *et al*., 1995), in marine fish *in vivo*, EPA could be the main substrate due to its high presence in diet (Ganga *et al*., 2005). ARA also constitutes a good substrate for several lypoxigenases, its derivative hydroxi-fatty acids having important physiological functions in marine fish.

However, excess amount of this fatty acid can also exert a negative impact in marine fish larvae (Copeman *et al*., 2002). In Japanese flounder (*Paralycthys olivaceous*) broodstock diet (Furuita *et al*., 2003) increase up to 0.6% ARA improved reproductive performance (egg production and quality and larval survival), but further increase up to 1.2 % reduced egg and larval quality. This negative effect has been suggested to be partly related to the competition of this fatty acid with EPA for different enzymes such as lipoxigenases and cycloxigenases (Izquierdo *et al*., 2000; Izquierdo, 2005; Furuita *et al*., 2003). Thus, the importance and effect of dietary ARA its being studied as a function of its relative value in relation to dietary EPA (McEvoy *et al*., 1998; Bessonart *et al*.,1999; Estévez *et al*., 1999; Sargent *et al*., 1999a; Copeman *et al*.,

2002), and definition of optimum dietary ratios among them will be important to determine their incorporation into the tissue lipids, their effect in membrane functioning, the energy values obtain from their beta-oxidation and the production of metabolically active compounds (Izquierdo, 2005). For instance, Estévez *et al*. (1999) observed that EPA:ARA ratios ≥1 in phosphatidyl inositol are associated with normal pigmentation in turbot larvae and suggested that, given a sufficiency of dietary DHA, the optimum dietary EPA level is a function of dietary ARA. The objective of the present study was to determine the effect of different ARA dietary contents at several dietary EPA levels on growth, survival and biochemical composition of gilthead seabream larvae, to better define the importance of this fatty acid as a function of EPA.

Materials and methods

Gilthead Sea Bream (*Sparus aurata)* larvae were obtained from natural spawnings from broodstocks at the GIA facilities (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain), where the experiment was carried out. Larvae were previously fed enriched rotifers (DHA Protein Selco, INVE, Dendermonde, Belgium), until they reached 18 days old (standard length, mean ± SD, 4.29 ± 0.50 mm, dry body weight 37.5 μg) and then they were randomly distributed into 24 tanks at a density of 1400 larvae/tank and were fed one of the eight experimental diets tested in triplicates for 14 days, at a water temperature of 19.2 to 21 ºC.

All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L/min along the feeding trial. Water get in the tank from the bottom and get out from the top, water quality was daily tested and no deterioration was observed. Water was continuously aerated (125ml/min) attaining 5-8 ppm dissolved O_2 and saturation ranged between 60 and 80% in all tanks. Photoperiod by fluorescent lights was kept at 12h light: 12h dark. Eight isonitrogenous and isolipidic (71.10/20.41 protein/lipid) experimental microdiets (pellet size < 250 μm) containing 4 different EPA amounts (0. 3, 2, 3 and 4 %) and 4 different ARA amounts (0.1, 0.3, 0.6 and 1.2 %) (Table 4.1, Fig. 4.1) were formulated using EPA45, DHA45 and ARA44 (Polaris, Pleuven, France) as sources of EPA, DHA and ARA. The desired lipid content was completed with a non essential fatty acid source, oleic acid (Oleic acid vegetable, Merck, Darmstadt, Germany). The protein source used (squid meal) was defatted in all diets (3 consecutive times with a

chloroform:meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiet. The microdiets were prepared by mixing squid powder and water soluble components, then the lipid and fat soluble vitamins and finally, warm water solved gelatin. The paste was pelleted and dried in oven at 38 ºC for 24 h. Pellets were ground and sieved to obtain particle size below 250 µm. Diets were analyzed for proximate and fatty acid composition of dry basis (d.w.) (Tables 4.1 and 4.2).

Diets were manually supplied fourteen times per day each 45 min from 9:00-19:00. To assure feed availability, daily feed supplied was 2 and 2.5 g/tank during the first and second week of feeding, respectively. Larvae were observed under the binocular microscope to determine feed acceptance.

Table 4.1. Lipid sources (% total ingredients), crude lipid (% dry basis), crude protein (% dry basis) and moisture (% wet basis) content of the experimental diets

Diet (EPA/ARA)	EPA45[1]	ARA44[1]	DHA45[1]	Oleic acid[2]	Crude lipids	Crude protein	Moisture
0.3/0.1	0	0	0	14.9	19.82	69.92	7.96
2/0.6	0	0.9	9.2	4.8	20.71	71.72	10.73
2/1.2	0	2	9.2	3.7	20.47	71.72	10.83
3/0.3	2.2	0	8.7	4.0	20.46	71.84	10.63
3/1.2	1.9	2	8.6	2.4	20.09	70.47	8.40
4/0.3	4.5	0	8	2.4	20.72	71.65	11.42
4/0.6	4.5	0.7	8	1.7	20.47	70.56	8.91
4/1.2	4.3	2	7.9	0.7	20.50	70.17	8.94

[1] Polaris, Pleuven, France.
[2] Merck, Darmstadt, Germany.

Final survival was calculated by individually counting all the alive larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 hour) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish/tank at the beginning in the middle and at the end of the trial. Besides, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition after 12 hours of starvation at the end of the experiment. Moisture (A.O.A.C., 1995), crude protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

Table 4.2. Fatty acid composition of experimental diets for larval gilthead seabream (g/100 g diet dry weight)

Dietary (EPA/ARA)	0.3/0.1	2/0.6	2.0/1.2	3/0.3	3/1.2	4/0.3	4/0.6	4/1.2
12:0	0.011	0.010	0.011	0.017	0.006	0.108	0.015	0.007
14:0	0.403	0.346	0.286	0.287	0.244	0.228	0.220	0.207
14:1n-7	0.036	0.005	0.018	0.019	0.013	n.d.	0.016	0.008
14:1n-5	0.061	0.042	0.021	0.023	0.014	0.02	0.014	0.008
15:0	0.069	0.029	0.050	0.049	0.038	0.04	0.041	0.031
15:1n-5	0.039	0.060	0.016	0.017	0.012	n.d.	0.011	0.006
16:0ISO	0.008	0.022	0.012	0.010	0.010	n.d.	0.013	0.011
16:0	1.370	1.796	1.812	1.526	1.693	1.446	1.530	1.770
16:1n-7	0.877	0.622	0.462	0.511	0.407	0.427	0.375	0.315
16:1n-5	0.027	0.023	0.024	0.025	0.016	n.d.	0.024	0.020
16:2n-6	0.075	0.072	0.054	0.058	0.037	0.015	0.031	0.020
16:2n-4	0.064	0.054	0.043	0.046	0.036	0.025	0.050	0.048
17:0	0.051	0.072	0.067	0.064	0.054	0.038	0.064	0.071
16:3n-4	0.297	0.173	0.138	0.151	0.111	0.063	0.097	0.079
16:3n-1	n.d.	0.008	0.017	0.020	n.d.	0.015	0.022	0.020
16:4n-3	n.d.	0.036	0.018	0.016	n.d.	0.022	0.022	n.d.
16:4n-1	n.d.	0.563	0.037	0.042	0.039	0.048	0.046	0.046
18:0	0.261	0.013	0.691	0.557	0.741	0.686	0.775	0.914
18:1n-9	13.271	5.529	4.839	5.131	3.793	3.763	3.176	2.487
18:1n-7	0.107	0.042	0.449	0.586	0.444	0.598	0.539	0.452
18:1n-5	0.079	0.041	0.034	0.039	0.023	0.03	0.023	0.019
18:2n-6	1.574	1.613	1.528	1.377	1.464	1.333	1.324	1.448
18:3n-6	n.d.	0.069	0.111	0.043	0.113	0.05	0.080	0.130
18:3n-4	n.d.	0.009	0.019	0.013	n.d.	0.055	0.018	0.020
18:3n-3	0.159	0.258	0.185	0.203	0.194	0.223	0.214	0.217
18:4n-3	n.d.	0.099	0.097	0.180	0.167	n.d.	0.276	0.274
20:0	n.d.	0.050	0.064	0.058	n.d.	0.081	0.110	0.117
20:1n-9	0.129	0.371	0.256	0.395	0.388	0.485	0.480	0.452
20:1n-7	n.d.	0.025	n.d.	n.d.	n.d.	0.039	0.041	0.035
20:2n-9	n.d.	n.d.	0.048	0.039	0.050	0.043	0.049	0.057
20:3n-9	n.d.	0.044	0.120	0.022	0.125	0.045	0.083	0.143
20:4n-6	0.052	0.609	1.137	0.240	1.159	0.3	0.616	1.251
20:4n-3	n.d.	0.077	0.071	0.122	0.116	0.05	0.184	0.180
20:5n-3	0.258	1.839	1.842	2.794	2.679	4.057	3.970	3.867
22:1n-11	n.d.	0.189	0.194	0.184	0.191	0.187	0.194	0.187
22:1n-9	n.d.	0.048	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
22:4n-6	n.d.	0.037	0.162	0.158	0.159	0.035	0.156	0.158
22:5n-6	n.d.	0.167	n.d.	n.d.	n.d.	0.16	n.d.	n.d.
22:5n-3	n.d.	0.361	0.365	0.387	0.392	0.439	0.431	0.419
22:6n-3	0.491	5.154	5.158	5.030	5.143	5.161	5.045	4.925
Saturated	2.165	2.316	2.981	2.558	2.776	2.627	2.755	3.117
Monoenoics	14.626	6.997	6.313	6.93	5.301	5.549	4.893	3.989
n-3	0.908	7.824	7.736	8.732	8.691	9.952	10.14	9.882
n-6	1.701	2.567	2.992	1.876	2.932	1.893	2.207	3.007
n-9	13.4	5.992	5.263	5.587	4.356	4.336	3.788	3.139
n-3HUFA	0.749	7.431	7.436	8.333	8.33	9.707	9.63	9.391

ARA/EPA	0.202	0.331	0.617	0.086	0.433	0.074	0.155	0.324
EPA/DHA	0.525	0.357	0.357	0.555	0.521	0.786	0.787	0.785
n-3/n-6	0.534	3.048	2.586	4.655	2.964	5.257	4.595	3.286

*n.d. not detected.

All data were treated using one-way ANOVA and means were compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results

All diets were well accepted by seabream larvae and the average survival rate was 22.5% (Fig. 4.2). Increasing dietary ARA when EPA levels were high (3 and 4 %) markedly increased survival, being significantly different when EPA was kept at 4%. Hence, increasing of ARA dietary ratio form 0.3 to 1.2 % at 4% EPA was significantly correlated to larval survival (r^2 =0.973). Highest survival rate was found in larvae fed diet the highest EPA and ARA contents (diet 4/1.2), whereas dietary increase of either

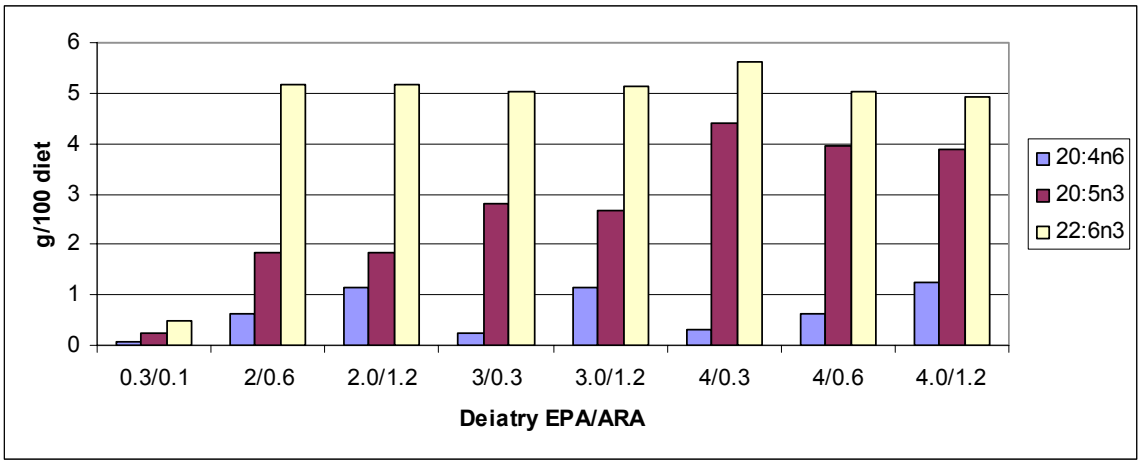

Figure 4.1. Dietary ARA. EPA and DHA % dry basis.

ARA or EPA alone did not significantly improved survival in comparison with larvae fed the lowest ARA and EPA contents (Diet 0.3/0.1). Highest whole body weight was found in fish fed the highest EPA and ARA contents (Diet 4/1.2) even after only seven

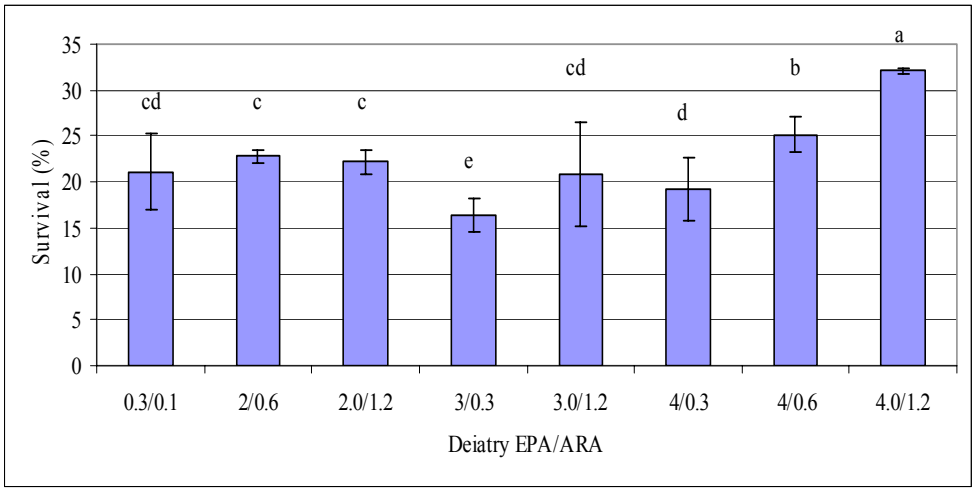

Figure 4.2. The survival rate of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

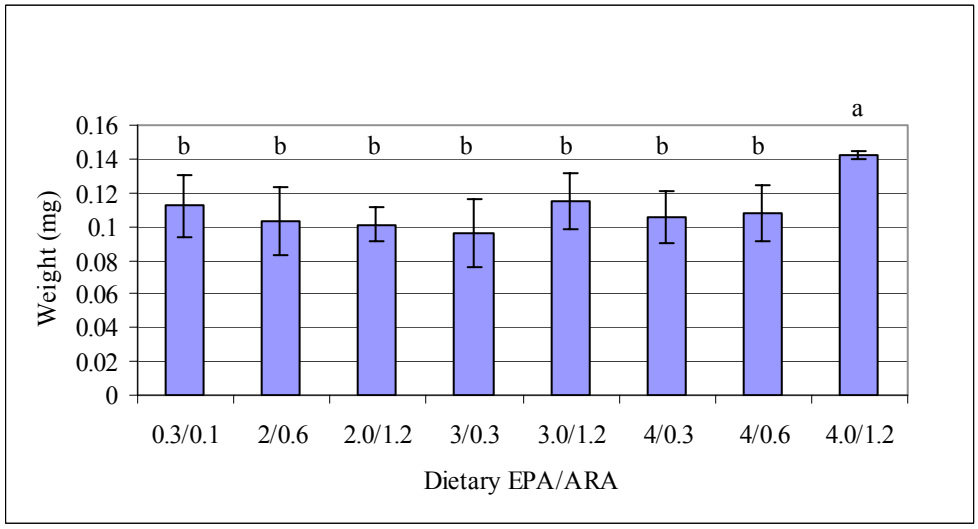

Figure 4.3. Dry whole body weight after 7 days of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

days of feeding (Fig. 4.3). At the end of the experiment, larvae fed the diet with the lowest EPA/ARA contents (Diet 0.3/0.1) showed the lowest whole body weight (Fig. 4.4). Increase of both EPA and ARA significantly improved growth, when dietary EPA/ARA ratios were higher than 2. Highest body weights were found in larvae fed diet 4/1.2 (Fig. 4.4). Similar results were obtained for SGR (Fig. 4.5). Larval growth in

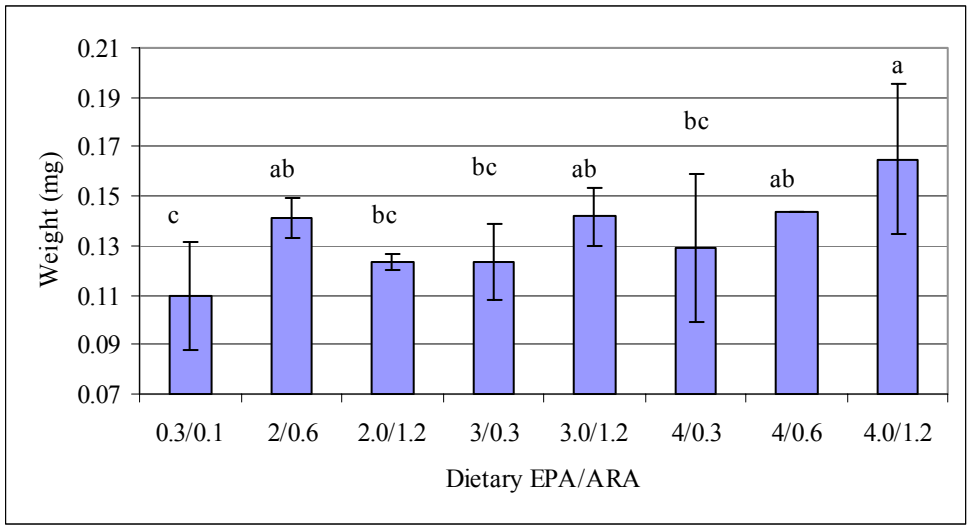

Figure 4.4. Final dry whole body weight of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

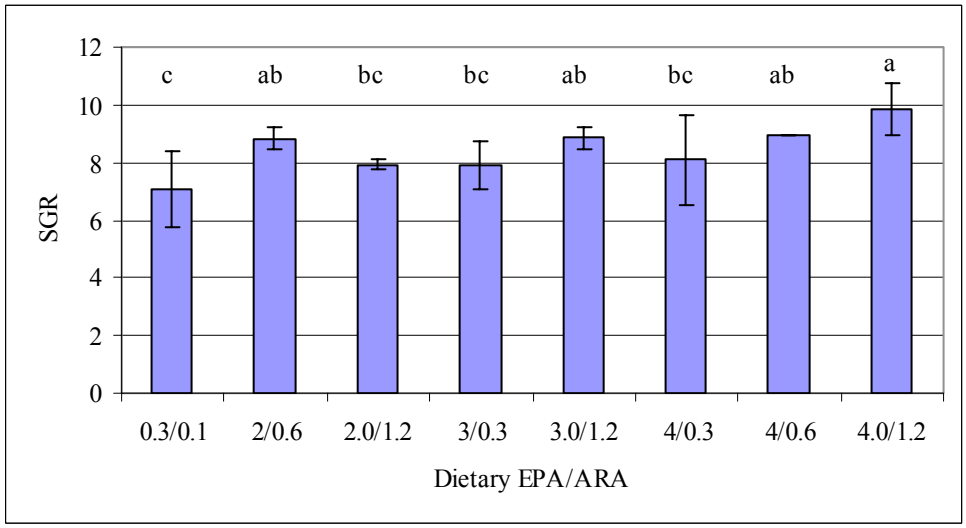

Figure 4.5. Specific growth rate of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

terms of standard length followed also a similar tendency, larvae fed diet 0.3/0.1 being significantly smaller and those fed diet 4/1.2 being the biggest (Fig. 4.6). Final total length of larvae fed 4% EPA (4/0.3, 4/0.6 and 4/1.2) was significantly correlated to the dietary ARA contents (r^2=0.974). As a consequence of the survival and growth results, final larvae biomass produced with diet 4/1.2 was significantly highest (Fig. 4.7). No significant differences were found in the larval resistance to the handling test.

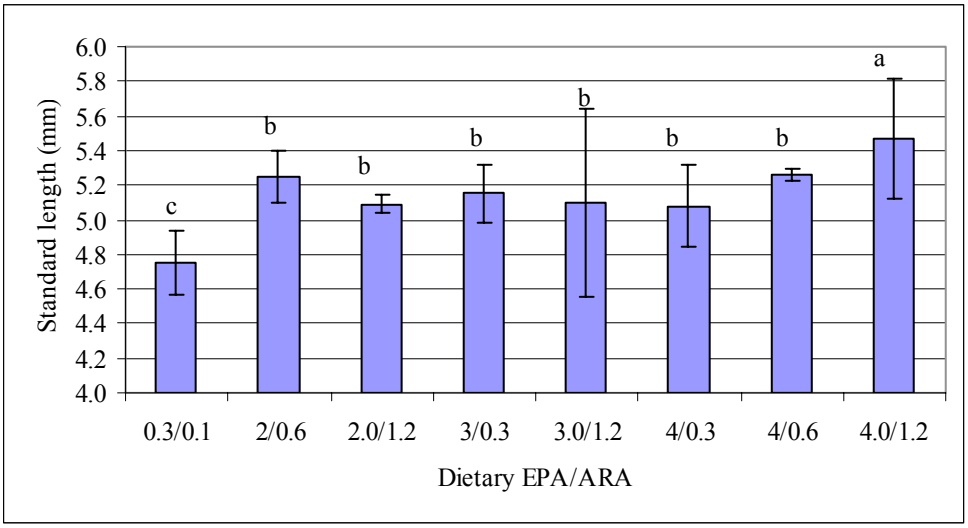

Figure 4.6. Final standard length of fish fed the different experimental diets (n=90; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

Dietary fatty acid composition showed 4 different EPA and ARA levels according to the dietary design. Diet 0.3/0.1 showed the lowest n-6, n-3 fatty acids and n-3 HUFA contents, particularly due to its low ARA, EPA and DHA contents. Besides, this diet showed the highest n-9 and monoenoic fatty acids, due to its high content in oleic acid. Oleic acid content in the other diets was inversely proportional to the ARA levels. Linoleic acid and EPA/DHA were very similar among the diets. Whole body lipid content of the larvae was significantly affected by the experimental diets (Table 4.3). Fatty acid composition of total lipids from whole larval body lipids (Table 4.4) reflected the dietary fatty acid profiles (Table 4.2), where increased dietary ARA levels were followed by increased ARA and reduced monounsaturated and n-9 fatty acids and 18:1n-9. Increased ARA in larval lipids was also followed by a reduction in EPA contents when dietary EPA was kept at higher levels (3 and 4 %), whereas DHA content was only reduced in larvae fed the diet containing the highest EPA and ARA levels (4/1.2). In a similar way, increased EPA contents from 3 to 4 in diets 3/0.3 and 4/0.3 reduced ARA incorporation into larval tissues, despite the similar ARA levels in both diets. Nevertheless, the lowest n-3 HUFA and DHA content was found in larvae fed diet 0.3/0.1, although EPA and ARA levels were also reduced but no so much affected as DHA. These larvae also showed the highest oleic and linoleic acid contents.

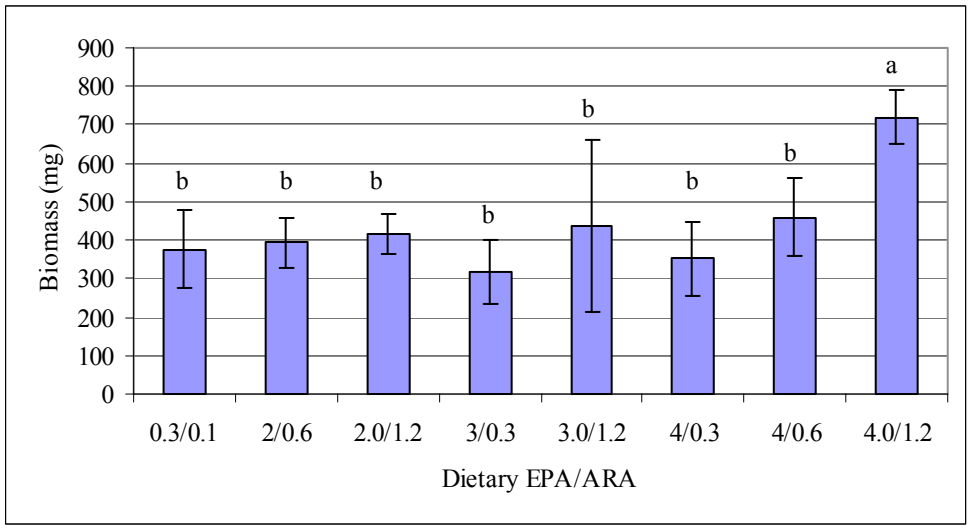

Figure 4.7. Final biomass of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

Discussion

Several studies have indicated that both EPA and ARA are essential fatty acids for marine fish larvae (Watanabe *et al*., 1989; Bessonart *et al*., 1999; Estévez *et al*., 1999; Koven *et al.*, 2001). However, less attention has been paid to their effects when their relative proportions vary in the diets. In the present study - when the dietary DHA was administrated with a sufficient amount (5%) - dietary increase of either ARA or EPA alone did not improved survival, whereas the increase in both fatty acids significantly enhanced growth and survival. Moreover, increase in ARA up to 1.2 % only improved growth and survival when EPA was as high as 4, suggesting the beneficial effect of the former fatty acid in relation to the requirements of the later. These results suggest that not only each of these fatty acids is important for the larvae, but also an optimum dietary ratio close to 4:1.2 EPA:ARA must be kept between them for this particular species. Both fatty acids are well known substrates competing between them for several enzymes such as lypoxigenases and cycloxigenases, among many others, and hence their relative proportions in the tissue may determine the type of derivative products synthesised, such as the different eicosanoids. In agreement, Estévez *et al*. (1999) found that in PI of turbot larva EPA:ARA ratios ≥1 are required for normal skin pigmentation.

Regarding ARA dietary levels, former studies in gilthead seabream have shown that increased up to 1.8 % improved growth and survival when both DHA and EPA

were provided in the diet at 1.3 and 0.7%, respectively (Bessonart *et al*.,1999). Similarly, in European seabass (*Dicentrarchus labrax*) (Atalah *et al*., submitted) elevation of dietary arachidonic acid up to 1.2 % showed a positive correlation with larval survival and a significant improvement in growth. However, despite elevation of dietary ARA up to 0.6% in Japanese flounder broodstock diets improved survival of the larvae produced, further elevation up to 1.2 % markedly reduced larval quality (Furuita *et al*., 2003). The possible negative effects of too high ARA levels were not determined by the present experiment.

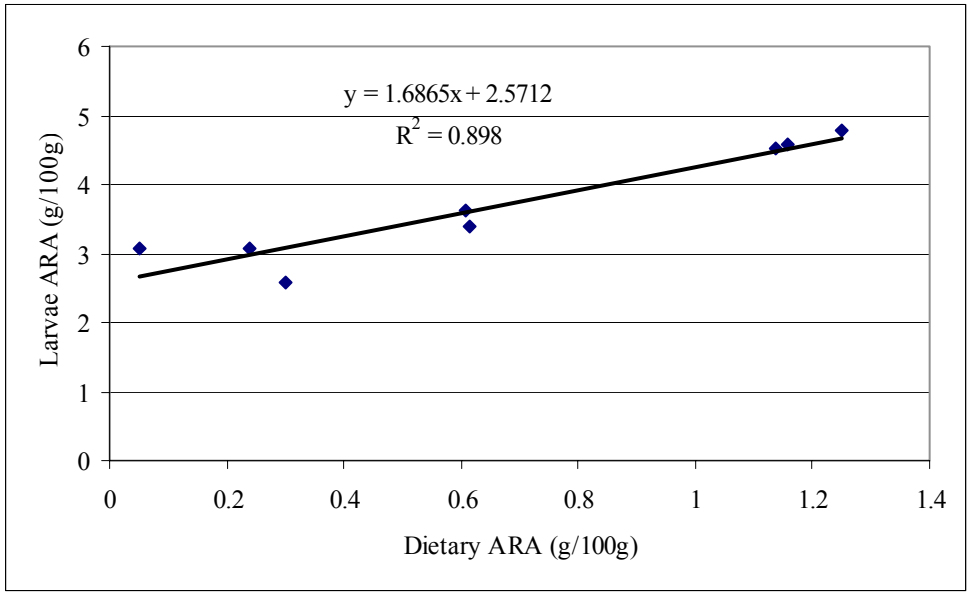

Figure 4.8. Incorporation of dietary ARA in larvae tissues.

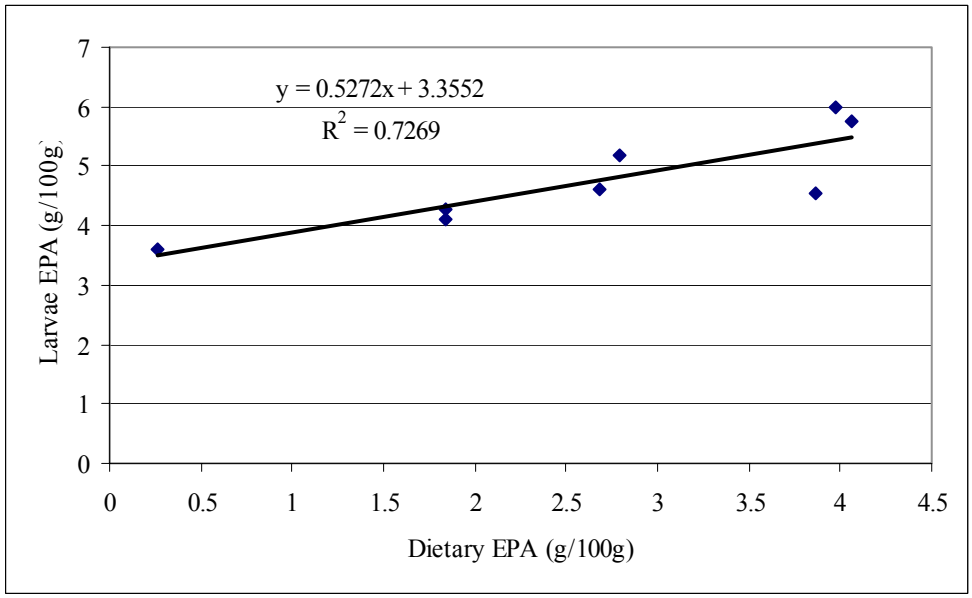

Figure 4.9. Incorporation of dietary EPA in larvae tissue.

Table 4.3. Lipid content and fatty acid composition of total lipids from gilthead seabream larvae after 14 days of feeding several EPA/ARA levels (n=3; g/100g total fatty acids)

Diets EPA/ARA	0.3/0.1	2/0.6	2.0/1.2	3/0.3	3/1.2	4/0.3	4/0.6	4/1.2
Lipid content (w.b.)	2.38^{bcd}	2.41^{bcd}	2.51^{bc}	3.14^{a}	2.99^{ab}	2.37^{bcd}	1.85^{d}	2.29^{cd}
12:0	0.172	0.196	0.118	0.211	0.171	0.375	0.137	0.071
14:0	0.727	1.081	0.768	0.744	1.019	0.983	0.743	0.797
14:1n-5	0.550	0.489	0.596	0.541	0.436	0.622	0.540	0.520
14:1n-7	n.d. *	n.d.	0.125	0.172	0.162	n.d.	0.104	0.094
15:0	0.448	0.516	0.423	0.550	0.610	0.558	0.493	0.434
15:1n-5	n.d.	0.171	0.159	0.155	0.157	0.197	0.146	0.138
16:0ISO	0.298	0.271	0.250	0.228	0.245	0.279	0.252	0.294
16:0	15.287	15.533	15.009	14.478	15.390	16.184	15.991	18.372
16:1n-9	1.451	1.242	1.250	1.155	1.091	1.229	1.137	1.145
16:1n-7	3.910	3.709	3.323	3.522	3.426	3.981	3.098	3.207
16:1n-5	0.241	0.218	0.212	0.192	0.205	0.219	0.190	0.210
16:2n-6	0.920	0.862	0.935	0.996	0.837	1.037	0.894	0.938
16:2n-4	0.350	0.253	0.270	0.239	0.245	0.247	0.225	0.237
17:0	0.860	0.150	0.166	0.151	0.778	0.923	0.862	0.970
16:3n-4	1.015	0.755	0.725	0.772	0.666	0.740	0.654	0.652
16:4n-3	0.820	0.865	0.819	0.800	0.850	0.882	0.874	1.002
16:4n-1	0.446	0.457	0.402	0.386	0.412	0.430	0.434	0.501
18:0	11.005	10.732	10.341	10.612	10.390	11.239	11.473	12.998
18:1n-9	24.365	18.496	18.191	17.504	16.679	17.852	16.026	15.251
18:1n-7	5.175	4.662	4.510	5.489	4.258	5.261	4.560	4.729
18:1n-5	0.293	0.189	0.185	0.179	0.184	n.d.	0.199	0.185
18:2n-9	1.444	1.201	1.033	1.058	1.028	0.987	0.968	0.906
18:2n-6	4.408	3.128	3.143	2.864	2.933	2.802	2.972	3.059
18:2n-4	0.403	0.209	0.210	0.244	0.139	0.169	0.102	0.082
18:3n-6	0.438	0.282	0.320	0.345	0.308	0.288	0.299	0.373
18:3n-3	0.269	0.268	0.252	0.203	0.223	0.229	0.259	0.234
20:0	n.d.	0.189	0.198	0.218	0.218	0.206	0.252	0.291
20:1n-9	0.793	0.787	0.730	1.168	0.839	0.905	0.880	0.950
20:1n-7	0.338	0.334	0.310	0.384	0.319	0.366	0.325	0.353
20:2n-9	0.610	0.407	0.436	0.420	0.351	0.361	0.394	0.304
20:3n-6	0.207	n.d.	0.101	0.112	0.088	n.d.	n.d.	n.d.
20:3n-9	0.493	n.d.	0.345	0.295	0.328	0.201	0.214	0.318
20:4n-6	3.074	3.609	4.527	3.067	4.582	2.578	3.403	4.776
20:4n-3	n.d.	0.166	0.170	0.231	0.247	n.d.	n.d.	0.205
20:5n-3	3.617	4.105	4.274	5.172	4.625	5.740	5.993	4.547
22:1n-11	n.d.	n.d.	n.d.	n.d.	0.211	n.d.	0.139	0.182
22:4n-6	0.798	0.956	0.912	0.853	0.875	0.814	0.856	0.758
22:5n-3	1.282	1.438	1.489	1.681	1.588	1.577	1.676	1.325
22:6n-3	13.493	22.072	22.721	22.511	22.540	19.543	22.236	18.463
Saturated	28.499	28.398	27.024	26.963	28.575	30.466	29.951	33.933
Monoenoics	37.117	30.297	29.591	30.461	27.968	30.631	27.344	26.964
n-3	19.481	28.914	29.671	30.597	30.323	27.971	31.039	25.901
n-6	9.845	8.837	9.937	8.237	9.623	7.518	8.425	9.905
n-9	29.157	22.134	21.985	21.601	20.316	21.536	19.618	18.875

n-3HUFA	18.392	27.781	28.654	29.595	29	26.86	29.905	24.54
ARA/EPA	0.850	0.879	1.059	0.593	0.9908	0.449	0.568	1.05
EPA/DHA	0.268	0.186	0.188	0.230	0.205	0.294	0.27	0.246
n-3/n-6	1.979	3.272	2.986	3.715	3.151	3.72	3.684	2.615

*n.d. not detected.

Dietary ARA was more efficiently incorporated into larval tissues (y=1.6865x+2.5712) than EPA (y=0.5272x+3.3552) (Fig. 4.8, 4.9 respectively). Accordingly, EPA/ARA ratios were lower in larval tissues than in diets. Moreover, increased incorporation of ARA in larval lipids slightly reduced EPA incorporation when the later was high in the diet. These results suggested a preferential incorporation or retention of ARA into larval tissues which could be related to a higher affinity of triglycerides and phospholipids biosynthesis enzymes, particularly those of the glycerol-3-phosphate pathway which have been shown to be stimulated by n-6 fatty acids (Caballero *et al*., 2006). Nevertheless, this proportionally higher incorporation of ARA, could be also related to a higher mitochondrial beta-oxidation of EPA as it occurs in rats (Froyland *et al*., 1997). In fact, EPA has been found to be an excellent substrate for mitochondrial carnitine acyltransferase-I.

Also, increased EPA contents from 3 to 4 in diets 3/0.3 and 4/0.3 reduced ARA incorporation into larval tissues, despite the similar ARA levels in both diets. The results of the present study suggest that, since DHA is more efficient as an essential fatty acid than EPA (Watanabe *et al*., 1989), being dietary levels of this fatty acid high enough to cover the EFA requirements, the optimum EPA dietary levels are not a function of dietary DHA, but of dietary ARA, in view of their competition as substrates for different enzymes. Moreover, competition for incorporation into the different lipid classes will probably show differences for each tissue since fatty acid composition of each lipid class markedly differs among cellular types (Lie *et al*., 1992). For instance, in juvenile turbot, the elevation of dietary ARA up to 0.78% reduced the incorporation of EPA into the liver and brain PI (Bell *et al*., 1995). The effect of further elevation of dietary ARA and its possible competition with EPA for phosphatidylinositol synthesis deserves further studies in marine fish larvae.

In summary, the results of this study have shown that when DHA is not a limiting factor in larval diets, only the increase in both ARA and EPA enhanced growth and survival, suggesting an optimum dietary ratio close to 4 for gilthead sea bream. Dietary ARA, being more efficiently incorporated into larval tissues may reduce EPA deposition, denoting the competition among both fatty acids.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Bell, J.G., Castell, J.D., Tocher, D.R., MacDonald, F.M. and Sargent, J.R., 1995. Effects of different dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid fatty acid compositions and prostaglandin production in juvenile turbot (*Scophthalmus maximus*). Fish Physiol. Biochem., 14, 139– 151.

Bell, J.G. and Sargent, J.R., 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture, 218, 491– 499.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M., González, M.M. and Fernández-Palacios, H., 1999. Effect of dietary arachidonic acid levels on growth and survival of gilthead seabream (*Sparus aurata* L.) larvae. Aquaculture, 179, 265-275.

Caballero, M.J., Torstensen, B.E., Robaina, L., Montero, D. and Izquierdo, M.S., 2006. Vegetable oils affect the composition of lipoproteins in sea bream (*Sparus aurata*). British Journal of Nutrition, 96, 830–839.

Castell, J.D., Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). Aquaculture, 128, 315– 333.

Copeman, L.A., Parrish C.C., Brown, J.A. and Harel, M., 2002. Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. Aquaculture, 210, 285–304.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Estévez, A., McEvoy, L.A., Bell, J.G. and Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot larvae fed live-prey enriched in arachidonic (ARA) and eicosapentaenoic (EPA) acids. Aquaculture, 180, 321–343.

Folch, J., Lees, M. and Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. ,226, 497–509.

Froyland, L., Madsen, L., Vaagenes, H., Totland, G.K., Auwerx, J., Kryviv, H., Staels, B. and Berge, R.K., 1997. Mitochondrion is the principal target for nutritional and pharmacological control of triglyceride metabolism. Journal of Lipid Research, 38 (9), 1851-1858.

Furuita, H., Yamamoto, T., Shima, T., Suzuki, N. and Takeuchi, T., 2003. Effect of arachidonic acid levels in broodstock diet on larval and egg quality of Japanese flounder *Paralichthys olivaceus*. Aquaculture, 220, 725-735.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S., 2005. Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 142, 410–418.

Ganga, R., Tort. L., Acerote, L., Montero, D. and Izquierdo, M.S., 2006. Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream (*Sparus aurata*). Journal of Endocrinology, 190, 39–45.

Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish larvae. Review article. Aquacult. Nutr., 2, 183–191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Options Mediterr., 63, 91-102

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989a. Optimal EFA levels in Artemia to meet the EFA requirements of red seabream *Pagrus major*.. In: Takeda, M., Watanabe, T. _Eds.., The Current Status of Fish Nutrition in Aquaculture. Japan Translation Centre, Tokyo, Japan, pp. 221–232.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989b. Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi, 55, 859–867.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem., 22 (2), 97-107.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A., 2001. The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture, 193, 107– 122.

Koven, W., Van Anholt, R., Lutzky, S., Ben Atia, I., Nixon, O., Ron, B. and Tandler, A., 2003. The effect of dietary arachidonic acid on growth, survival, and cortisol levels in different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or daily salinity change. Aquaculture, 228, 307–320.

Lie, O., Hemre G.-I. and Bjornsson, B. 1992. Fatty acid composition og glycerophospholipids and neutral lipids in six different tissues of halibut (*Hippoglossus hypoglossus*) fed capelin at constant temperature. Fisk. Dir. Skr. Ser. Ernaering, 5, 99–109.

McEvoy, L.A., Estévez, A., Bell, J.G., Shields, R.J., Gara, B. and Sargent, J.R., 1998. Influence of dietary levels of eicosapentaenoic and arachidonic acids on the pigmentation success of turbot (*Scophthalmus maximus* L.) and halibut (*Hippoglossus hippoglossus* L.). Bull. Aquacult. Assoc. Can., 4, 17– 20.

Mourente, G., Rodríguez, A., Tocher, D.R. and Sargent, J.R., 1993. Effects of dietary docosahexaenoic acid DHA; 22:6n-3.on lipid and fatty compositions and growth in gilthead sea bream *Sparus aurata* L.. larvae during first feeding. Aquaculture, 112, 79–98.

Rodríguez, C., 1994. Estudio de los requerimientos de acidos grasos esenciales de la dorada europea *Sparus aurata* L. durante las dos primeras semanas de alimentacion. PhD Thesis, University of La Laguna, Spain.

Rodríguez, C., Pérez, J.A., Izquierdo, M.S., Mora, J., Lorenzo, A. and Fernández-Palacios, H., 1993. Essential fatty acid requirements of larval gilthead seabream, *Sparus aurata* L.. Aquacult. Fish. Manage., 24, 295–304.

Salhi, M., Izquierdo, M.S., Hernández-Cruz, C.M., Gonzalez, M. and Fernández-Palacios, H., 1994. Effect of lipid and n-3 HUFA levels in microdiets on growth survival and fatty acid composition of larval gilthead seabream *Sparus aurata*. Aquaculture, 124, 275–282.

Sargent, J., Bell, G., McEnvoy, L., Tocher, D. and Estevez A., 1999a. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177,191-199.

Sargent, J.R., McEvoy, L.A., Estevez, A., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1999b. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture, 179, 217–229.

Wantanabe, T., 1993. Importance of docosahexaenoic acid in marine larval fish. J. World Aquacult. Soc., 24,152– 161.

Watanabe, T. and Kiron, Y., 1994. Prospects in larval fish dietetics. Aquaculture, 124, 223–251.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C., 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. Nippon Suisan gakkaishi, 55(9), 1635-1640.

CHAPTER 5

IMPORTANCE OF DIETARY ARACHIDONIC ACID FOR SURVIVAL, GROWTH AND STRESS RESISTANCE OF LARVAL EUROPEAN SEA BASS (*Dicentrarchus labrax*) FED HIGH DIETARY DOCOSAHEXAENOIC AND EICOSAPENTAENOIC ACIDS

E. Atalah*,[1], C. M. Hernández-Cruz[1], E. Ganuza[1], T. Benítez-Santana[1], R. Ganga[1], J. Roo[1], D. Montero[1] and M.S. Izquierdo[1].

Abstract

Information about essential fatty acid requirements for larval European sea bass is still incomplete. Together with docosahexaenoic and eicosapentaenoic acids, arachidonic acid is being considered important in larval diets, particularly in relation to the levels of other essential fatty acids. The objective of the present study was to determine the importance of dietary arachidonic acid levels for larval sea bass performance and stress resistance, when eicosapentaenoic and docosahexaenoic acids are provided in sufficient amounts.

Eighteen day old larvae were fed gelatine based microdiets with 5% docosahexaenoic acid and 3% eicosapentaenoic acid, and containing graded arachidonic acid levels: 0.3, 0.6 or 1.2%, for 14 days. Elevation of dietary arachidonic acid up to 1.2% showed a positive correlation with larval survival and a significant improvement in specific growth rates, body weight and total length. Dietary arachidonic acid was efficiently incorporated into larval lipids, although they remained in a lower proportion (0.53-0.92%) than they were in the diet. Increased accumulation of arachidonic acid did not affect the incorporation of docosahexaenoic or eicosapentaenoic acids from the diet into larval lipids. A significant positive correlation was found between dietary

arachidonic acid levels and survival after handling stress, denoting the importance of this fatty acid in seabass larvae response to stressors, in agreement with its role in regulation of ACTH induced cortisol release by interrenal cells. The results denoted the importance of arachidonic acid for sea bass larvae, but higher dietary levels should be tested to determine if there is a negative effect of this fatty acid in sea bass as reported for other species.

Keywords: *Dicentrarchus labrax*; larval nutrition, arachidonic acid; stress resistance; EPA; DHA.

Abbreviations: ARA: arachidonic acid, DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, d.w.: dry weight basis, PC: phosphatidylcoline.

Introduction

It is well recognized that essential fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, constitute one of the main nutritional factors that influence growth and survival of marine fish larvae (Izquierdo *et al*., 1989; Sargent *et al*., 1997; Izquierdo, 2005). Recently, more attention has been paid to the importance of arachidonic acid (ARA), as an essential fatty acid for marine fish, due to its potential as eicosanoid precursor (Bell *et al*., 1994; Sargent *et al*., 1994; Bessonart *et al*., 1999). Eicosanoids constitute a group of highly active compounds, once known as local hormones, which, among other molecules, include prostaglandins, thromboxanes and leukotrienes, involved in many different physiological functions related with reproduction, growth and development, immune system or stress response (Sargent *et al*., 1995). Thus, dietary ARA seems to be important along the whole life cycle of fish. For instance, ARA deficiencies in juvenile turbot have resulted in high mortality and obvious pathology (Bell *et al*., 1985). In larvae, dietary ARA improves survival (Bessonart *et al*., 1999) and handling stress resistance (Koven *et al*., 2001), while high dietary ARA produced a malpigmentation in Japanese flounder (Estévez *et al*., 2001) and Senegal sole (*Solea senegalensis*) (Vilalta *et al*., 2005). Finally, ARA has been suggested to be required for reproduction success in broodstock of several species such as gilthead seabream (*Sparus aurata*) (Fernández-Palacios *et al*., 1995) and European (*Dicentrarchus labrax*) (Sargent *et al*.,1999a) or striped sea bass (*Morone saxatilis*) (Harel *et al*., 2002). Moreover, several studies have shown that ARA is preferentially retained in various species during starvation, denoting a metabolic priority for its

conservation which enhances the importance of this fatty acid (Castell *et al*., 1994; Rainuzzo *et al*., 1994; Izquierdo, 1996).

Since EPA is also recognized as a precursor of eicosanoids (Ganga *et al*., 2005), competing with ARA for the same eicosanoid synthetizing enzymes, the ratio between both fatty acids ARA and EPA may influence eicosanoid production in different tissues (Sargent *et al*., 1995, 1999b; Ganga *et al*., 2005). Evidences of competition among the three essential fatty acids (ARA, EPA and DHA) have been suggested for digestive enzymes (Iijima *et al*., 1998, Izquierdo *et al*., 2000), fatty acid binding proteins, phosphoacylglicerides synthetases (Bell *et al*., 1995; Izquierdo *et al*., 2002), lipoxygenases and cyclooxygenases (Hwang, 1989; Ganga *et al*., 2005) and probably in beta-oxidation (Totland *et al*., 2000). Hence, not only absolute dietary values for each of these essential fatty acids but also optimum dietary ratios among them must be define since both factors will affect at least to their incorporation into the tissue lipids and hence membrane fluidity and function, the energy values obtain from their beta-oxidation and the production of metabolically active compounds (Izquierdo *et al*., 2000; Izquierdo, 2005). Thus, several studies have been focused on the importance of the relative amounts of DHA, EPA, and ARA (McEvoy *et al*., 1998; Bessonart *et al*., 1999; Estévez *et al*., 1999; Sargent *et al*., 1999a; Copeman *et al*., 2002; Bell and Sargent, 2003; Koven *et al*., 2003). For instance, Bessonart *et al*. (1999) showed that increase in dietary ARA levels up to 1% improves gilthead seabream (*Sparus aurata*) survival and growth in presence of adequate dietary EPA and DHA contents, whereas Sargent *et al*. (1999b) suggest a beneficial DHA/EPA/ARA ratio of about 10:5:1 for marine fish larvae and Castell *et al*. (2001) of 40:5:4 for haddock (*Melanogrammus aeglefinus*) larvae.

Despite the European sea bass is the second more important marine fish produced by European aquaculture, the information about its nutritional requirements is still incomplete compared to other fish species such as salmonids and carps (Oliva-Teles, 2000; Kaushik, 2002), particularly regarding dietary essential fatty acids (Izquierdo, 2005). The objective of the present study was to determine the importance of dietary ARA levels for larval sea bass when DHA and EPA are not limiting factors, and its effect in larval performance and stress resistance.

Materials and methods

Sea bass larvae were obtained from natural spawnings from France (Ecloserie Marine de Gravelines, Gravelines, France) and the experiment was carried out in the GIA facilities (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, Spain). Larvae (standard length 6.77 ± 0.71 mm (mean±SD), dry body weight 350 μg), previously fed a commercial microdiet until they reached 18 days old, were randomly distributed in the experimental tanks at a density of 1200 larvae/tank and fed one of the experimental diets tested in triplicates for 14 days, at a water temperature of 19.6 to 20.9 ºC.

All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L/min to assure enough water quality along the whole trial. Water entered by the tank basement and outlet from the top to maintain a high water quality which was daily tested. Water was continuously aerated (125ml/min) attaining 5-8 ppm dissolved O_2 and 60-80% saturation in all tanks. Photoperiod was kept at 12h light: 12h dark, by fluorescent lights.

Three isonitrogenous and isolipidic (70.27/20.56 protein/lipid) experimental microdiets (pellet size < 250 μm) similar in their DHA and EPA content (5 and 3%, respectively) and with increasing ARA levels (Table 5.1) were formulated using EPA45, DHA45 and ARA44 (Polaris, Pleuven, France) as sources of EPA, DHA and ARA. The desired lipid content was completed with a non essential fatty acid source, oleic acid (Oleic acid vegetable, Merck, Darmstadt, Germany). The protein source used (squid meal) was defatted in all diets (3 consecutive times with a chloroform: squid meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiet. The microdiets were prepared by mixing squid powder and water soluble components, then the lipids and fat soluble vitamins and finally, gelatin dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain a particle size below 250 μm. Diets were analyzed for proximate and fatty acid composition (Tables 5.1 and 5.2) at GIA facilities.

Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00 for 14 days. To assure feed availability, daily feed supplied was maintained at 2

and 2.5 g/tank during the first and second week of feeding, respectively. Larvae were observed under the binocular microscope to determine feed acceptance.

Table 5.1. Main lipid ingredients composition and analyzed lipid and protein contents of the experimental diets (g/100g diet d.w.)

	DIETS		
	0.3ARA	0.6ARA	1.2ARA
DHA50[1]	8.00	8.00	7.90
EPA50[1]	4.50	4.50	4.30
ARA44[1]	0.00	0.70	2.00
Oleic acid[2]	2.40	1.70	0.70
Lipid content (d.w.)	20.72	20.46	20.50
Protein content (d.w.)	70.38	70.27	70.17

[1] Polaris, Pleuven, France.
[2] Merck, Darmstadt, Germany.

Before the end of the experiment an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and subsequently allocating them in another tank supplied with clean seawater and aeration, to determine survival after 24 hours. Final survival was calculated by individually counting all the alive larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 h) and total length (Profile Projector V-12A Nikon, Tokyo, Japan) of 30 fish/tank at the beginning, in the middle and at the end of the trial. The specific growth rate (SGR) was determined according to the equation: SGR={(ln final body weight) – (ln initial body weight)} X 100/t, where t = days of the feeding period. In addition, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition after 12 h of starvation at the end of the trial.

Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

All data were treated using one-way ANOVA and means were compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Table 5.2. Fatty acid composition of experimental diets for larval European sea bass (g/100 g diet d.w.)

Diet	0.3 ARA	0.6 ARA	1.2 ARA
12:0	0.108	0.015	0.007
14:0	0.228	0.220	0.207
14:1n-7	n.d.*	0.016	0.008
14:1n-5	0.020	0.014	0.008
15:0	0.040	0.041	0.031
15:1n-5	n.d.	0.011	0.006
16:aiso	n.d.	0.013	0.011
16:0	1.446	1.530	1.770
16:1n-7	0.427	0.375	0.315
16:1n-5	n.d.	0.024	0.0200
16:2n-6	0.015	0.031	0.0200
16:2n-4	0.025	0.050	0.048
17:0	0.038	0.064	0.071
16:3n-4	0.063	0.097	0.079
16:3n-3	0.114	n.d.	n.d.
16:3n-1	0.015	0.022	0.020
16:4n-3	0.022	0.022	n.d.
16:4n-1	0.048	0.046	0.046
18:0	0.686	0.775	0.914
18:1n-9	3.763	3.176	2.487
18:1n-7	0.598	0.539	0.452
18:1n-5	0.030	0.023	0.019
18:2n-6	1.333	1.324	1.448
18:2n-4	n.d.	0.048	0.046
18:3n-6	0.050	0.080	0.130
18:3n-4	0.055	0.018	0.020
18:3n-3	0.223	0.214	0.217
18:4n-3	n.d.	0.276	0.274
18:4n-1	0.282	0.029	0.028
20:0	0.081	0.110	0.117
20:1n-9	0.485	0.480	0.452
20:1n-7	0.039	0.041	0.035
20:2n-9	0.043	0.049	0.057
20:3n-9	0.045	0.083	0.143
20:4n-6	0.300	0.616	1.251
20:4n-3	0.050	0.184	0.180
20:5n-3	4.057	3.970	3.867
22:1n-11	0.187	0.194	0.187
22:4n-6	0.035	0.156	0.158
22:5n-6	0.160	n.d.	n.d.
22:5n-3	0.439	0.431	0.419
22:6n-3	5.161	5.045	4.925
Saturated	2.627	2.768	3.128
Monoenoics	5.549	4.893	3.989
n-3	10.066	10.142	9.882
n-6	1.893	2.207	3.007

n-9	4.336	3.788	3.139
n-3HUFA	9.707	9.63	9.391
ARA/EPA	0.074	0.155	0.324
EPA/DHA	0.786	0.787	0.785
oleic a./DHA	0.729	0.630	0.505
oleic a./ n-3HUFA	0.408	0.352	0.283
n-3/n-6	5.317	4.595	3.286

*n.d. not detected.

Results

From the beginning of the feeding trial all diets were well accepted by fish larvae. Despite final survival rate was no significantly different among larvae fed the different ARA levels, it proportionally increased with the elevation of dietary ARA level (Table 5.3). Moreover, elevation of dietary ARA markedly improved growth along the experiment. Thus, average SGR was significantly ($P<0.05$) higher for larvae fed diet 1.2ARA than for those fed diet 0.3ARA (Table 5.3). Accordingly, at the end of the experiment, body weight and total length of larvae fed diet 1.2ARA was significantly ($P<0.05$) higher than those fed 0.3ARA for the weight, and than those fed 0.3ARA and 0.6ARA for the length, respectively (Figs. 5.1 and 5.2). Besides, a positive significant correlation was found between dietary ARA and whole body weight (r^2 =0.9325).

However, after only one week of feeding the experimental diets, no significant differences were found in weight nor in length (Figs. 5.1 and 5.2). Increased dietary ARA levels were positively correlated to larval biomass produced (r^2=0.967) and survival of the larvae after the stress resistance test (r^2=0.8466) (Fig. 5.3, Table 5.3).

Whole body lipid content of the larvae was not significantly affected by the experimental diets. Fatty acid composition of total lipids from whole larval body (Table 5.4) reflected the dietary fatty acid profiles (Table 5.2), where increased dietary ARA

Table 5.3. Effect of dietary ARA levels on survival, growth and stress resistance of European sea bass larvae after 14 days of feeding (Mean ±SD; n=3; different letters in the same column denote significant differences)

Diet	Survival %	SGR	Biomass mg	Survival % after handling
0.3 ARA	42.44±1.76	4.03±0.38b	310.39±26.21	56.67±12.58
0.6 ARA	43.64±6.38	4.71±0.62ab	354.34±80.66	63.33±12.58
1.2 ARA	46.44±2.72	5.26±0.24a	403.04±24.71	66.67±5.77

Table 5.4. Lipid content and fatty acid composition of total lipids from European sea bass larvae after 14 days of feeding several ARA levels (n=3; % total fatty acids, mean±SD)

Diet	0.3ARA	0.6ARA	1.2ARA
Lipid content (d.w.)	17.84±4.07	20.510±4.20	16.165±2.94
12:0	0.136±0.04	0.116±0.10	0.099±0.01
14:0	0.637±0.01	0.973±0.56	0.604±0.04
14:1n-7	0.094±0.02	0.100±0.01	0.066±0.01
14:1n-5	0.077±0.03	n.d.*	0.087±0.01
15:0	0.322±0.03	0.341±0.04	0.296±0.03
15:1n-5	0.131±0.01	0.126±0.01	0.112±0.02
16:0ISO	0.229±0.02	0.222±0.03	0.245±0.06
16:0	15.836±1.73	15.201±0.90	19.745±4.48
16:1n-9	0.792±0.35	1.274±1.20	0.645±0.21
16:1n-7	0.904±0.72	0.889±0.66	1.087±0.05
16:1n-5	0.141±0.09	0.180±0.14	0.145±0.12
16:2n-6	0.281±0.01	0.285±0.04	0.238±0.04
16:2n-4	0.338±0.01	0.409±0.17	0.286±0.01
17:0	0.663±0.09	0.616±0.07	0.854±0.26
16:3n-4	0.438±0.03	0.451±0.10	0.315±0.04
16:4n-3	0.469±0.30	0.681±0.10	0.703±0.12
16:4n-1	0.526±0.15	0.403±0.06	0.359±0.11
18:0	7.510±6.67	8.700±1.02	14.052±6.49
18:1n-9	11.842±2.38	12.759±1.08	10.514±2.12
18:1n-7	6.852±6.42	3.184±0.13	2.742±0.51
18:1n-5	1.254±1.91	0.169±0.02	0.173±0.01
18:2n-6	2.321±1.89	3.520±0.27	3.178±0.61
18:2n-4	1.861±2.35	0.196±0.02	0.150±0.03
18:3n-6	0.287±0.10	0.349±0.01	0.4960.09±
18:3n-3	1.017±0.05	1.080±0.02	0.936±0.21
18:4n-3	0.527±0.02	0.841±0.54	0.452±0.04
20:0	0.448±0.15	0.345±0.05	0.584±0.35
20:1n-9	1.793±0.05	1.765±0.08	1.463±0.19
20:1n-7	0.135±0.04	0.104±0.00	0.092±0.02
20:2n-9	0.734±0.01	0.701±0.05	0.687±0.09
20:3n-9	0.113±0.05	0.188±0.05	0.218±0.08
20:4n-6	2.964±0.15	4.063±0.38	5.796±1.22
20:4n-3	0.358±0.01	0.422±0.12	0.293±0.02
20:5n-3	10.176±0.64	11.325±2.21	7.918±1.17
22:1n-11	0.219±0.04	0.341±0.19	0.207±0.04
22:4n-6	0.718±0.05	0.691±0.05	0.614±0.11
22:5n-3	1.132±0.11	1.274±0.29	1.043±0.11
22:6n-3	26.256±2.31	25.735±0.90	23.086±4.48
Saturated	25,23±8,10	25,95±1,52	35,94±11,57
Monoenoics	24,10±6,24	20,77±0,39	17,22±3,17
n-3	39,93±3,08	41,36±2,22	34,43±6,04
n-6	6,57±1,87	8,91±0,66	10,32±1,87
n-9	15,27±2,50	16,69±0,18	13,53±2,25

n-3HUFA	37.92±3.03	38.76±1,79	32,34±5,75
AA/EPA	0,29±0,01	0,36±0,09	0,73±0,07
EPA/DHA	0,39±0,01	0,44±0,10	0,34±0,02
oleic a./DHA	0,07	0,07	0,06±0,01
oleic a./ n-3HUFA	0,05	0,05	0,05
n-3/n-6	6,08±2,24	4,64±0,61	3,34±0,03

*n.d. not detected.

levels were followed by increased 18:0, 18:2n-6 and ARA and reduced monounsaturated and n-9 fatty acids, 18:1n-9, EPA and DHA. Besides, in comparison with dietary profiles, larval fatty acids also showed an increase in saturated fatty acids with increased ARA levels, and, generally higher ARA/EPA, EPA/DHA and n-3/n-6.

Discussion

The results of this study suggested the importance of ARA to promote European sea bass performance along larval development, even when EPA and DHA dietary contents were not limiting as essential fatty acids for this species. In particular, dietary ARA levels were positively correlated to seabass larval survival (r^2 0.9997; Fig. 5.4). These results are in agreement with those found in other species such as seabream

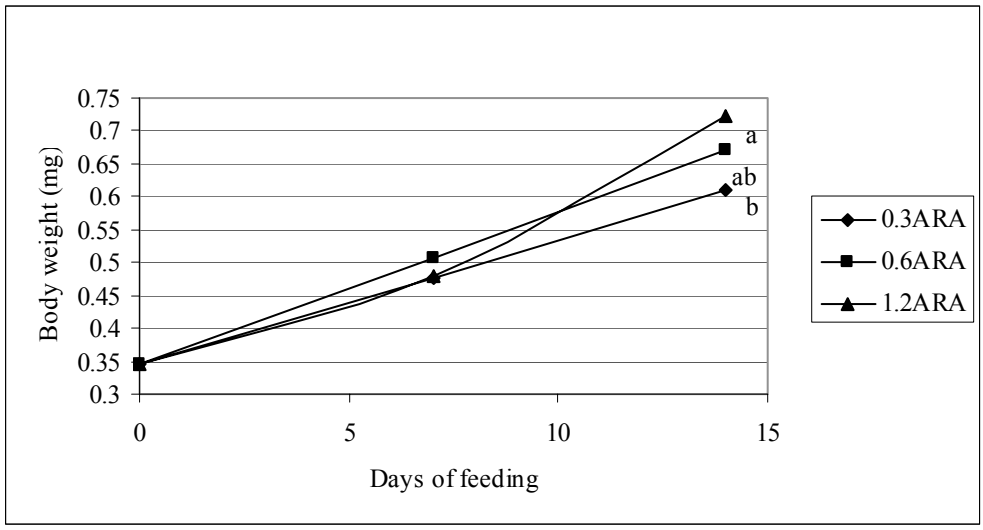

Figure 5.1. Evolution of body weight of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences among larvae fed different diets).

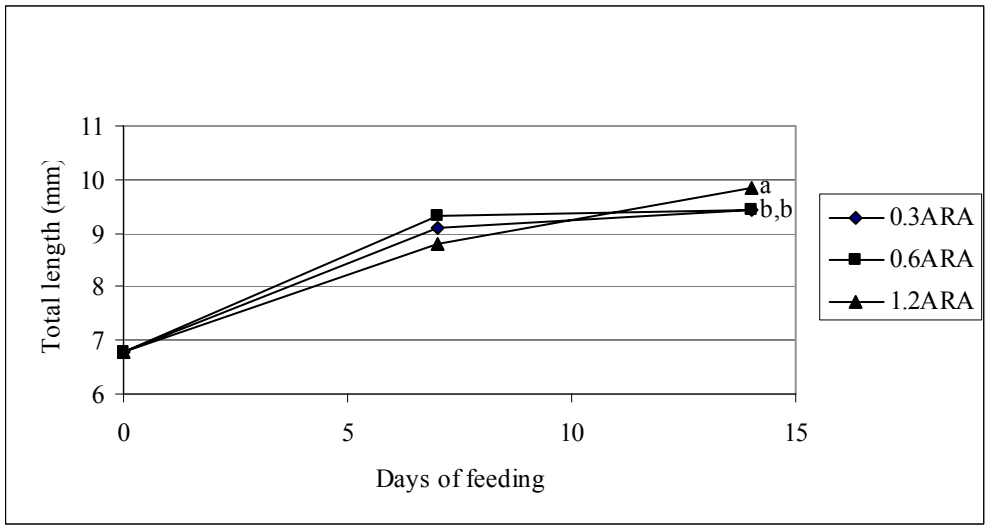

Figure 5.2. Evolution of standard length of fish fed the different experimental diets (n=90; different letters for a given date denote significant differences among larvae fed different diets).

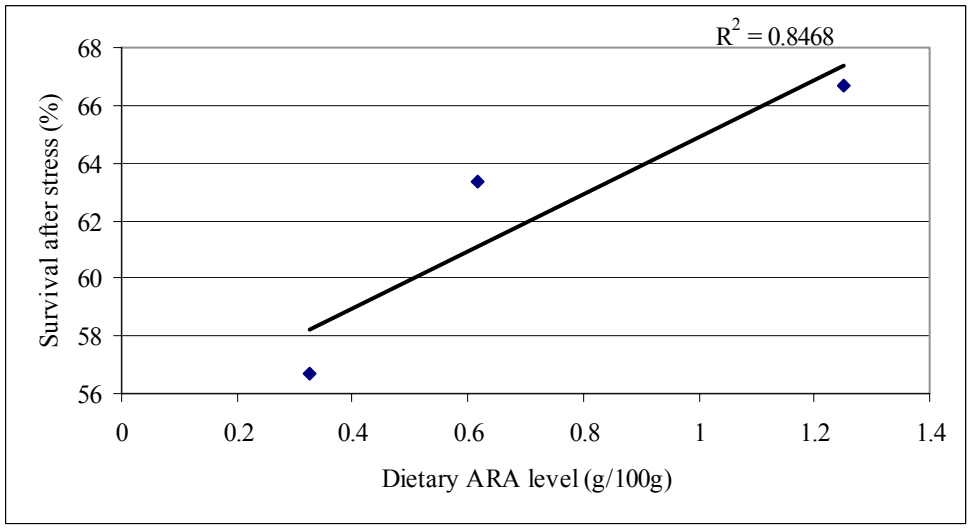

Figure 5.3. Effect of dietary ARA level on larval survival after handling stress at the end of the experiment.

whose larval survival was improved by ARA levels in diets containing EPA and DHA (Bessonart *et al*., 1999) or turbot juvenile were ARA improved survival when fed as the sole essential fatty acid in comparison with those diets containing DHA (Castell *et al*., 1994).

Dietary ARA also had a marked effect in growth of sea bass larvae, in terms of body weight, total length and SGR, showing that for this species at least a 1.2 % dietary ARA is required in first feeding diets to promote larval growth. Similarly, studies in

gilthead sea bream (Bessonart *et al*., 1999) suggested a minimum requirement of about 1 % dietary ARA to improve larval growth and in juvenile turbot (Castell *et al*., 1994) inclusion of ARA is also necessary to improve fish growth.

Dietary ARA was very efficiently incorporated into sea bass larval lipids, although its content in dry weight basis of the larvae (0.53-0.92 %) was slightly lower than in the diet. Elevation of dietary ARA up to 0.6% did not affect the incorporation of DHA or EPA from the diet into larval lipids, however further elevation up to 1.2% markedly reduced EPA incorporation into larval lipids. Similarly, elevation of dietary EPA up to 1.1% in diet did not affected DHA incorporation into larval lipids (Izquierdo *et al*., 2000), but further elevation of dietary EPA to 1.7% at constant dietary DHA contents of 1.1, displaced DHA from the sn-2 position of the phosphatidylcholine (PC) and markedly reduced growth (Izquierdo *et al*., 2000). Hence, dietary ARA levels, ARA/DHA or ARA/EPA higher than those tested in the present study could also affect incorporation into larval lipids of other dietary essential fatty acids (Izquierdo *et al*., 2000) and cause negative effects as in other species (Copeman *et al*., 2002). Nevertheless, arachidonic acid is a main component of a minor but very important polar lipid class, phosphatidylinositol, which is particularly rich in 20 C fatty acids, and is possibly responsible of eicosanoid synthesis regulation.

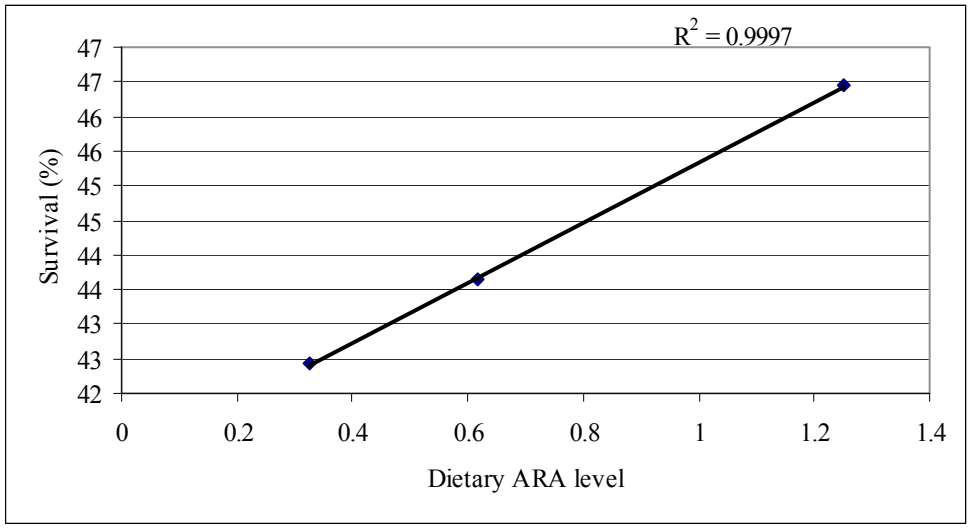

Figure 5.4. Effect of dietary ARA level on larval survival.

A significant positive correlation was found between dietary ARA levels and larval survival after handling stress, denoting the importance of this fatty acid in seabass

larvae response to stressors. Dietary ARA is necessary to improve stress resistance after handling (Koven *et al*. 2003, Van Anholt *et al*. 2004), whereas high levels of ARA in diet seem to be detrimental to chronic stress resistance (Koven *et al*. 2003). Dietary ARA levels close to those used by these authors did not affect the handling of stress resistance in Japanese flounder, whereas higher levels reduced larval stress resistance (Furuita *et al*. 1998).

Several mechanisms may be involved in the beneficial effect of ARA in larval growth and survival. In vitro, ARA is a preferred substrate for most cyclooxygenases, being the main precursor for prostaglandin synthesis, although in some marine fish in vivo, EPA is also a main substrate due to its high presence in the diet (Ganga *et al*., 2005). Cyclooxygenases derived prostaglandins are known to have several functions in the organism including promotion of larval development. Besides, ARA constitutes a good substrate for several lipoxygenases, its derivative hydroxi-fatty acids having important physiological functions in marine fish related with immune-competence (Montero *et al*., 2003) and hence with survival and development (Izquierdo *et al*., 2000). Moreover, recently, ARA has been found to affect ACTH-induced cortisol production in sea bream interrenal cells (Ganga *et al*., 2006) and its effect was inhibited by indomethacin denoting that ARA regulation of stress is mediated by their cyclooxygenase-derived metabolites.

The importance of ARA in marine fish aquaculture has been largely neglected (Bell and Sargent, 2003). The requirements of marine fish larvae for EPA and DHA as essential fatty acids have been known for two decades, only recently more attention has been paid to ARA in marine fish aquaculture (Bessonart *et al*.1999; Bell and Sargent, 2003; Koven *et al*., 2003). The results of the present study contributed to denote the importance of ARA for marine fish larvae and have shown that when fed high DHA levels, European sea bass larvae seem to have a requirement for at least 1.2% ARA at EPA/ARA ratios close to 4 to show good growth performance, as well as high survival and stress resistance. Dietary ARA was very efficiently incorporated into sea bass larval lipids but it reduced the incorporation of EPA. Hence, optimum dietary EPA/ARA ratio or the effect of higher dietary ARA levels should be determined in this and other species of commercial importance in order to promote optimum survival, growth and resistance in production systems.

Acknowledgements

The authors want to acknowledge the provision of fish by the Ecloserie Marine de Gravelines (Gravelines, France). This study was partly supported by a grant from Spanish International Cooperation Agency (programme ll-A) to Eyad Atalah, and by the Canary Islands Government through the project (Efectos de los ácidos grasos esenciales en el desarrollo de las respuestas endocrina e inmune temprana en larvas de lubina *Dicentrarchus labra* 2004).

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Bell, J.G., Henderson, R.J., Pirie, B.J.S. and Sargent, J.R., 1985. Effect of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gill structure in the turbot *Scophthalmus maximus*. J. Fish Biol., 26, 181–191.

Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of supplementation with 20:3n-6, 20:4n-6 and 20:5n-3 on the production of the prostaglandins E and F of the 1, 2 and 3 series in turbot *Scophthalmus maximus* L. brain astroglial cells in primary culture. Biochim. Biophys. Acta., 1211, 335–342.

Bell, J.G., Castell, J.D., Tocher, D.R., MacDonald, F.M. and Sargent, J.R., 1995. Effects of different dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid fatty acid compositions and prostaglandin production in juvenile turbot (*Scophthalmus maximus*). Fish Physiol. Biochem., 14, 139– 151.

Bell, J.G. and Sargent, J.R., 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture, 218, 491– 499.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M., González, M.M. and Fernández-Palacios, H., 1999. Effect of dietary arachidonic acid levels on growth

and survival of gilthead seabream (*Sparus aurata* L.) larvae. Aquaculture, 179, 265-275.

Castell, J.D., Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). Aquaculture, 128, 315– 333.

Castell, J.D., Blair, T., Neil, S., Howes, K., Mercer, S., Reid, J., Young-Lai, W., Gullison, B., Dhert, P. and Sorgeloos, P., 2001. The effect of different HUFA enrichment emulsions on the nutritional value of rotifers (*Brachionus plicatilis*) to larval haddock (*Melanogrammus aeglefinus*). In: Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloos, P. (Eds.), Larvi 01 Fish and Shellfish Larviculture Symposium European Aquaculture Society, Special Publication, vol. 30. Oostende, Belgium, pp. 111 –114.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Copeman, L.A., Parrish C.C., Brown, J.A. and Harel, M., 2002. Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. Aquaculture, 210, 285–304.

Estévez, A., McEvoy, L.A., Bell, J.G. and Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot larvae fed live-prey enriched in arachidonic (ARA) and eicosapentaenoic (EPA) acids. Aquaculture, 180, 321–343.

Estévez, A., Kaneko, T., Seikai, T., Tagawa, M. and Tanaka, M., 2001. ACTH and MSH production in Japanese flounder (*Paralichthys olivaceus*) larvae fed arachidonic acid-enriched live prey. Aquaculture, 192, 309– 319.

Fernández-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M. and Vergara, J., 1995. Effect of n-3 HUFA level in broodstock diets on egg quality of gilthead seabream *Sparus aurata* L.. Aquaculture, 132, 325–337.

Folch, J., Lees, M. and Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497– 509.

Furuita, H., Takeuchi, T. and Uematsu, K., 1998. Effects of eicosapentaenoic and docosahexaenoic acids on growth, survival and brain development of larval Japanese flounder (*Paralichthys olivaceus*). Aquaculture, 161, 59–69.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S., 2005. Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 142, 410–418.

Ganga, R., Tort. L., Acerote, L., Montero, D. and Izquierdo, M.S., 2006. Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream (*Sparus aurata*). Journal of Endocrinology, 190, 39–45.

Harel, M., Koven, W., Lein, I., Bar, Y., Behrens, P., Stubblefield, J., Zoha, Y. and Place, A.R., 2002. Advanced DHA, EPA and ArA enrichment materials for marine aquaculture using single cell heterotrophs. Aquaculture, 213, 347–362.

Hwang, D., 1989. Essential fatty acids and immune responses. FASEB J., 3, 2052–2061.

Iijima, N., Tanaka, S. and Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem., 18, 59–69.

Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish larvae. Review article. Aquacult. Nutr., 2, 183–191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Options Mediterr., 63, 91-102.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989. Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi, 55, 859–867.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem., 22, 97–107.

Izquierdo, M.S., Indrasena, W.M., Milley, J.E. and Lall, S.P. 2002. Identification of leukotrienes, hydroxy fatty acids and prostaglandins in haddock and halibut leucocytes. In: 10th International Symposium on Nutrition and Feeding in Fish, Rodhes (Greece), 2-7 June 2002, pp. 55.

Kaushik, S.J., 2002. European sea bass, *Dicentrarchus labrax*. In: Webster, C.D., Lim, C. (Eds.), Nutrient Requirements and Feeding of Finfish for Aquaculture. CABI Publishing, New York, pp. 28–39.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A., 2001. The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture, 193, 107– 122.

Koven, W., Van Anholt, R., Lutzky, S., Ben Atia, I., Nixon, O., Ron, B. and Tandler, A., 2003. The effect of dietary arachidonic acid on growth, survival, and cortisol

levels in different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or daily salinity change. Aquaculture, 228, 307–320.

McEvoy, L.A., Estévez, A., Bell, J.G., Shields, R.J., Gara, B. and Sargent, J.R., 1998. Influence of dietary levels of eicosapentaenoic and arachidonic acids on the pigmentation success of turbot (*Scophthalmus maximus* L.) and halibut (*Hippoglossus hippoglossus* L.). Bull. Aquacult. Assoc. Can., 4, 17– 20.

Montero, D., Kalinowski, T., Obach, A., Robaina, L., Tort, L., Caballero, M.J. and Izquierdo, M.S., 2003. Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on fish health. Aquaculture, 225, 270– 353.

Oliva-Teles, A., 2000. Recent advances in European sea bass and gilthead sea bream nutrition. Aquac. Int., 8, 477–492.

Rainuzzo, J.R., Reitan, K.I., Jorgensen, L. and Olsen, Y., 1994. Lipid composition in turbot fed live feed cultured by emulsions of different lipid classes. Comp. Biochem. Physiol., 107A, 699–710.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1994. The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: Lahlou, B., Vitiello, P. (Eds.), Aquaculture: Fundamental and Applied Research. American Geophysical Union, pp. 103–113.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol. 11, 183–198.

Sargent, J.R., McEnvoy, L.A., Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEnvoy, L., Tocher, D. and Estevez A., 1999a. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177, 191-199.

Sargent, J.R., McEvoy, L.A., Estevez, A., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1999b. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture, 179, 217–229.

Totland, G.K., Madsen, L., Klementsen, B., Vaagenes, H., Kryvi, H., Frøyland, L., Hexeberg, S. and Berge R.K., 2000. Proliferation of mitochondria and gene expression of carnitine palmitoyltransferase and fatty acyl-CoA oxidase in rat skeletal muscle, heart and liver by hypolipidemic fatty acids. Biology of the Cell, 92 (5), 317-329.

Van Anholt, R., Spanings, F., Koven, W. and Wendelaar Bonga, S., 2004. Dietary supplementation with arachidonic acid in tilapia (*Oreochromisi mossambicus*) reveals physiological effects not mediated by prostaglandins. General and Comparative Endocrinology, 139, 215–226.

Villalta, M., Estévez, A. and Bransden, M.P., 2005. Arachidonic acid enriched live prey induces albinism in Senegal sole (Solea senegalensis) larvae. Aquaculture, 245, 193-209.

CHAPTER 6

THE EFFECT OF VITAMIN E AND DIFFERENT DHA LEVELS ON GROWTH, SURVIVAL, AND STRESS RESISTANCE IN SEA BASS (*Dicentrarchus labrax*) LARVAE

E. Atalah, C. M. Hernández Cruz, E. Ganuza, T. Benítez-Santana, R. Ganga, J. Roo, Fernández-Palacios, H. and M.S. Izquierdo

Abstract

Most marine fish larvae require high amounts of n-3 HUFA, which are very sensitive to peroxidation and require the action of antioxidants to protect them intra- and extra-cellularly from free radical compounds. The objective of the present study was to investigate the combined effect of dietary vitamin E and polyunsaturated fatty acids on growth, survival and health of sea bass larvae along early development. Five isonitrogenous and isolipidic microdiets different in DHA (1, 3, 5 % D. W) and vitamin E (1500, 3000 mg/kg) content were tested in triplicate during 14 days.

The elevation of dietary DHA up to 5% d.w. significantly reduced larval survival. Increase of dietary vit E levels (from 1500 to 3000 mg/kg) for a given DHA level significantly improved resistance to stress. Besides, dietary vitamin E also significantly improved larval growth.There was a positive combined effect of elevation of dietary vitamin E and DHA (from 1 to 3%) whereas further elevation of dietary DHA levels significantly reduced total length. Similarly, increasing of both DHA and vitamin E significantly improved the whole body weight.

Keywords: *Dicentrarchus labrax*; larval nutrition; stress resistance; EPA; DHA; vitamin E.

Abbreviations: DHA (docosahexaenoic acid, 22:6n-3); d.w. (dry weight); EPA (eicosapentaenoic acid, 20:5n-3); HUFA (high unsaturated fatty acids); PUFA (polyunsaturated fatty acid). vit E: vitamin E.

Introduction

Most marine fish larvae require high amounts of n-3 HUFA (highly unsaturated fatty acids with 20 or more carbon atoms) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in their diets as essential fatty acids for normal growth and development (Watanabe, 1982; Watanabe and Kiron 1994; Izquierdo *et al*., 1989a, b, 1992; Izquierdo, 1996; Sargent *et al*. 1999). These fatty acids are very sensitive to peroxidation and they are even more exposed in formulated diets for marine fish larvae which are formed by micro-particles with a large surface area to volume ratio. Indeed, fish larval tissues are known to be very rich in those polyunsaturated fatty acids (PUFA) and require the action of antioxidants to protect them intra- and extra-cellularly from free radical compounds. Moreover, larval tissues have also much higher water contents than juveniles, and hence a functional defence against oxidised fat is more important for the larvae than at later stages of fish life cycle. Lipid and PUFA peroxidation will damage the biomembranes, produce several pathological conditions in fish (Kawatsu 1969; Watanabe *et al*. 1970; Murai and Andrews 1974; Sakai *et al*., 1989) and could cause irreversible changes in the developing tissues of marine fish larvae. Thus, lipid oxidation in marine fish larvae could be at least partly responsible for the higher disease incidence and subsequent larval mortalities as suggested by some authors (Tocher *et al*., 2002). Even increasing vitamin E contents in broodstock diets markedly improves embryo and early larval development in gilthead sea bream (*Sparus aurata*) (Fernández-Palacios *et al*., 1998).

Vitamin E, α-tocopherol is a structural component of cell membranes which has an important role as an antioxidant, controlling peroxidation of unsaturated fatty acids (Putnam and Comben, 1987). α-tocopherol functions together with selenium and ascorbic acid in the radical scavenging enzymes conferring fish with an efficient physiological antioxidant protection (Blazer, 1982) which prevent the chain reactions of PUFAs peroxidation. Thus, for physiological protection against oxidation, the increasing in dietary PUFA must be combined to an increase in vitamin E (Watanabe *et al*., 1981a, b; Sargent *et al*., 1997). Indeed, tissue vitamin E contents are closely relate

with tissue polyunsaturated fatty acid levels (Izquierdo and Fernandez-Palacios, 1997), both nutrients showing a synergistic effect on the non-specific immune responses and disease resistance in Japanese flounder (*Paralychthis olivaceous*) (Wang *et al*., 2006).

Vitamin E is recognize as an important nutrient to regulate fish immune response (Putnam and Comben, 1987), enhancing the activity of non-specific immune system parameters in sea bream (Montero *et al*., 1998). Besides, dietary vitamin E is necessary to overcome stress situations in cultured fish as shown by Montero *et al*. (2001). Vitamin E deficiency is a well-described disease in fish and has been reported in adults and fingerlings in many fish species as chinook salmon (Woodall *et al*., 1964), brown trout (Hashimoto *et al*., 1966) and carp (Watanabe *et al*., 1970). Numerous pathological signs have been observed in vitamin E deficient fishes, including ascites, erythrotcytic fragility, anemia, muscular dystrophy, lordosis and ceroid deposition in liver (Woodall *et al*., 1964; Murai and Andrews, 1974; Poston *et al*., 1976; Smith, 1979; Roald *et al*., 1981). The severity and occurrence of these lesions seems to vary according to the species, maybe related to variations in the site of lipids metabolism. The histopathological changes produced when vitamin E deficiency have not been studied in fish larvae. Moreover, the combined effect of dietary PUFA and vitamin E and its effect on pathological damages on *Dicentrachus labrax* larvae has not been already studied. Early nutritional studies showed that vitamin E is essential for several fish species (Watanabe *et al*., 1970; Murai and Andrews, 1974; González *et al*., 1995). In gilthead sea bream larvae, González *et al*. (1995) found that increase in microdiet α-tocopherol content (22.27 mg/kg to 136 mg/k) improves larval growth and survival, whereas further elevation of this nutrient significantly reduced larval survival. In juveniles of the same species, kept under chronic stress or acute stress conditions, vitamin E supplementation also improves growth and survival (Montero *et al*., 2001). Moreover, Tocher *et al*. (2003) have shown that supplementation of vitamin E to an oxidized diet improves seabream juveniles growth and reduces lipid peroxidation products content in sea bream and turbot (*Psetta maxima*) tissues. However, much less information is available on the effect of vit E along larval development and its relation with dietary polyunsaturated levels in marine fish larvae.

The objective of the present study was to investigate the combined effect of dietary vitamin E and polyunsaturated fatty acids on growth, survival and health of sea bass larvae along early development.

Materials and methods

Sea bass (*Dicentrarchus labrax)* larvae were obtained from natural spawnings from France (Ecloserie Marine de Gravelines, Gravelines, France), the experiment was carried out in the GIA facilities (Grupo de Investigacion en Acuicultura, Las Palmas de Gran Canaria, Spain). A trial was conducted to test 5 microdiets in triplicates. Larvae were previously fed a commercial microdiet (SKRETTING) until they reached 34 days old. Then, larvae (total length 12.11 ± 1.06 mm, dry body weight 1.9 mg) were randomly distributed into the experimental tanks at a density of 600 larvae/tank and were fed one of the experimental diets tested in triplicates for 14 days, at a water temperature of 19.5 to 20ºC. All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (37 ppm salinity) at an increasing rate of 1.0 - 1.5 L/min along the feeding trials. Water entered the tank from the bottom and get out from the top; water quality was daily tested and no deterioration was observed. Water was continuously aerated (125 ml/min), attaining 5-8 ppm dissolved O_2 and saturation ranged between 60 and 80%.

Table 6.1. Main lipid ingredients composition and vitamin E contents of the experimental diets (g/100g diet d.w.)

Dietary DHA/vit E	1/1500	1/3000	3/1500	3/3000	5/1500
DHA50[1] g/100g (DW)	0.00	0.00	4.00	4.00	8.20
EPA50[1] g/100g (DW)	4.00	4.00	3.00	3.00	1.90
vit E[2] mg/kg (d.w.)	1500	3000	1500	3000	1500

[1] CRODA, East Yorkshire, England, UK.
[2] Sigma-Aldrich, Madrid, Spain.

Five isonitrogenous and isolipidic (70.07/17.71 protein/lipid) experimental microdiets (Pellet size < 250 μm) similar in their EPA content (2 % DW) and different in DHA (1, 3, 5 % D. W) and vitamin E (1500, 3000 mg/kg) content were formulated using EPA50 and DHA50 (CRODA, East Yorkshire, England, UK) as sources of EPA and DHA (Table 6.1). The vitamin E (DL- α- Tocoferol Acetate) contents were tested in 1500 and 3000 mg/kg (Sigma-Aldrich, Madrid, Spain). The protein source used (squid meal) was defatted (3 consecutive times with a chloroform:squid meal ratio of 3:1) to allow a complete control of the fatty acid profile of the microdiet. The microdiet based on defatted squid meal (2.6 % lipid content), EPA50 and DHA50 were added in different quantity to obtain the desired ratios, and the oleic acid was added to equalize

Table 6.2. Fatty acid composition of the experimental diets for European sea bass (g/100 g diet dry weight)

Dietary DHA/vit E	1/1500	1/3000	3/1500	3/3000	5/1500
12:0	0.008	0.008	0.007	0.006	0.004
14:0	0.262	0.276	0.228	0.228	0.138
14:1n-7	0.023	0.005	0.018	0.018	0.010
14:1n-5	0.040	0.064	0.030	0.030	0.017
15:0	0.042	0.041	0.046	0.045	0.030
15:1n-5	0.024	0.045	0.021	0.021	0.011
16:0ISO	0.011	0.025	0.011	0.011	0.012
16:0	0.973	1.038	1.162	1.162	1.033
16:1n-9	0.578	0.500	0.440	0.445	0.254
16:1n-7	0.029	0.602	0.023	0.023	0.013
16:1n-5	0.015	0.022	0.018	0.017	0.012
16:2n-6	0.087	0.091	0.073	0.072	0.039
16:2n-4	0.011	0.012	0.009	0.014	0.015
17:0	0.037	0.039	0.042	0.049	0.047
16:3n-4	0.199	0.203	0.152	0.153	0.089
18:0	0.199	n.d*	0.338	0.338	0.429
18:1n-9	8.260	8.808	6.403	6.397	3.745
18:1n-7	0.517	0.620	0.416	0.454	0.366
18:1n-5	0.054	0.054	0.043	0.031	0.021
18:2n-6	1.370	1.409	1.386	1.417	1.342
18:2n-4	0.054	0.052	n.d	0.047	0.034
18:3n-6	n.d	0.024	n.d	n.d	0.048
18:3n-3	0.180	0.192	0.192	0.197	0.196
18:4n-3	0.183	0.183	0.172	0.173	0.139
18:4n-1	n.d	0.020	0.017	n.d	0.013
20:0	n.d	n.d	0.031	0.032	0.058
20:1n-9	0.162	0.170	0.174	0.216	0.280
20:1n-7	n.d	n.d	n.d	n.d	0.025
20:2n-9	n.d	0.012	0.032	n.d	0.049
20:3n-9	n.d	0.017	n.d	0.023	n.d
20:4n-6	0.079	0.180	0.234	0.236	0.297
20:4n-3	0.089	0.090	0.100	0.101	0.105
20:5n-3	2.729	2.741	2.705	2.708	2.440
22:1n-11	n.d	0.027	0.026	n.d	0.060
22:4n-6	n.d	n.d	0.153	0.158	0.314
22:5n-3	0.076	0.082	0.188	0.191	0.323
22:6n-3	0.760	0.853	3.080	3.148	5.870
Saturated	1.533	1.428	1.865	1.869	1.750
Monoenoics	9.700	9.909	7.611	7.652	4.815
n-3	4.018	4.141	6.437	6.517	9.074
n-6	1.536	1.705	1.846	1.884	2.040
n-9	8.999	9.058	7.049	7.081	4.328
n-3HUFA	3.654	3.766	6.073	6.147	8.738
ARA/EPA	0.029	0.066	0.087	0.087	0.122
EPA/DHA	3.591	3.214	0.878	0.860	0.416

Oleic a./DHA	10.87	10.33	2.079	2.033	0.638
Oleic a./n-3HUFA	2.367	2.451	1.107	1.093	0.451
n-3/n-6	2.615	2.429	3.488	3.460	4.448

*n.d. not detected

the lipid content in each diet. Diets were analyzed for proximate and fatty acid composition of dry basis (d.b.) (Table 6.2). Diets were manually supplied; fourteen times per day each 45 min from 9:00-19:00. Daily feed supplied was 2 and 2.5 g/tank during the first and second week of feeding, respectively.

Before the end of the experiment an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and, subsequently, allocating them in another tank supplied with clean seawater and aeration, to determine survival after 24 hours. Final survival was calculated by individually counting all the alive larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish/tank at the beginning, in the middle and at the end of the trial. Besides, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition after 12 hours of starvation at the end of the trial. Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

All data were treated using one-way ANOVA and means were compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA). For Analysis of two-ways for growth data (weight, length, SGR, biomass), normality and homogeneity of variance were checked, and following general linear model was used:

$$\mathbf{Y}_{ijk} = \mu + \mathbf{F}_i + \mathbf{H}_j + (\mathbf{FH})_{ij} + \varepsilon_{ijk}$$

Where $\mathbf{Y}_{ijk}$ is the mean value of the tank, μ is the mean population, $\mathbf{F}_i$ is the fixed effect of the first factor (vitamin for example), $\mathbf{H}_j$ the fixed effect of the second factor (PUFA for example), $(\mathbf{FH})_{ij}$ the interaction between fixed effects, and ε_{ij} is the residual error.

Results

All experimental microdiets were well accepted by larvae. At the end of the trial larvae fed with 1/3000 diet showed the highest survival. However, the elevation of dietary DHA up to 5% d.w. significantly reduced larval survival (Fig. 6.1). Dietary vit E at 1% dietary DHA slightly but not significantly improved the survival.

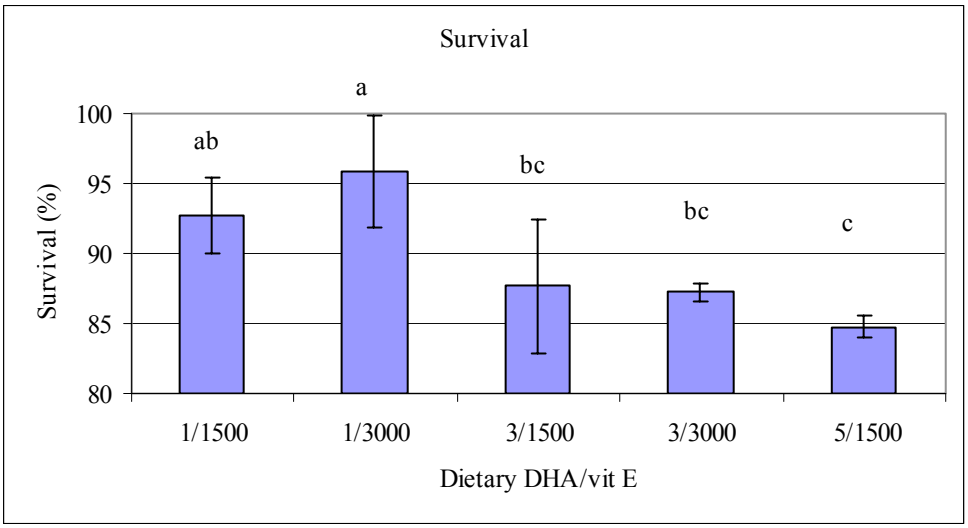

Figure 6.1. Survival rate of fish fed the experimental diets containing several vit E and DHA (n=3; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

Elevation of dietary vit E from 1500 to 3000 mg/kg for a given DHA level significantly improved survival 24 hours after the stress test (Fig. 6.2).

Despite after one week of feeding there were no significant differences in growth in terms of total length, at the end of the experiment dietary vit E elevation significantly ($P = 0.0001$) improved larval growth (Fig. 6.3), regardless the DHA dietary levels. Increase in DHA did not improved total length at the lowest dietary vit E contents, but it significantly improved ($P = 0.003$) total length at dietary vit E values of 3000 mg/kg. Lowest growth was found in larvae fed the highest DHA levels. The two way ANOVA (Table 6.3) analysis showed a significant and positive effect of the elevation of DHA from 1 to 3%, being larvae fed this later level a 4% bigger. But further elevation of dietary DHA levels significantly reduced total length, being larvae fed 5% DHA 4% smaller. This analysis also showed a positive significant effect of vit E, being larvae fed with 3000 mg/kg a 6% bigger.

Table 6.3. Effect of different dietary DHA/vit E contents on larval growth, and results of the two-way anova statistical analysis

	Experimental Diets DHA/vit E												
	1/1500		**1/3000**		**3/1500**		**3/3000**		**5/1500**		Probability of contrasts		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	DHA	vit E	DHA X vit E
Whole body weight (mg)	4.11	0.72	4.75	0.84	4.54	0.74	5.84	1.07	3.50	0.30	NS	0.055	NS
Total length (mm)	13.89	0.36	14.51	0.44	14.25	0.56	15.11	0.37	13.67	0.29	0.003	0.0001	NS
Biomass (g)	213.6	50.8	263.9	62.0	211.6	52.2	266.9	52.9	150.7	15.3	0.073	NS	NS
SGR	5.44	1.21	6.48	1.22	6.15	1.21	7.94	1.26	4.34	0.60	NS	0.055	NS

*NS: Not significant

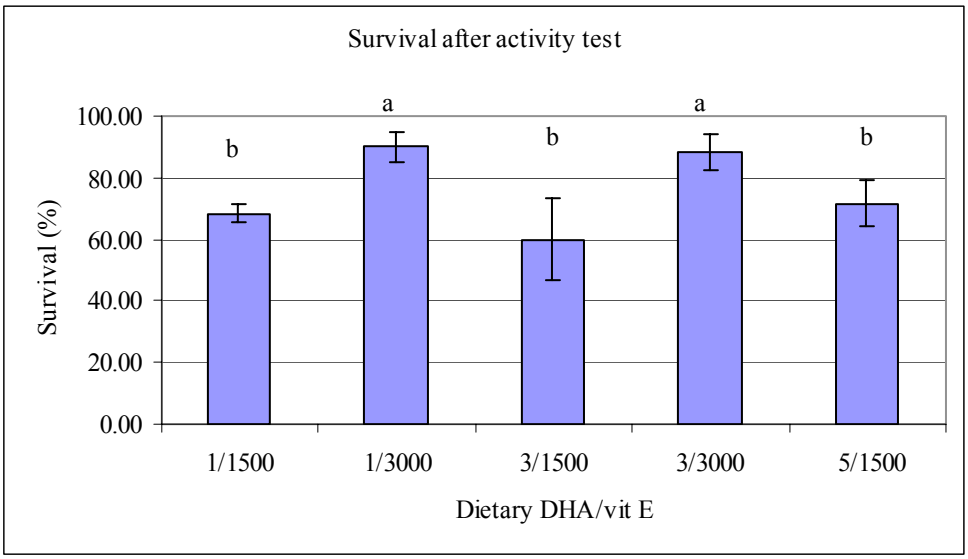

Figure 6.2. Survival after 24h of air activity test of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

The increase in both DHA and vit E from 1/1500 to 3/3000 (DHA/Vit E) significantly improved whole body dry weight (Fig. 6.4), whereas neither the elevation of dietary DHA alone nor vit E alone significantly improved body weight. However, further increase of DHA showed a negative effect, larvae fed with 3% DHA being 45% heavier than those fed 5% DHA.

In SGR, only the elevation of both DHA from 1 to 3 and vit E from 1500 to 3000 significantly improved specific growth rates (fig. 6.5). The two way ANOVA analysis showed a significantly negative effect of dietary DHA over SGR.

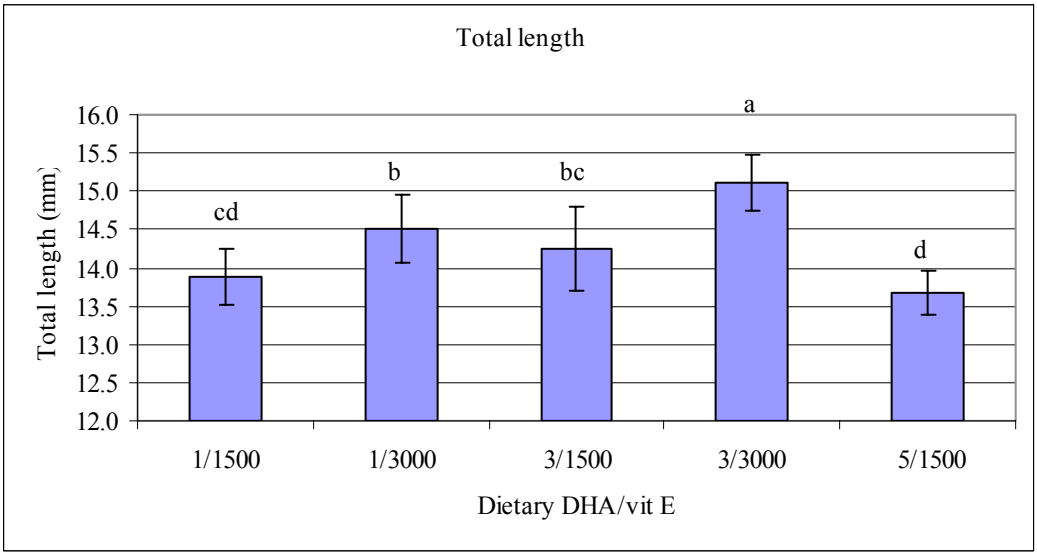

Figure 6.3. Final total length of fish fed the different experimental diets (n=90; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

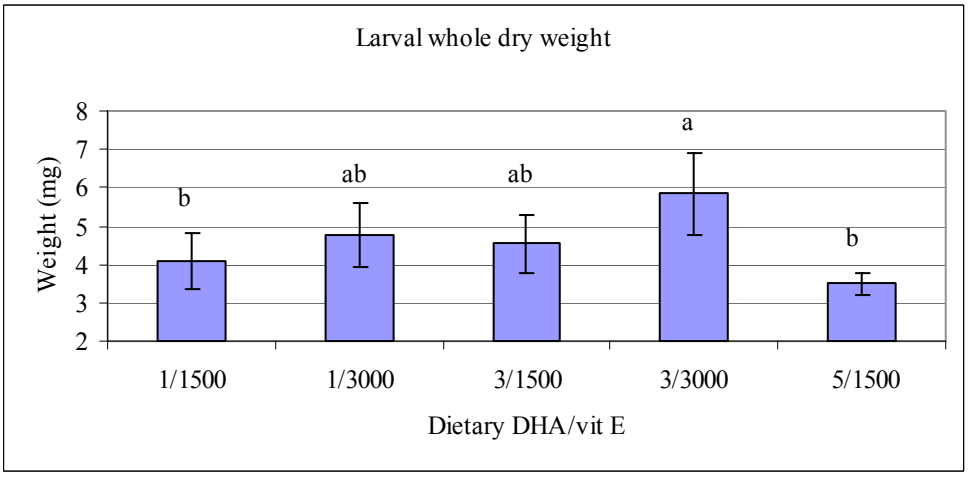

Figure 6.4. Final dry whole body weight of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

As a consequence of the lowest survival and body weight, the elevtion of dietary DHA up to 5% significantly reduced larval total biomass. (Fig. 6.6). The two way Anova analysis showed a negative significant effect of dietary DHA, being average biomass of larvae fed 3% DHA 53% bigger than those fed 5% DHA.

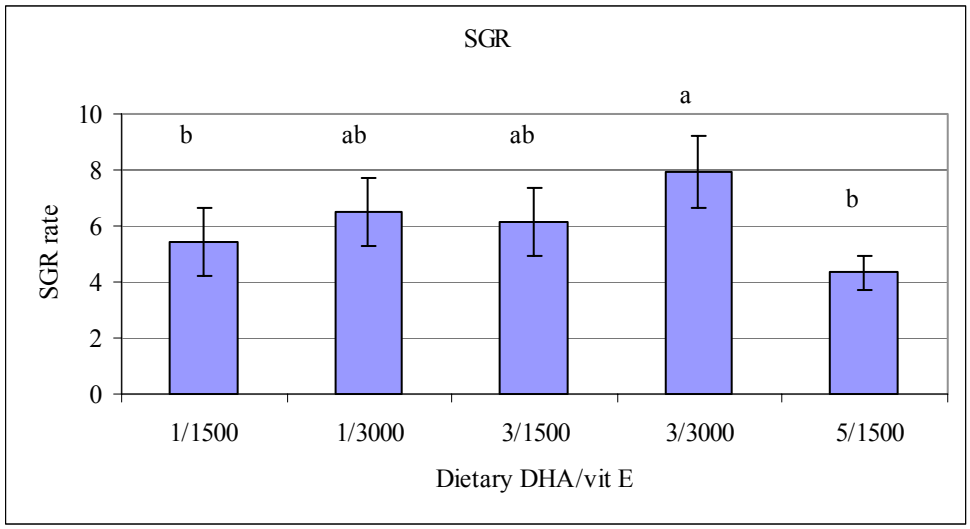

Figure 6.5. Specific growth rate (SGR) of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

Dietary fatty acid compositions were similar in saturated content, whereas they differed in their monounsaturated and polyunsaturated fatty acids contents (Table 6.2). Diets contain about 1% DHA (1/1500 and 1/3000) showed high monoenoic (28.47 % increament) and n-9 (27.79 % increament) fatty acid than diet contain 3 or 5 DHA (3/1500, 3/3000 and 5/1500) due to higher oleic acid in the former. Increasing n-3, n-6 and n-3 HUFA was found with the increase in dietary DHA.

In comparison with larvae fed diets containing about 1% DHA (1/1500, 1/3000), larvae fed diets (3/1500, 3/3000) showed increment in saturated (13.67 % increase), n-3 (13.88 % increase), 18:3n-6 (21.5 % increase), 22:4n-6 (71.82 % increase), ARA (11.25 % increase), DHA (42.46 % increase) and n-3 HUFA (14.76 % increase) fatty acids, in agreement with their diet composition. Besides, larvae fed 3% DHA diets (3/1500, 3/3000) were lower in 18:1n-9 (11.44 % reduction) 20:3n-9 (48.93 % reduction), 20:4n-3 (13.62% reduction), 22:5n-3 (5.76 % reduction), EPA (57.65 % reduction) than larvae fed 1% DHA (1/1500,1/3000) diets.

Regarding fatty acid composition of total lipids from larvae (Table 6.4), elevation of vit E from 1500 to 3000 mg/kg, slightly increased EPA (12.79 % increase), n-3 (5.20 % increase), and n-3 HUFA (5.82 % increase) in larvae fed 1% DHA diets and slightly increased ARA (1.44 % increase), DHA (1.62 % increase), n-6 (23.78 % increase) in larvae fed 3% DHA.

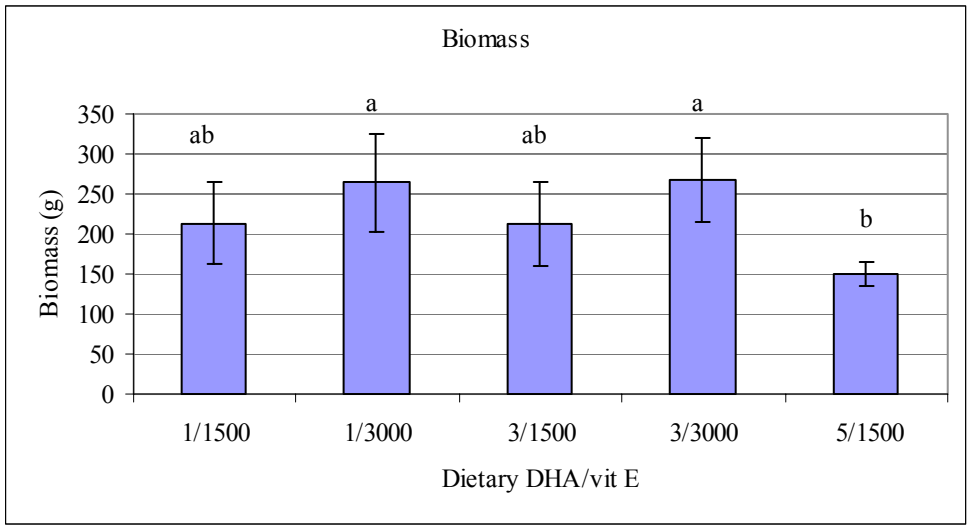

Figure 6.6. Final biomass of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

Discussion

Elevation of DHA contents from 1 to 3 % in diets containing 3000 mg/ kg vit E, significantly improved larval growth in terms of total length. DHA is more essential as EFA than EPA in marine fish larvae (Watanabe *et al*., 1989; Watanabe, 1993), being essential for fish growth (Izquierdo, 1996). Its incorporation into cell membrane regulates membrane integrity and function (Izquierdo, 2005) and this fatty acid is an important component of phosphoglycerides, particularly phosphatidyl ethanolamine and phosphatidyl choline in fish larvae. It is specifically retained in starved or low-EFA fed fish, due to the lower cell oxidation rates than other fatty acids (Madsen *et al*., 1999).

However, when the diet contained only 1500 mg/ kg vit E, the elevation of DHA dietary levels from 1 to 3 % did not improved total length, and the further increase up to 5% significantly reduced sea bass growth. This different effect of dietary DHA at different vitamin E levels suggest the formation of DHA derived peroxide compounds toxic for sea bass. Indeed, increase in dietary vitamin E markedly increased several polyunsaturated fatty acids in seabass total lipids. In the same diets which contained only 1500 vit E mg/kg, the elevation of DHA decrease the survival (Fig. 6.7).

Lipid peroxidation, is recognized as being highly deleterious, resulting in damage to cellular biomembranes (Kanazawa, 1991, 1993) and sub-cellular membranes, such as those of mitochondria, and causing several pathological conditions in fish (Kawatsu, 1969; Watanabe *et al*., 1970; Murai and Andrews, 1974; Sakai *et al*., 1989).

Table 6.4. Fatty acid composition of total lipid of the larval sea bass (% total fatty acid)

Dietary DHA/vit E	1/1500	1/3000	3/1500	3/3000	5/1500
12:0	0.084± 0.07	0.042±0.00	0.139±0.18	0.084±0.03	0.081±0.03
14:0	0.886±0.08	0.796±0.14	0.854±0.08	0.867±0.14	0.676±0.06
14:1n-7	0.097±0.01	0.065±0.04	0.073±0.01	0.081±0.01	0.057±0.00
14:1n-5	0.099±0.00	0.087±0.00	0.076±0.01	0.073±0.01	0.056±0.00
15:0	0.376±0.05	0.223±0.14	0.349±0.03	0.328±0.05	0.322±0.05
15:1n-5	0.181±0.12	0.153±0.07	0.097±0.01	0.097±0.02	0.066±0.01
16:0ISO	0.186±0.06	0.157±0.08	0.214±0.01	0.213±0.04	0.203±0.02
Me16:0	0.134±0.16	0.090±0.07	0.041±0.00	n.d*	0.052±0.00
16:0	14.024±2.07	12.073±1.65	14.804±1.45	15.323±2.73	13.868±1.36
16:1n-9	0.903±0.08	0.782±0.16	0.768±0.08	0.813±0.11	0.625±0.04
16:1n-7	2.239±0.16	2.032±0.49	1.904±0.21	1.932±0.30	1.287±0.15
16:1n-5	0.173±0.03	0.158±0.02	0.157±0.03	0.162±0.04	0.103±0.03
16:2n-6	0.507±0.05	0.480±0.13	0.428±0.06	0.484±0.02	0.281±0.04
16:2n-4	0.207±0.06	0.154±0.03	0.240±0.06	0.257±0.09	0.310±0.07
17:0	0.426±0.06	0.382±0.06	0.505±0.08	0.522±0.09	0.524±0.08
16:3n-4	0.956±0.07	0.854±0.21	0.772±0.10	0.784±0.12	0.528±0.06
16:3n-1	0.114±0.02	0.094±0.02	0.120±0.02	0.121±0.02	0.116±0.01
16:4n-3	0.644±0.08	0.500±0.07	0.589±0.07	0.583±0.13	0.526±0.05
16:4n-1	0.730±0.05	0.635±0.13	0.572±0.02	0.568±0.10	0.446±0.03
18:0	6.163±1.04	5.102±0.63	6.497±0.43	6.602±1.16	6.626±0.65
18:1n-9	31.256±3.33	27.460±7.00	26.305±2.97	26.382±4.15	18.458±2.13
18:1n-7	4.087±0.47	3.408±0.83	3.652±0.25	3.686±0.51	2.062±1.72
18:1n-5	0.331±0.03	0.277±0.08	0.262±0.03	0.266±0.04	0.184±0.04
18:2n-9	0.216±0.00	0.148±0.00	0.169±0.00	0.166±0.02	0.130±0.00
18:2n-6	5.825±0.21	5.610±0.86	5.959±0.17	5.857±0.50	5.811±0.45
18:2n-4	0.245±0.02	0.207±0.13	0.264±0.05	0.220±0.02	0.188±0.01
18:3n-6	0.199±0.05	0.184±0.03	0.240±0.02	0.248±0.03	0.333±0.03
18:3n-4	0.164±0.01	0.125±0.03	0.137±0.00	0.079±0.01	0.000
18:3n-3	0.553±0.04	0.542±0.07	0.574±0.03	0.577±0.03	0.628±0.02
18:3n-1	0.139±0.00	0.174±0.04	0.199±0.09	0.149±0.04	0.093±0.02
18:4n-3	0.411±0.15	0.508±0.18	0.400±0.03	0.386±0.07	0.400±0.02
20:0	0.202±0.04	0.168±0.02	0.266±0.02	0.258±0.05	0.356±0.05
20:1n-9	1.978±0.27	1.581±0.42	2.026±0.25	1.927±0.27	1.989±0.25
20:1n-7	n.d	n.d	0.145±0.01	0.072±0.00	0.142±0.03
20:2n-9	0.809±0.07	0.712±0.15	0.866±0.05	0.865±0.12	0.963±0.08
20:3n-9	0.145±0.02	0.139±0.04	0.096±0.00	0.095±0.01	0.076±0.00
20:4n-6	2.058±0.28	1.963±0.33	2.249±0.27	2.282±0.29	2.518±0.16
20:4n-3	0.272±0.05	0.296±0.11	0.251±0.02	0.248±0.03	0.257±0.01
20:5n-3	10.322±3.60	11.642±2.72	8.266±1.80	8.457±2.25	7.876±1.22
22:1n-11	0.126±0.10	0.073±0.00	0.139±0.04	0.138±0.01	0.187±0.03
22:4n-6	0.266±0.02	0.223±0.04	0.856±0.16	0.879±0.17	1.476±0.11
22:5n-3	0.907±0.34	0.963±0.22	0.884±0.25	0.884±0.24	1.063±0.15
22:6n-3	11.048±3.74	10.961±2.97	18.973±7.01	19.279±6.90	28.457±6.32
Saturated	22.283±3.32	18.991±2.49	23.642±1.96	24.169±4.20	22.672±2.30

Monoenoics	41.403±4.41	35.978±8.99	35.508±3.98	35.552±5.49	24.953±4.75
n-3	24.156±7.79	25.411±6.12	29.936±9.07	27.623±10.62	39.206±7.64
n-6	8.855±0.09	8.460±1.24	7.746±3.11	9.588±0.72	10.418±0.26
n-9	34.305±3.67	30.012±7.53	29.275±3.25	29.296±4.55	21.001±2.85
n-3HUFA	22.548±7.33	23.861±5.61	28.373±8.79	26.077±10.46	37.652±7.54
ARA/EPA	0.209±0.04	0.171±0.02	0.276±0.03	0.277±0.04	0.323±0.03
EPA/DHA	0.932±0.03	1.074±0.11	0.452±0.06	0.449±0.04	0.279±0.02
Oleic a./DHA	3.091±1.17	2.677±1.25	1.553±0.70	1.548±0.76	0.679±0.21
Oleic a./n-3HUFA	1.603±0.62	1.273±0.52	1.058±0.44	1.209±0.54	0.529±0.16
n-3/n-6	2.729±0.89	3.027±0.68	4.937±4.02	2.932±1.31	3.777±0.84

* n.d. not detected

PUFA oxidation produces compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons which are implicated in several pathological conditions in fish (Kawatsu, 1969; Watanabe *et al*., 1970; Murai and Andrews, 1974; Sakai *et al*., 1989).

Elevation of dietary vitamin E levels for a given DHA level significantly improved larval growth in terms of total length. Studies conducted with the same type of microdiets showed that elevation of dietary vitamin E levels from 500 mg/kg to 3000 mg/kg, significantly improve growth of larval gilthead seabream (Atalah *et al*., in prep). Similar results have been found in juveniles of species such as Atlantic salmon (*Salmo salar*) (Hamre *et al*., 1994) or rainbow trout (*Onchorhynchus mykiss*) (Cowey *et al*., 1984). Besides, increase in vitamin E dietary contents improves juvenile gilthead sea bream growth, particularly when oxidized oils are present in the diet (Tocher *et al*., 2003).

There was a clear positive effect of vitamin E on the resistance to stress. Larvae fed with high vitamin E showed a significantly improved stress resistance (Fig. 6.2). In agreement with this finding, Montero *et al*., (1998) showed in sea bream juveniles a reduction in alternative complement pathway (ACP - a pathway as a non-specific immune mechanism) activity in fish fed with deficiencies in α-tocopherol. Under both chronic and acute stress Montero *et al*., (2001) observed decrease resistance to stress by increasing mortality in sea bream juveniles fed vitamin E-deficient diet.

Nevertheless, the positive effect of vitamin E on fish growth is not only related to its antioxidant properties, but also to other functions of this molecule in cellular metabolism, signal transduction (Traber and Parker, 1995) or modulation of eicosanoid synthesis (Cornwell and Parganamala, 1993). Indeed, analyzed α-tocopherol fish body

contents along larval development showed a steady increase from the first days of feeding until the end of metamorphosis sea bass (Guerriero *et al*., 2004), suggesting the high requirement of vit E during the larval stage.

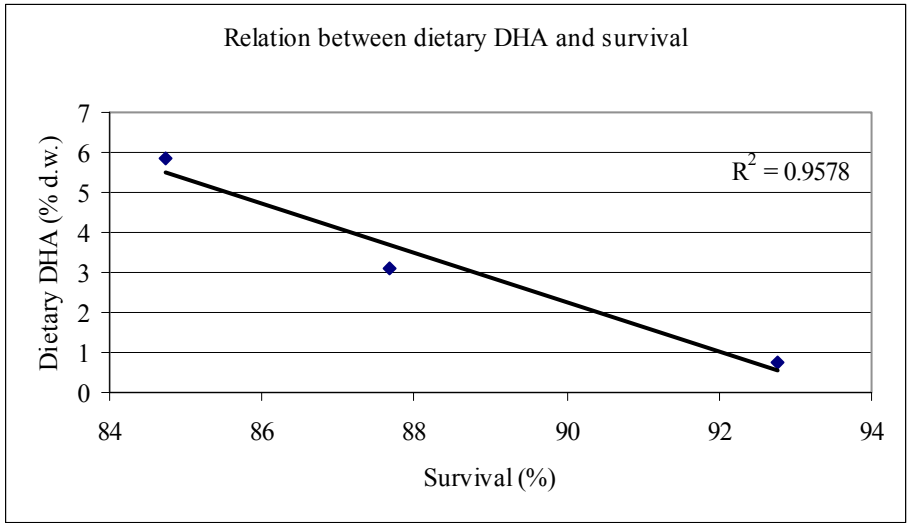

Figure 6.7. Correlation between dietary DHA levels (in the 1500 mg vit E diets) and survival of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

In this experiment in sea bass survival was not affected by the elevation of dietary vitamin E from 1500 to 3000 mg/kg, In gilthead sea bream, Gonzalez *et al*., (1995) showed improved survival with the elevation of vitamin E form 22.27 to 136 mg/kg and a reduction in survival when dietary vitamin E level was increased up to 136mg/kg (Gonzalez *et al*., 1995). However, feeding with high dietary level of HUFA and oxidized oil, Tocher *et al*. (2003) demonstrated that the vitamin E improve the survival in juvenile of turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*) and gilthead sea bream (*Sparus aurata*).

In summary, the results of the present study have shown that despite European sea bass requires DHA to promote growth, high dietary levels of this fatty acid have a negative impact on seabass survival, particularly when dietary antioxidants such as vitamin E are insufficient. Besides, dietary vitamin E showed a marked positive effect on fish growth and stress resistance.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Blazer, V. S., 1982. The ffect of marginal deficiencies of ascorbic acid and α-tocopherol on the natural resistance and immune response of rainbow trout (*Salmo gairdneri*). Ph.D. dissertation. University of Rhode Island.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Cornwell, D. G. and Panganamala, R. V., 1993. Vitamin E action in modulation the arachidonic acid cascade. In: L. Packer, & J. Fuchs (Eds.), *Vitamin E in Health and Disease*. Marcel Dekker, Inc., New York, U.S., 385-410.

Cowey, C. B., Degener, E., Tacon, A. G. J., Youngson, A. and Bell, J. G., 1984. The effect of vitamin E and oxidized fish oil on the nutrition of rainbow trout (*Salmo gairdneri*) growth at natural, varying water temperatures. British Journal of Nutrition, 51**,** 443–451.

Fernández-Palacios, H., Izquierdo, M.S., Gonzalez, M., Robaina, L. and Valencia, A., 1998. Combined effect of dietary a-tocopherol and n-3 HUFA on egg quality of gilthead seabream broodstock (*Sparus aurata*). Aquaculture, 161, 475–476.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

González, M.M., Izquierdo, M.S., Salhi, M., Hernández- Cruz, C.M. and Fernández-Palacios, H., 1995. Dietary vitamin E for *Sparus aurata* larvae. Europ. Aqua. Soc. Spec. Publ., 24, 239–242.

Guerriero, G., Ferroa, R., Russob, G.L. and Ciarcia, G., 2004. Vitamin E in early stages of sea bass (*Dicentrarchus labrax*) development. Comparative Biochemistry and Physiology, Part A, 138, 435– 439.

Harel, M., Tandler, A. and Kissil, G.W., 1994. The kinetics of nutrient incorporation into body tissues of gilthead seabream (*Sparus aurata*) females and the subsequent effects on egg composition and egg quality. Br. J. Nutr., 72, 45– 58.

Hashimoto, Y., Okaichi, T., Watanabe, T., Furukawa. A. and Umezu, T., 1966. Muscle dystrophy of carp due to oxidized oil and the preventive effect of vitamin E. Bull. Jpn. Sci. Fish, 32, 64-69.

Izquierdo, M. S., 1996. Review article: essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr., 2, 183–191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Options Mediterr., 63, 91-102.

Izquierdo, M. and Fernández-Palacios, H., 1997. Nutritional requirements of marine fish larvae and broodstock. Cah. Options Mediterr., 22, 243–264.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989a. Optimal EFA levels in *Artemia* to meet the EFA requirements of red seabream *Pagrus major*. In: Takeda, M., Watanabe, T. _Eds., The Current Status of Fish Nutrition in Aquaculture. Japan Translation Centre, Tokyo, Japan, pp. 221–232.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989b. Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi, 55, 859–867.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Izquierdo, M.S., Arakawa, T., Takeuchi, T, Haroun, R. and Watanabe, T., 1992. Effect of n-3 HUFA levels in Attemia on growth of larval Japanese flounder (*Paralichthys olivaceous*). Aquaculture, 105, 73-82.

Kanazawa, K., 1991. Hepatotoxicity caused by dietary secondary products originating from lipid peroxidation. In: Friedman, M. (Ed.), Nutritional and Toxicology Consequences of Food Processing. Plenum, New York, pp. 237–253.

Kanazawa, K., 1993. Tissue injury induced by dietary products of lipid peroxidation. In: Corongiu, F. (Ed.), Free Radicals and Antioxidants in Nutrition. Richelieu Press, London, pp. 383– 399.

Kawatsu H., 1969. Studies on the anemia of fish-III. An example of macrocytic anemia found in brook trout, Salvelinus fontinalis. Bulletin of the Freshwater Research Laboratory, 19, 161–167.

Madsen, L., Rustan-Arild, C., Vaagenes, H., Berge, K., Dyroy, E. and Berge, R.K., 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipid, 34 (9), 951-963.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L. and Vergara, J. M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiology and Biochemistry, 18, 399–407.

Montero D, Tort L, Robaina L, Vergara JM and Izquierdo M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish and Shellfish Immunology, 11, 473–490.

Murai T. and Andrews J.W., 1974. Interaction of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 104, 1416–1431.

Roald, S. O., Armstrong, D. and Landsverk, T., 1981. Histochemical, fluorescent and electron microscopical appearance of hepatocellular ceroidosis in the Atlantic salmon (*Salmo salar* L.). J. Fish. Dis. 4: 1-14.

Poston, H.A., Combs Jr., G.F. and Leibovitz, L., 1976. Vitamin E and selenium interrelations in the diet of Atlantic salmon (*Salmo salar*): gross, histological and biochemical deficiency signs. J. Nutr., 106, 892–904.

Putnam M.E. and Comben M., 1987. Vitamin E. Veterinary Record, 121, 541-545.

Sakai T., Murata H., Endo M., Yamauchi K., Tabata N. and Fukudome M., 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola aquiqueradiata*. Agricultural and Biological Chemistry, 53, 1739–1740.

Sargent, J.R., McEvoy, L.A. and Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEvoy, L., Tocher, D. and Estevez, A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177, 191–199.

Smith, C.E., 1979. The prevention of liver lipoid degeneration ceroidosis and microcytic anemia in rainbow trout *Salmo gairdneri* Richarson fed rancid diets: a preliminary report. J. Fish Dis., 2, 429–437.

Tocher D.R., Mourente G., Van der Eecken A., Evjemo J.O., Diaz E., Bell J.G., Geurden I., Lavens P. and Olsen Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquaculture Nutrition, 8, 195–207.

Tocher D.R., Mourente G., Van der Eecken A., Evjemo J.O., Diaz E., Wille M., Bell J.G., and Olsen Y., 2003. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International, 11, 195–216.

Traber, M.G. and Packer, L., 1995. Vitamin E: beyond antioxidant function. *Am. J. Clin. Nutr.*, 62 (suppl.), 1501S-1509S.

Wang Z., Mai K., Liufu Z., Ma H., Xu W., Ai Q., Zhang W., Tan B. and Wang X., 2006. Effect of high dietary intakes of vitamin E and n-3 HUFA on immune responses and resistance to Edwardsiella tarda challenge in Japanese flounder (*Paralichthys olivaceus*, Temminck and Schlegel) Aquaculture Research, 37, 681-692.

Watanabe, T., 1982. Lipid nutrition in fish. Comp. Biochem. Physiol., 73, 3-15.

Watanabe, T., 1993. Importance of docosahexanoic acid in marine larval fish. J. World Aquacult. Soc., 24, 152– 161.

Watanabe, T. and Kiron, V., 1994. Prospects in larval fish dietetics. Aquaculture, 124, 223–251.

Watanabe T., Takashima F., Ogino C. and Hibiya T., 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi, 36, 623–630.

Watanabe, T., Takeuchi, T. and Wade, M., 1981a. Dietary lipid levels and α-tocopherol requirement of carp. Bulletin of the Japanese Society of Scientific Fisheries, 47(12), 1585–1590.

Watanabe, T., Takeuchi, T., Wada, M. and Uehara, R., 1981b. The relationship between dietary lipid levels and α-tocopherol requirement of rainbow trout. Bulletin of the Japanese Society of Scientific Fisheries, 47(11), 1463–1471.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C., 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. Nippon Suisan gakkaishi, 55(9), 1635-1 640.

Woodall, A.N., Ashley, L.M., Halver, J.E., Scott, H.S. and Van de Veen, J., 1964. Nutrition of salmonid fishes: XIII. The α-tocopherol requirement of chinook salmon. J. Nutr., 84, 125– 135.

CHAPTER 7

ENHANCEMENT OF GILTHEAD SEABREAM (*Sparus aurata*) LARVAL GROWTH BY DIETARY VITAMIN E IN RELATION TO TWO DIFFERENT LEVELS OF ESSENTIAL FATTY ACIDS

E. Atalah, C. M. Hernández Cruz, E. Ganuza, T. Benítez-Santana, R. Ganga, J. Roo, Fernández-Palacios, H. and M.S. Izquierdo

Abstract

Despite the particular importance of vitamin E as a defence against oxidised fat in fish, studies aimed to determine its significance along larval development and its relation with dietary polyunsaturated fatty acid levels are very scarce. The objective of the present study was to determine the effect of dietary vitamin E on gilthead seabream growth and survival, at two different PUFAs levels. Eighteen days old gilthead seabream larvae were fed five experimental diets formulated combining two different dietary levels of PUFAs with three different levels of vitamin E (2.5/1.5 DHA/EPA with 500 and 3000 mg/kg vitamin E and 5/2.5 DHA/EPA with 500, 3000 and 6000 mg/kg vitamin E). Increase in vitamin E levels up to 3000 mg/kg markedly improved larval growth, particularly when dietary PUFA levels were lower, suggesting a higher protection value when these fatty acids are more limiting. At higher dietary PUFA levels, increase in vitamin E from 500 to 3000 mg/kg enhanced overall larval performance in terms of growth parameters and survival, increasing PUFAs content in the larval polar lipids, despite they were identical in both diets and denoting the anti-oxidative effect of vitamin E. However, increase in vitamin E from 3000 to 6000 mg/kg not only did not further improved larval performance showed, but also reduced PUFAs contents in larval polar lipids, suggesting the pro-oxidant effect of excess vitamin E. In summary, the results of the present study suggest that elevation of dietary vitamin E up to 3000 mg/kg with 2.5/1.5 DHA/EPA gives a better performance in seabream larvae

than increasing DHA/EPA dietary levels to 5/2.5, denoting the importance of dietary vitamin E for larval growth and its interrelation to dietary PUFA levels.

Keywords: sea bream (*Sparus aurata*) larvae; DHA; EPA; vitamin E; microdiet.

Abbreviattions: DHA: Docosahexaenoic acid, EPA: Eicosapentaenoic acid, HUFA: High unsaturated fatty acid, PUFA: Polyunsaturated fatty acid, ARA: Arachidonic acid, EFA: Essential fatty acids, n-3 HUFA: highly unsaturated fatty acids with 20 or more carbon atoms and 3 or more double bonds, vitE: vitamin E, DPA: Docosapentaenoic acid.

Introduction

Fish feeds, and particularly those for the larval stages, are rich in polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic (22:6n-3; DHA) acids, which are molecules with a great susceptibility to oxidative damage (Halliwell and Gutteridge, 1996). When PUFAs are exposed to either oxygen or reactive oxygen species form lipid radicals, lipid peroxy radicals and other derivatives, which are toxic and damage various biomolecules, such as protein and DNA. Peroxidation risk is even higher in larval diets since they contain micro-particles with a large surface area to volume ratio, and frequently remain in the water for a longer period of time (Izquierdo, 2005). Thus, functional defence against oxidised fat is particularly important in fish larvae. Besides, fish larvae tissue lipids are also very high in PUFA, what implies a higher risk of peroxidation (Sargent *et al*. 1999) and, hence, cellular damage (Sakai *et al*. 1989; Kanazawa, 1991, 1993).

Endogenous enzymes play an important role in physiological antioxidant protection (Blazer, 1982) including radical scavenging enzymes, such as catalase and superoxide dismutase, acting on hydrogen peroxide (H_2O_2) and superoxide (O^{-2}), respectively, and glutathione peroxidase, which scavanges H_2O_2 and lipid hydroperoxides (Halliwell and Gutteridge, 1996). α-tocopherol functions together with selenium and ascorbic acid in these enzymes to stop the chain reactions of PUFAs peroxidation. Thus, vitamin E (vit E) functions as a chain breaking antioxidant, reacting with the lipid peroxide radical produced and preventing the further reaction with a new PUFA. In addition, vit E plays an important role in the fish immune response (Montero *et al*., 1998; Wang *et al*., 2006), reproduction (Izquierdo *et al*., 2001), stress resistance

(Montero *et al*., 2001), larval growth (González *et al.*, 1995a), etc. Vit E also improves growth in juveniles fed oxidized oil, reducing the lipid peroxidation products in sea bream and turbot (Tocher *et al*., 2003).

In seabream (*Sparus aurata*), vit E requirements were found to be close to 250 mg α-tocopherol/kg in broodstock diets (Fernández-Palacios *et al*., 1998) and at least 136 mg/kg in larval diets (González *et al*., 1995a). Indeed, further elevation of vit E up to 136 mg/kg markedly reduced larval survival (González *et al*., 1995a). But, vit E requirements can be strikingly affected by the dietary levels of other nutrients (Hamre and Lie, 1995a, b, Hamre *et al.*, 1994), such as selenium, vitamin C, PUFAs, etc. In fact, vit E oxidation is accelerated by PUFAs presence, since the nature of these fatty acids makes them prone to oxidation and, thus, they have the potential to increase the need for vit E (Watanabe, 1982). For instance, increasing the PUFA level in diets for Atlantic salmon (*Salmo salar*) reduced the liver vit E concentration (Waagbø *et al*., 1993). Thus, increasing dietary PUFAs seem to require increasing vit E to avoid lipid peroxidation (Watanabe *et al*., 1981a, b; Sargent *et al*., 1997; Izquierdo *et al*., 2001), although this fact has not been yet studied in gilthead seabream. Hence, the objective of the present study was to determine the effect of dietary vit E on gilthead seabream growth and survival, at two different PUFAs levels.

Materials and methods

Gilthead Sea Bream (*Sparus aurata)* larvae were obtained from natural spawnings from broodstocks at the GIA facilities (Grupo de Investigacíon en Acuicultura, Las Palmas de Gran Canaria, Spain) where the experiment was carried out. A trial was conducted to test 5 microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco, INVE, Dendermonde, Belgium) until they reached 18 days old. Gilthead seabream larvae (total length 5.20 ± 0.077 mm, dry body weight 144 ±0.08 μg) were randomly distributed into the experimental tanks at a density of 1800 larvae/tank and were fed one of the experimental diets tested in triplicates for 14 days, at a water temperature of 18.8 to 20.3 ºC. All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L/min along the feeding trial. Water entered the tank from the bottom and get out from the top; water quality was daily tested and no deterioration was observed. Water was continuously aerated (125 ml/min), attaining 5-8 ppm dissolved O_2

and saturation ranged between 60 and 80% in all tanks. Photoperiod was kept at 12h light: 12h dark by fluorescent lights.

Five isonitrogenous and isolipidic (70.22/16.95) formulated protein/lipid) experimental microdiets (Pellet size < 250 μm) (Table 7.1) were formulated using DHA50 and EPA50 (CRODA, East Yorkshire, England, UK) as sources of DHA and EPA, respectively. The desired lipid content was completed with a non essential fatty acid source, oleic acid (Oleic acid vegetable, Merck, Darmstadt, Germany). The protein source used (squid meal) was defatted (3 consecutive times with a chloroform: squid meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiet. Two different dietary levels of PUFAs were formulated: 2.5/1.5 and 5/2.5 (DHA/EPA). For the first PUFAs level (2.5/1.5) two vit E (Sigma-Aldrich, Madrid, Spain) contents were tested: 500 and 3000 mg/kg; whereas for the second PUFAs level (5/2.5) three vit E contents were tested: 500, 3000 and 6000 mg/kg. Diets were analyzed for proximate and fatty acid composition of dry weight (d.w.) (Tables 7.2). Diets were manually supplied; fourteen times per day each 45 min from 9:00-19:00. Daily feed supplied was 2 and 2.5 g/tank during the first and second week of feeding, respectively.

Table 7.1. Lipid sources (% total ingredients), α-tocopherol (mg/kg) and crude lipid (% dry basis) content of the experimental diets

Diet (DHA/EPA/vit E)	EPA 50*	DHA 50*	Oleic acid**	α-Tocopherol***	Crude protein
2.5/1.5/500	2	3.4	7.6	500	70.5
2.5/1.5/3000	2	3.4	7.6	3000	70.3
5/2.5/500	4.2	7.4	1.4	500	70.1
5/2.5/3000	4.2	7.4	1.4	3000	70.2
5/2.5/6000	4.2	7.4	1.4	6000	70

* CRODA, East Yorkshire, England, UK.
** Merck, Darmstadt, Germany.
*** Sigma-Aldrich, Madrid, Spain.

Table 7.2. Fatty acid composition of the experimental diets for gilthead seabream (g/100 g diet dry weight)

Dietary DHA/EPA/vit E	2.5/1.5/500	2.5/1.5/3000	5/2.5/500	5/2.5/3000	5/2.5/6000
12:0	0.152	0.151	0.008	0.017	0.001
14:0	0.083	0.079	0.089	0.094	0.005
14:1n7	n.d.*	n.d.	n.d.	n.d.	0.003
14:1n5	n.d.	n.d.	0.011	0.012	0.023
15:0	n.d.	n.d.	n.d.	n.d.	0.002
15:1n5	0.017	0.011	0.027	0.027	0.028
16:0ISO	0.011	0.011	0.013	0.013	0.011

16:0	1.032	0.981	0.84	0.845	0.001
16:1n7	0.044	0.041	0.162	0.161	0.971
16:1n5	n.d.	n.d.	n.d.	n.d.	0.239
16:2n6	n.d.	n.d.	0.019	0.019	0.013
16:2n4	0.008	0.008	0.019	0.019	0.014
17:0	0.023	0.021	0.041	0.038	0.037
16:3n4	0.015	0.013	0.054	0.052	0.014
16:3n3	0.007	0.007	0.015	0.015	0.044
16:3n1	0.007	0.006	0.008	0.008	0.084
16:4n-3	n.d.	n.d.	0.009	0.01	0.014
18:0	0.307	0.296	0.373	0.365	0.403
18:1n9	8.108	7.712	2.261	2.159	3.524
18:1n7	0.000?	0.145	0.275	0.276	0.350
18:1n5	0.008	0.009	0.018	0.018	0.026
18:2n9	0.006	n.d.	0.012	0.012	0.026
18:2n6	2.238	2.154	1.191	1.189	1.261
18:2n4	0.020	0.017	0.046	0.046	0.031
18:3n6	0.023	0.023	0.053	0.053	0.045
18:3n4	0.014	n.d.	0.035	0.035	0.012
18:3n3	0.154	0.142	0.180	0.182	0.184
18:4n3	0.101	0.101	0.237	0.24	0.131
18:4n1	0.010	0.011	0.025	0.025	0.012
20:0	0.041	0.040	0.064	0.063	0.060
20:1n9	0.182	0.177	0.304	0.293	0.298
20:2n9	0.012	0.012	0.028	0.027	0.025
20:2n6	n.d.	n.d.	n.d.	n.d.	0.012
20:3n9	0.009	0.007	0.020	0.023	0.046
20:3n-6	0.023	0.022	0.051	0.052	0.032
20:3n3	0.020	0.020	0.048	0.048	0.036
20:4n6	0.159	0.158	0.367	0.363	0.279
20:4n3	0.064	0.064	0.152	0.150	0.099
20:5n3	1.656	1.681	3.797	3.813	2.320
22:1n11	0.033	0.035	0.080	0.083	0.083
22:1n9	n.d.	n.d.	n.d.	n.d.	0.064
22:4n6	0.108	0.109	0.257	0.254	0.059
22:5n6	n.d.	n.d.	0.007	0.007	0.296
22:5n3	0.140	0.143	0.339	0.334	0.318
22:6n3	2.344	2.461	5.312	5.256	5.583
Saturated	1.639	1.568	1.421	1.428	0.460
Monoenoics	8.392	8.130	3.148	3.036	5.610
n-3	4.487	4.620	10.088	10.046	8.729
n-6	2.551	2.465	1.946	1.944	1.997
n-9	8.317	7.908	2.624	2.515	3.983
n-3HUFA	4.224	4.369	9.648	9.601	8.356
ARA/EPA	0.100	0.090	0.100	0.100	0.120
EPA/DHA	0.710	0.680	0.710	0.730	0.415
n-3/n-6	1.760	1.870	5.180	5.170	4.371

*n.d. not detected

Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish/tank at the beginning, in the middle and at the end of the trial. In the last day of the experiment an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and subsequently allocating them in another tank supplied with clean seawater and aeration to determine survival after 24 hours. Other 20 larvae/tank were translated to 15^{o}C sea water tanks, to determine survival after 24 hours. Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment. Besides, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition after 12 hours of starvation at the end of the trial. Moisture (A.O.A.C., 1995), crude protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Neutral and polar fractions of the larval total lipid were separated by adsorption chromatography on silica cartridges (Sep-pak; Waters S.A., Massachussets, USA) using 30 ml chloroform and 20 ml chloroform/methanol (49: 1, v/v) as solvent for neutral lipid, then 30 ml methanol for polar fractions according to Juaneda and Rocquelin (1985). Fatty acid methyl esters were obtained by transmethylation of polar lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

Data were treated using both, one-way and two way ANOVA and means were compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

For Analysis of two-ways for growth data (weight, length, SGR, biomass), normality and homogeneity of variance were checked, and following general linear model was used:

$$\mathbf{Y}_{ijk} = \mu + \mathbf{F}_i + \mathbf{H}_j + (\mathbf{FH})_{ij} + \varepsilon_{ijk}$$

Where $\mathbf{Y}_{ijk}$ is the mean value of the tank, μ is the mean population, $\mathbf{F}_i$ is the fixed effect of the first factor (vitamin for example), $\mathbf{H}_j$ the fixed effect of the second factor (PUFA for example), $(\mathbf{FH})_{ij}$ the interaction between fixed effects, and ε_{ij} is the residual error.

Results

All experimental microdiets were well accepted by larvae. At the end of the trial the average survival rate was 52.63% and there were no significant differences among treatments (Fig. 7.1). Despite no significant differences were found in survival, a significant correlation (r^2= 0.96) (Fig. 7.2) was found between larval survival and dietary vit E level in diets with the higher HUFA content (5/2.5 DHA/EPA).

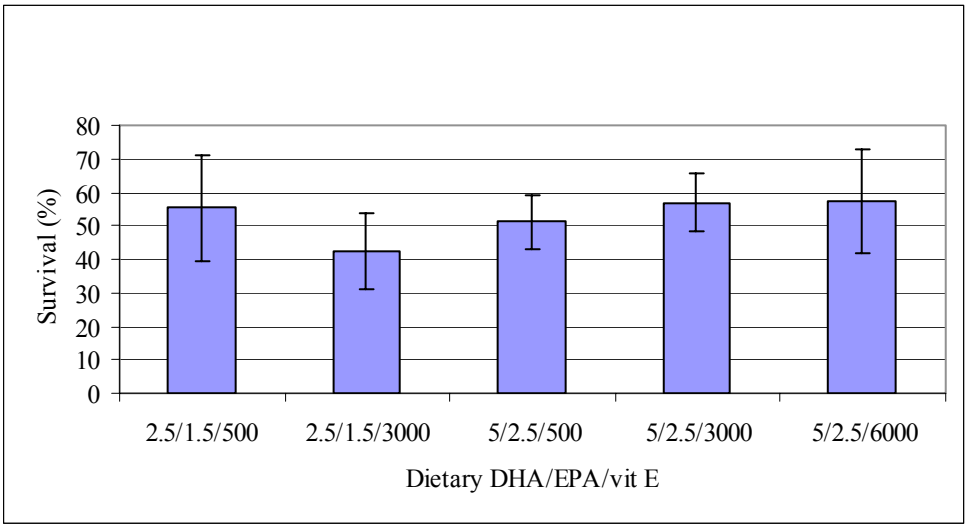

Figure 7.1. Survival rate of fish fed the experimental diets containing several vit E and DHA/EPA (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

After only one week of feeding, there was no significant difference among treatments in any of the growth related parameters. However, at the end of the experiment, growth, in terms of total length, was improved in larvae fed 3000 mg/kg vit E, being significantly higher at 2.5/1.5 DHA/EPA dietary levels (Fig. 7.3). The two way ANOVA analysis showed that dietary vit E significanlty improved larval total length (P=0.04) and there was a significant interaction between vit E and DHA/EPA dietary levels (P=0.015) (Table 7.3), 2.5/1.5/3000 diet giving the best growth results. Similarly, whole body weight of larvae fed 3000 mg/kg vit E tent to be higher, being significantly improved when larvae were fed a 2.5/1.5 DHA/EPA levels (Fig. 7.4). The two way ANOVA showed that vit E significantly improves whole body weight (P=0.01), and there was a significant interaction between vit E and DHA/EPA dietary levels (P=0.044). SGR followed the same trend, with larvae fed diet 2.5/1.5/500 showing significantly lower SGR than larvae from the other treatments (Fig. 7.5) and the dietary increase of vit E or DHA/EPA with respect to this diet significantly improved SGR.

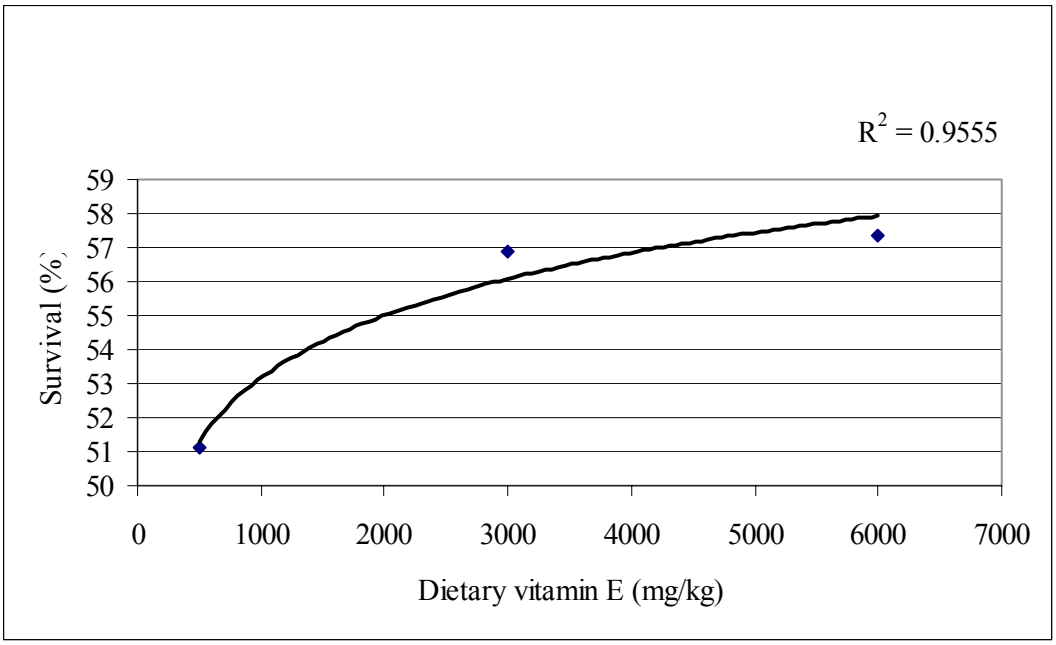

Figure 7.2. Correlation between the vitamin E levels (in the 5/2.5 diets) and survival of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

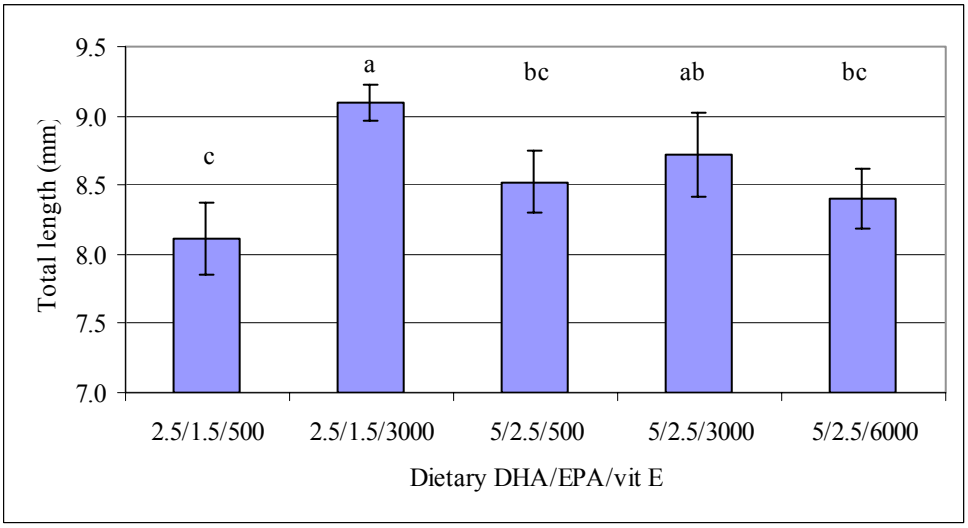

Figure 7.3. Final total length of fish fed the different experimental diets (n=90; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

The elevation of vitamin E up to 3000 mg/kg in presence of 2.5/1.5 DHA/EPA significantly improved final larval biomass (Fig. 7.6). There were no significant differences among treatments in the survival after air or low water temperature exposure (15 ºC) (Figs. 7.7 & 7.8 respectively).

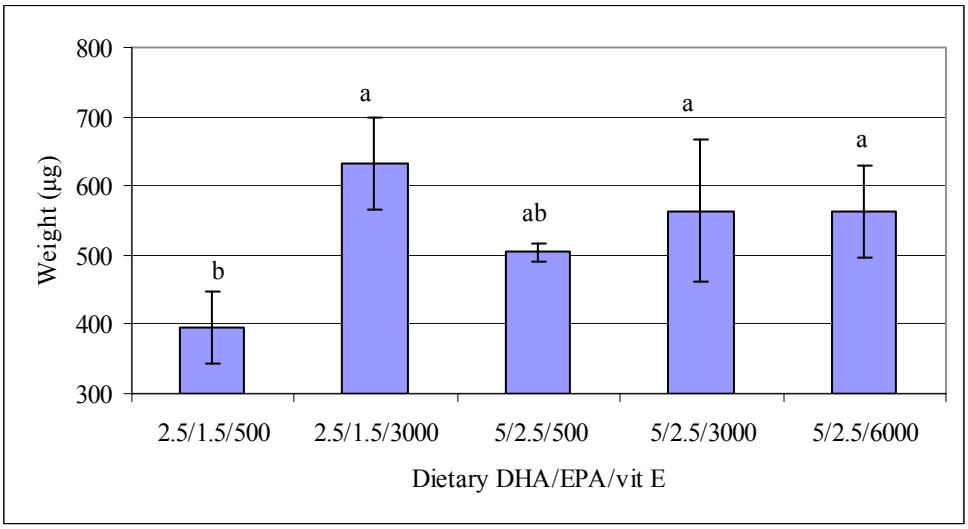

Figure 7.4. Final dry whole body weight of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

Table 7.3. Effect of different dietary DHA/EPA and vit E contents on larval growth, and results of the two-way anova statistical analysis

	Experimental Diets DHA/EPA/vit E										Probability of contrasts		
	2.5/1.5/500		2.5/1.5/3000		5/2.5/500		5/2.5/3000		5/2.5/6000				
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	vit E	DHA/ EPA	vit E x DHA/EPA
Weight (µg)	394	52	632	67	504	13	564	103	563	67	0.010	NS*	0.044
Total length (mm)	8.11	0.26	9.09	0.13	8.52	0.23	8.72	0.30	8.40	0.22	0.04	NS	0.015
Biomass (mg)	311	98	656	161	443	63	444	151	425	132	NS	NS	0.04
SGR	7.1	0.9	10.5	0.8	8.9	0.2	9.7	1.3	9.7	0.8	0.07	NS	0.024

* Not significant

Fatty acid compositions of diets were similar in their levels of saturated fatty acids, whereas they differed in their monounsaturated and polyunsaturated fatty acids contents (Table 7.2). Diets containing 2.5/1.5 DHA/EPA showed higher monoenoic and n-6 fatty acids than diets containing 5/2.5 DHA/EPA, particularly due to higher oleic and linoleic acids, respectively and n-3 DPA (23.88% increasing), where lower levels of PUFA, particularly ARA (52.9% reduction in 2.5/1.5 DHA/EPA diets), EPA (49.6% reduction), DHA (55.37 % reduction). In comparison with the initial larvae, fatty acid composition of polar lipids from larvae at the end of the experiment showed a reduction in saturated fatty acids, particularly 14:0 and 16:0, and in polyunsaturated fatty acids, such as ARA, EPA and DPA, being higher in 16:1n-7 and other minor fatty acids (Table 7.4). In comparison with larvae fed diets containing 5/2.5 DHA/EPA, larvae fed diets

2.5/1.5, which showed a very similar fatty acid composition between them, were higher in monounsaturated and n-6 fatty acids, particularly oleic and linoleic acid, in agreement with their diet composition. Besides, larvae fed 2.5/1.5 diets were lower in 17:0, 18:1n-7, 18:4n-1, ARA (8.8 % reduction), 20:4n-3 (4.8 % reduction), 22:4n-6 and DHA (9.9 % reduction), than larvae fed 5/2.5 diets, but only slightly lower in EPA (0.7 % reduction). Regarding fatty acid composition of polar lipids from larvae fed 5/2.5 diets, elevation of vit E from 500 to 3000 mg/kg slightly increased polyunsaturated fatty acids, due to a slight increase in larval ARA (3.5 % increase), 20:4n-3, 20:3n-9, EPA, DPA and, particularly, DHA (2.3 % increase), despite these fatty acids were the same in both diets. Further elevation of vit E up to 6000 mg/kg in 5/2.5 diets was followed by an increase in larval monoenoic fatty acids, 16:1n-7, 18:1n-9, 20:1n-9, and 22:1n-11 and a reduction in EPA (31.9 % reduction), n-3 DPA and DHA. While the increasing of vit E

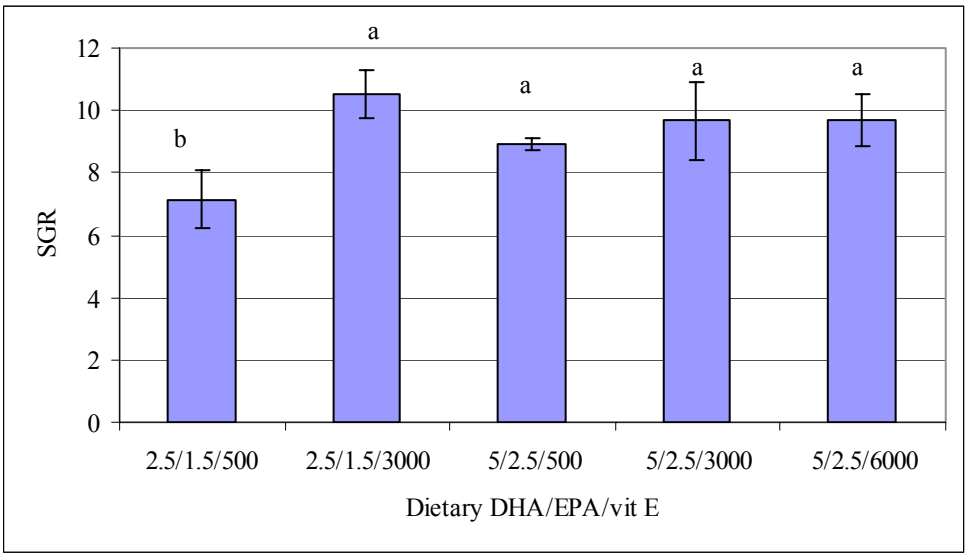

Figure 7.5. Specific growth rate of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

from 500 to 3000mg/kg was accompanied by a slight elevation in dietary EPA and DHA (1.5 and 4.99 % respectively) in 2.5/1.5 treatments, the larvae contents of EPA and DHA (1.5, 0.23 % increases respectively) were not affected by this increase. Between 5/2.5/500 and 5/2.5/3000 treatments, the increase of vit E from 500 to 3000 was accompanied by an elevation of dietary EPA (0.42 %) and a reduction in dietary DHA (1.05%), this increase in vit E produced a slightly increase in the larval contents of EPA and DHA (5.7, 2.3 % respectively). On the contrary, between 5/2.5/3000 and

5/2.5/6000 diets, the increase of vit E from 3000 to 6000 was followed by a slight reduction of dietary EPA (39.2 %) and an elevation in dietary DHA (6.2%), whereas these increase in vit E markedly reduced larval contents of both EPA and DHA (31.9 and 38 %, respectively).

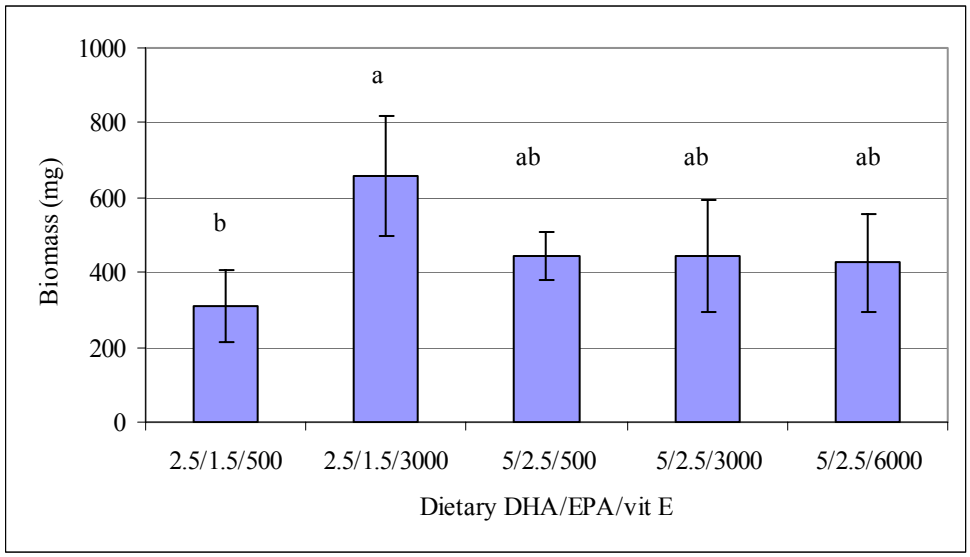

Figure 7.6. Final biomass of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

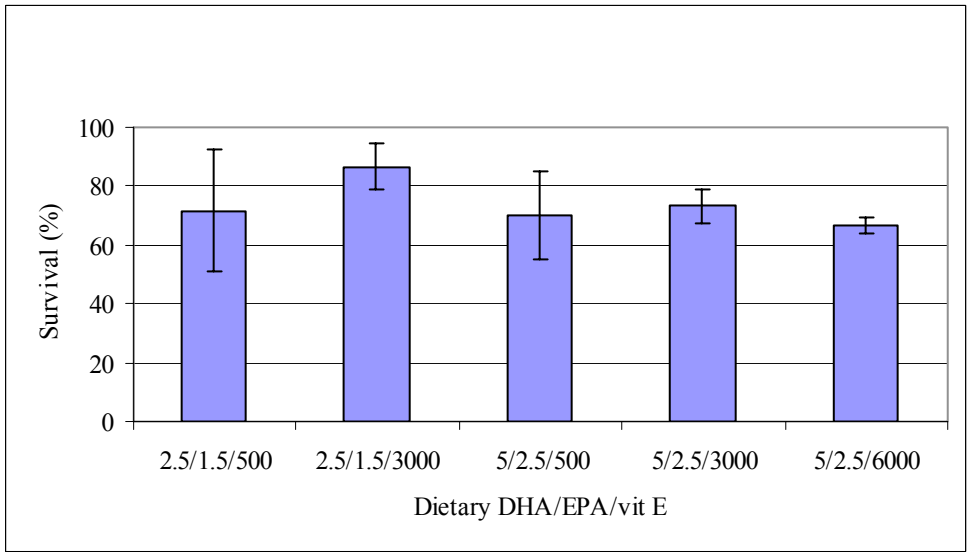

Figure 7.7. Survival after 24h of air activity test of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

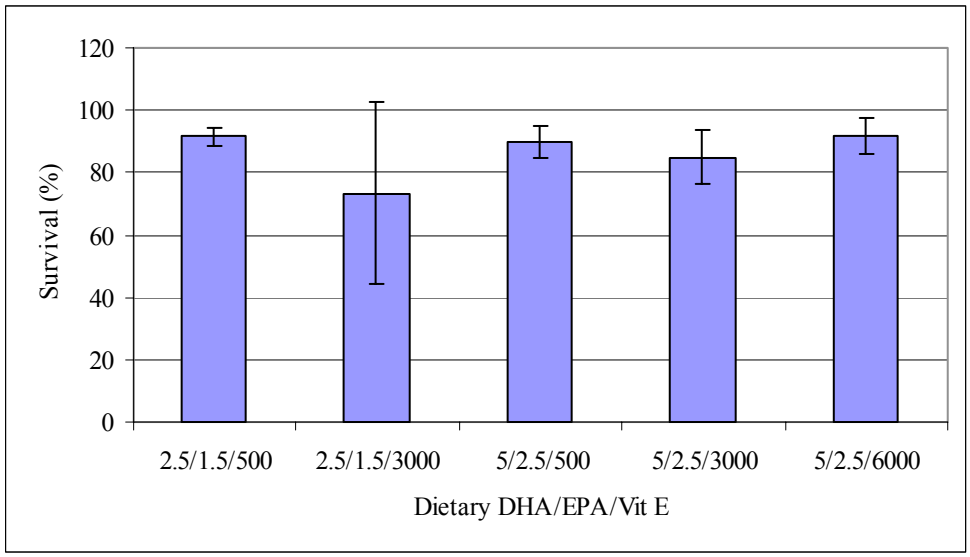

Figure 7.8. Survival after 24h of exposure to a temperature reduction in fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

Discussion

Despite the particular importance of vit E as a defense against oxidized fat in fish, studies aimed to determine its significance along larval development and its relation with dietary polyunsaturated fatty acid levels are very scarce. The present study pointed out the importance of dietary vit E for gilthead seabream larval growth and its interaction with dietary PUFA levels. Thus, increase in vit E levels up to 3000 mg/kg markedly improved larval growth, particularly when dietary PUFA levels were lower, suggesting a higher protection value when these fatty acids are more limiting. Previous studies in our laboratory have shown that elevation of dietary levels from 88 to 265 mg/kg α-tocopherol, significantly improved growth of larval gilthead seabream (González *et al*., 1995b) at 1.4/0.8 dietary DHA/EPA levels. In juveniles of the same species, dietary supplementation with vit E up to 1000 mg/kg increased fish growth (Tocher *et al*., 2002), particularly when fish were reared at high densities (Montero *et al*., 2001). In adult sea bream (150g) the dietary supplementation with 1200 mg/kg vit E produced a slightly higher (but not statistically significant) SGR compared with 100, 600 and 1800 mg/kg vit E supplemented diet (Ortuño *et al*., 2000). In other fish species, such as rohu (*Labeo rohita*) increase in dietary vit E levels over 100 mg/kg improved weight gain, feed efficiency and other nutritional indices (Sau *et al*., 2004), whereas the increase from 19 to 210 mg/kg improved weight gain and SGR in mrigal (*Cirrhinus mrigala)* fry (Paul *et al*., 2004).

Table 7.4 Fatty acid composition of polar lipid of the larval seabream (% total fatty acid)

Dietary DHA/EPA/vit E	Initial	Final				
		2.5/1.5/500	2.5/1.5/3000	5/2.5/500	5/2.5/3000	5/2.5/6000
12:0	0.12	0.15±0.05	0.13±0.08	0.15±0.02	0.14±0.04	0.14±0.07
14:0	1.12	0.6±0.05	0.60±0.04	0.69±0.11	0.69±0.08	1.48±1.21
14:1n-7	0.05	0.04±0.00	0.03±0.01	0.05±0.01	0.05±0.01	0.06±0.02
15:0	0.42	0.24±0.01	0.26±0.04	0.27±0.03	0.26±0.05	0.29±0.06
15:1n-5	0.77	0.51±0.04	0.51±0.04	0.6±0.08	0.61±0.08	0.90±0.42
16:0ISO	0.23	0.13±0.02	0.13±0.02	0.16±0.02	0.15±0.03	0.24±0.12
Me16:0	0.64	0.40±0.09	0.39±0.08	0.41±0.05	0.46±0.12	0.65±0.23
16:0	25.41	21.02±1.91	19.5±1.68	20.9±2.87	20.81±2.44	21.95±2.06
16:1n-9	0.95	0.58±0.40	0.79±0.06	0.70±0.09	0.72±0.06	0.74±0.09
16:1n-7	6.03	2.34±0.26	2.53±0.27	2.77±0.23	2.86±0.38	4.18±1.88
16:1n-5	1.00	0.69±0.06	0.75±0.15	0.78±0.09	0.77±0.13	0.77±0.15
16:2n-6	n.d.*	0.63±0.42	0.38±0.06	0.41±0.05	0.76±0.61	0.49±0.05
16:2n-4	1.27	1.20±0.05	1.18±0.12	1.30±0.14	1.19±0.03	1.36±0.14
17:0	0.95	0.68±0.08	0.76±0.07	0.87±0.08	0.91±0.11	1.01±0.13
16:3n-3	1.91	1.34±0.30	1.38±0.31	1.42±0.17	1.57±0.40	1.64±0.42
16:3n-1	0.43	0.98±0.18	0.96±0.14	0.71±0.05	0.78±0.16	0.63±0.38
16:3n-4	0.30	0.29±0.06	0.30±0.06	0.34±0.03	0.39±0.10	0.43±0.07
18:0	10.50	10.39±1.06	9.80±0.98	10.63±0.65	10.25±1.14	9.63±2.30
18:1n-9	14.01	19.62±1.58	19.87±1.22	13.73±1.82	13.23±1.29	19.44±10.63
18:1n-7	5.85	3.55±0.35	3.70±0.38	4.16±0.41	4.19±0.52	4.61±0.62
18:1n-5	0.35	0.20±0.02	0.22±0.03	0.26±0.01	0.26±0.02	0.25±0.10
18:2n-9	1.31	0.55±0.32	0.57±0.27	0.67±0.02	0.70±0.08	0.52±0.30
18:2n-6	3.96	5.23±0.26	5.31±0.32	3.22±0.22	3.14±0.24	2.91±0.40
18:2n-4	0.06	0.06±0.00	0.06±0.00	0.13±0.01	0.13±0.01	0.13±0.01
18:3n-6	0.30	0.34±0.02	0.34±0.03	0.41±0.03	0.41±0.03	0.42±0.03
18:3n-4	0.06	0.04±0.00	0.07±0.03	0.09±0.03	0.07±0.04	0.23±0.27
18:3n-3	0.23	0.27±0.25	0.46±0.43	0.46±0.44	0.44±0.47	0.27±0.07
18:3n-1	n.d.	0.07±0.02	0.08±0.03	0.13±0.03	0.14±0.04	0.11±0.07
18:4n-3	n.d.	0.06±0.00	0.08±0.00	0.07±0.01	0.08±0.01	0.10±0.02
18:4n-1	0.38	0.19±0.03	0.20±0.04	0.20±0.02	0.24±0.05	0.27±0.04
20:0	0.17	0.26±0.04	0.26±0.03	0.33±0.03	0.32±0.03	0.44±0.19
20:1n-9	0.90	0.91±0.05	0.99±0.08	0.97±0.11	0.92±0.09	1.75±1.39
20:1n-7	0.45	0.29±0.04	0.34±0.04	0.36±0.02	0.33±0.05	0.48±0.28
20:2n-9	0.16	0.16±0.04	0.18±0.07	0.14±0.05	0.12±0.01	0.12±0.01
20:2n-6	0.37	0.32±0.02	0.35±0.04	0.31±0.02	0.31±0.02	0.52±0.38
20:3n-9	0.07	0.05±0.00	0.08±0.05	0.10±0.07	0.19±0.00	n.d.
20:3n-6	0.38	0.23±0.03	0.26±0.03	0.23±0.02	0.22±0.05	0.15±0.10
20:4n-6	2.44	1.85±0.22	1.87±0.20	1.98±0.15	2.05±0.16	2.04±0.24
20:4n-3	0.28	0.17±0.04	0.18±0.01	0.19±0.04	0.21±0.03	0.15±0.08
20:5n-3	5.38	3.98±0.81	4.04±0.68	4.36±0.80	4.61±0.83	3.14±1.94
22:1n-11	0.24	0.11±0.02	0.13±0.01	0.15±0.03	0.12±0.01	0.43±0.52
22:4n-6	0.43	0.73±0.10	0.75±0.11	0.93±0.10	0.94±0.11	0.63±0.42
22:5n-6	0.11	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.02	0.07±0.02
22:5n-3	2.01	1.52±0.36	1.58±0.27	1.38±0.36	1.42±0.23	0.96±0.80
22:6n-3	8.01	17.7±4.28	17.66±4.57	21.93±5.33	22.43±5.39	13.91±11.78

*n.d. not detected.

Optimum dietary vit E levels seem to be higher during larval development. In agreement, in larvae of striped trumpeter (*Latris lineate*) fed rotifers enriched with 40000 mg/kg vit E significantly increased fish length in comparison to those fed 0 or 5000 mg/kg vit E enriched rotifers (Brown *et al*., 2005). Indeed, analyzed α-tocopherol fish body contents along larval development showed a steady increase from the first days of feeding until the end of metamorphosis in seabream (González *et al*., 1995b) and sea bass (Guerriero *et al*., 2004), suggesting the high requirement of vit E during the larval stage.

In juvenile grouper (*Epinephelus malabaricus*) increasing of dietary vit E over 100 mg/kg improved the growth in two dietary lipid levels either 4 or 9% (Lin and Shiau, 2005). In the present study, when gilthead seabream larvae were fed higher dietary PUFA levels (5/2.5), increase in vit E from 500 to 3000 mg/kg enhanced overall larval performance in terms of growth parameters and survival, and increased the PUFAs content in the larval polar lipids, despite there was the same content in these fatty acids in both diets. These results denote the anti-oxidative effect of vit E, which is positioned in the membrane and competes with PUFA located at the phospholipids in donating a hydrogen atom to the lipid peroxyl radical, breaking the chain of reactions involved in lipid auto-oxidation. For instance, in rainbow trout (*Oncorhynchus mykiss*), increased dietary vit E contents from 300 to 1500 mg/kg reduced the rate of lipid oxidation in fish fillets and the formation of off-flavors (Chaiyapechara *et al*., 2003). Nevertheless, the positive effect of vit E on fish growth is not only related to its antioxidant properties, but also to other functions of this molecule in cellular metabolism, signal transduction (Traber and Parker, 1995) or modulation of eicosanoid synthesis (Cornwell and Parganamala, 1993).

However, increase in vit E from 3000 to 6000 mg/kg not only did not further improved larval performance, but also reduced PUFAs contents in larval polar lipids, suggesting the pro-oxidant effect of excess vit E. Indeed, at high concentrations and under conditions where vitamin C and E radicals are allowed to accumulate, both antioxidants have been shown to act as pro-oxidants (Bowry *et al*., 1992, Ingold *et al*., 1993, Porter *et al*., 1995). Thus, under certain conditions vit E may be present as tocopheroxyl radicals, which capture hydrogen atoms from PUFA at low rates and initiates lipid oxidation. Previous studies in our laboratory have shown that the elevation of dietary vit E up to 1500 mg/kg in larval seabream diets containing free ascorbic acid significantly reduced larval survival, whereas the same increase in α-tocopherol when

vitamin C was supplemented as ascorbic acid polyphosphate caused a significant improvement in larval growth without affecting survival (González, 1997). In other species such as mrigal (*Cirrhinus mrigala*) increase of dietary vit E over 210 mg/kg also reduced growth markedly (Paul *et al*., 2004).

In the present experiment, neither dietary vit E nor PUFAs contents affected significantly larval survival, confirming that neither of these nutrients was present in deficient levels. In previous studies with similar rearing conditions, dietary vit E levels of 22 mg/kg were found to be deficient for gilthead seabream larvae, markedly reducing growth (González *et al*., 1995b). Similarly, low survival was also found in other species such as juvenile golden shiner (*Notemigonus crysoleucas*) fed diets without vit E supplementation (Chen *et al*., 2004). Moreover, in the present experiment, comparison of results from larvae fed 500 mg/kg vit E at the two PUFA levels assayed showed even a better performance of the higher PUFA content fed larvae, particularly in SGR, which showed a higher DHA content but similar ARA and DPA. This fact, besides pointing out the growth promoting effect of these essential fatty acids (Izquierdo *et al*., 1996), could be also related to the anti-oxidative effects of DHA reported in animal systems. DHA effectively decreases the levels of cellular lipid peroxide (Nishida *et al.,* 2006) contradicting the classical concept that an increase in DHA levels in biological systems has deleterious effects by enhancing lipid peroxidation.

Vit E is well known to play an important role in stress resistance in fish (Montero *et al.,* 2001). In the present study, there was not a significant effect of vit E levels in seabream larvae resistance to air exposure or low temperature. The magnitude of both stressors has been shown to be enough to cause different response in larvae fed different levels of fatty acids (Izquierdo *et al.,* 2000; Liu *et al.,* 2002). In juveniles of the same species, reduction of vit E levels from 167.5 to 18.5 mg/kg markedly reduced fish resistance to a chronic and acute stress (Montero *et al.,* 2001). Despite juvenile requirements are probably lower than those for larvae, the minimum vit E levels in the present study were 10 times higher than those assayed by Montero and co-workers and could be enough to prevent the damage of the type of stress assayed.

In summary, the results of the present study suggest that elevation of dietary vit E up to 3000 mg/kg with DHA/EPA levels of 2.5/1.5 give a better performance in seabream larvae than increasing DHA/EPA dietary levels to 5/2.5, showing the importance of dietary vit E for larval growth and its interrelation to dietary PUFA levels.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Blazer, V. S., 1982. The effect of marginal deficiencies of ascorbic acid and α-tocopherol on the natural resistance and immune response of rainbow trout (*Salmo gairdneri*). Ph.D. dissertation. University of Rhode Island.

Bowry, V. W., Ingold, K.U. and Stocker, R., 1992. Vitamin E in human low-density lipoprotein. When and how this antioxidant becomes a pro-oxidant. Biochemical Journal, 288, 341–344.

Brown, M.T., Dunstan, G.A., Nichols, P.D., Battaglene, S.C., Morehead, D.T., Anna L. and Overweter, A.L., 2005. Effects of a-tocopherol supplementation of rotifers on the growth of striped trumpeter *Latris lineata* larvae. Aquaculture, 246, 367–378.

Chaiyapechara, S., Casten, M.T., Hardy, R.W. and Dong, F.M., 2003. Fish performance, fillet characteristics, and health assessment index of rainbow trout (*Oncorhynchus mykiss*) fed diets containing adequate and high concentrations of lipid and vitamin E. Aquaculture, 219, 715–738.

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K. and Lee, K.J., 2004. Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). Aquaculture, 242, 553–569.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Cornwell, D. G. and Panganamala, R. V., 1993. Vitamin E action in modulation the arachidonic acid cascade. In: L. Packer, & J. Fuchs (Eds.), *Vitamin E in Health and Disease*. Marcel Dekker, Inc., New York, U.S., 385-410.

Fernández-Palacios, H., Izquierdo, M.S., Gonzalez, M., Robaina, L. and Valencia, A., 1998. Combined effect of dietary a-tocopherol and n-3 HUFA on egg quality of gilthead seabream broodstock (*Sparus aurata*). Aquaculture, 161, 475–476.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

Gonzalez, M.M., 1997. Dietary Vitamin E, Vitamin A and Carotenoids for Gilthead seabream (*Sparus aurata*) Larvae. Doctoral Thesis, Universidad de Las Palmas de Gran Canaria, Spain, pp. 54-59.

González, M.M., Izquierdo, M.S., Salhi, M., Hernández- Cruz, C.M. and Fernández-Palacios, H., 1995a. Dietary vitamin E for *Sparus aurata* larvae. Europ. Aqua. Soc. Spec. Publ., 24, 239–242.

González, M.M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M. and Fernández-Palacios, H., 1995b. The effect of vitamin-enriched rotifers on newly hatched and 15-d-old gilthead sea bream larvae (*Sparus aurata*). K.Pittman, R.S. Batty & J. Verreth (eds.) Mass rearing of Juvenile Fish, pp: 196-201.

Guerriero, G., Ferroa, R., Russob, G.L. and Ciarcia, G., 2004. Vitamin E in early stages of sea bass (*Dicentrarchus labrax*) development. Comparative Biochemistry and Physiology, Part A, 138, 435– 439.

Halliwell, B. and Gutteridge, J.M.C., 1998. Free Radicals in Biology and Medicine, 3rd edn, Oxford University Press, Oxford.

Halliwell, B. and Gutteridge, J.M.C., 1996. Lipid peroxidation: a radical chain reaction. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Clarendon Press, Oxford, pp. 188– 266.

Hamre, K. and Lie, Ø., 1995a. Minimum requirement of vitamin E for Atlantic salmon, *Salmo salar*, at first feeding. Aquacult. Res., 26, 175–184.

Hamre, K. and Lie, Ø., 1995b. Alpha-tocopherol levels in different organs of Atlantic salmon (*Salmo salar* L.) effect of smoltification, dietary levels of n-3 polyunsaturated fatty acids and vitamin E. Comp. Biochem. Physiol., 111A, 547–554.

Hamre, K., Hjeltnes, B., Kryvi, H., Sandberg, S., Lorentzen, M. and Lie, O., 1994. Decreased concentration of hemoglobin, accumulation of lipid oxidation products and unchanged skeletal muscle in Atlantic salmon (*Salmo salar*) fed low dietary vitamin E. Fish Physiology and Biochemistry, 12, 421–429.

Ingold, K. U., Bowry, V. W., Stocker, R. and Walling, C., 1993. Autooxidation of lipids and antioxidation by alpha-tocopherol and ubiquinol in homogeneous solution and aqueous dispersions of lipid: Unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein. *Proc. Natl. Acad. Sci. USA*, 90, 45-49.

Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish larvae. Review article. Aquacult. Nutr., 2, 183–191.

Izquierdo, M.S. 2005. Essential fatty acid requirements in Mediterranean fish species Cah. Opt. Mediterr., 63, 91-102.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem., 22, 97–107.

Izquierdo, M.S, Fernández-Palacios, H. and Tacon, A.G.J., 2001. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture, 197, 25-42.

Kanazawa, K., 1991. Hepatotoxicity caused by dietary secondary products originating from lipid peroxidation. In: Friedman, M. (Ed.), Nutritional and Toxicology Consequences of Food Processing. Plenum, New York, pp. 237–253.

Kanazawa, K., 1993. Tissue injury induced by dietary products of lipid peroxidation. In: Corongiu, F. (Ed.), Free Radicals and Antioxidants in Nutrition. Richelieu Press, London, pp. 383– 399.

Lin, Y-H. and Shiau, S-Y., 2005. Dietary vitamin E requirement of grouper, *Epinephelus malabaricus*, at two lipid levels, and their effects on immune responses. Aquaculture, 248, 235– 244.

Liu, J., Caballero, M.J., El-Sayed, Ali.T., Izquierdo, M.S., Hernández Cruz, C.M., Valencia, A. and Fernández-Palacios, H., 2002. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream (*Sparus aurata*). Fish Science, 68, 1165–1172.

Juaneda, P. and Rocquelin, G., 1985. Rapid and convenient separation of phospholipids and non-phosphorous lipids from rat heart using silica cartridges. Lipids, 20, 40–41.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L. and Vergara, J. M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiology and Biochemistry , 18, 399–407.

Montero, D., Tort, L., Robaina, L., Vergara, J.M. and Izquierdo, M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish and Shellfish Immunology, 11, 473–490.

Nishida, T., Orikasa, Y., Ito, Y., Yu, R., Yamada, A., Watanabe, K. and Okuyama, H., 2006. Escherichia coli engineered to produce eicosapentaenoic acid becomes resistant against oxidative damages. FEBS Letters, 580, 2731–2735.

Ortuño, J., ESTEBAN, M.A. and MESEGUER, J., 2000. High dietary intake of α-tocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish & Shellfish Immunology, 10, 293–307.

Paul, B.N., Sarkar, S. and Mohanty, S.N., 2004. Dietary vitamin E requirement of mrigal, *Cirrhinus mrigala* fry. Aquaculture, 242, 529-536.

Porter, N.A., Caldwell, S.E. and Mills, K.A., 1995. Mechanisms of free radical oxidation of unsaturated lipid. Lipids, 30, 277-290.

Sakai T., Murata H., Endo M., Yamauchi K., Tabata N. and Fukudome, M., 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola quiqueradiata*. Agricultural and Biological Chemistry, 53, 1739–1740.

Sargent, J.R., McEvoy, L.A. and Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEvoy, L., Tocher, D. and Estevez, A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177, 191–199.

Sau, S.K., Paul, B.N., Mohanta, K.N. and Mohanty, S.N., 2004. Dietary vitamin E requirement, fish performance and carcass composition of rohu, *Labeo rohita* fry. Aquculture, 240, 359–368.

Tocher, D.R., Mourente, G., Van der Eecken A., Evjemo J.O., Diaz E., Bell J.G., Geurden I., Lavens P. and Olsen Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquaculture Nutrition, 8, 195–207.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz E., Wille, M., Bell J.G. and Olsen, Y., 2003. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International, 11, 195–216.

Traber, M.G. and Packer, L., 1995. Vitamin E: beyond antioxidant function. *Am. J. Clin. Nutr*., 62 (suppl.), 1501S-1509S.

Waagbø, R., Sandnes, K., Torrissen, O.J., Sandvin, A. and Lie, Ø., 1993. Chemical and sensory evaluation of fillets from Atlantic salmon (*Salmo salar*) fed three levels of n_3 polyunsaturated fatty acids at two levels of vitamin E. Food Chem., 46, 361–366.

Wang Z., Mai K., Liufu Z., Ma H., Xu W., Ai Q., Zhang W., Tan B. and Wang X., 2006. Effect of high dietary intakes of vitamin E and n-3 HUFA on immune responses and resistance to Edwardsiella tarda challenge in Japanese flounder (*Paralichthys olivaceus*, Temminck and Schlegel). Aquaculture Research, 37, 681-692.

Watanabe, T., 1982. Lipid nutrition in fish. Comp. Biochem. Physiol., 73, (1) 3-15.

Watanabe, T., Takeuchi, T. and Wade, M., 1981a. Dietary lipid levels and α-tocopherol requirement of carp. Bulletin of the Japanese Society of Scientific Fisheries, 47(12), 1585–1590.

Watanabe, T., Takeuchi, T., Wada, M. and Uehara, R., 1981b. The relationship between dietary lipid levels and α-tocopherol requirement of rainbow trout. Bulletin of the Japanese Society of Scientific Fisheries, 47(11), 1463–1471.

CHAPTER 8

COMBINED EFFECT OF VITAMIN C AND VITAMIN E MICRODIETS FOR GILTHEAD SEA BREAM *Sparus aurata*

E. Atalah*, C. M. Hernández Cruz, E. Ganuza, T. Benítez-Santana, R. Ganga, J. Roo, Fernández-Palacios, H. and M.S. Izquierdo

Abstract

Vitamins C (AA) and vitamin E (α-T) are two of the most important micro-nutrients for fish aquaculture especially larvae aquaculture. An experiment was carried out to study the effect dietary AA with several levels of α-T. The nutritional effect of different combinations of α –T and AA in microdiets for gilthead sea bream larvae (total length 5.08 ± 0.54 mm, dry body weight 143 ± 4.71 μg) were investigated in five formulated diets contain two levels basal or high (1500, 3000 mg/kg microdiet) of DL-α-tocopheryl acetate (α-T) and without or with basal or high (0, 1800, 3600mg/kg microdiet) ascorbic acid (AA) respectively were tested, using triplicates.

The survival of fish fed 3000/0 diet was significantly lower than those fed 3000/1800 diet. After two weeks feeding, elevation of AA levels from 1800 to 3600 mg in presence of 1500 mg α-T, and the elevation of AA from 0 to 1800 or to 3600 mg in presence of 3000 mg α-T, respectively, significantly improve the final total length, whole body dry weight, SGR and final biomass.

In exposure to stressful water temperature (15 ºC), larvae fed with 1500/3600 diet showed a significantly higher survival than those fed with high level α-T with different levels of AA diets. In conclusion, the elevation of AA from 1800 to 3600 mg/kg in presence of 1500 mg/kg vitamin E and elevation of vitamin C (from none to 1800 or 3600 mg/kg) in high vitamin E diet (3000 mg/kg) significantly improved growth performance indicating the importance of different ration of vitamin E and C.

Keywords: n-3 HUFA; Vitamin E; Vitamin C, Gilthead sea bream larvae *Sparus aurata*

Introduction

Vitamins constitute important nutrients for fish production (Gaylord *et al*., 1998; Montero *et al*., 1988, 2001; Tocher 2002, 2003, Izquierdo and Fernandez-Palacios, 1997; Fernandez-Palacios *et al*., 1998).

Vitamin E is a structural component of cell membranes and has a specific role as an antioxidant, controlling peroxidation of unsaturated fatty acids (Putnam and Comben, 1987) and tissue lipid peroxidation which produces oxygen radicals. Sargent *et al*. (1999) suggest that the tissue lipid peroxidation is greater in marine finfish than other vertebrates resulting in damage to biomembrans lipid peroxidations is implicated in several pathological conditions in fish (Kawatsu 1969; Watanabe *et al*. 1970; Murai and Andrews 1974; Sakai *et al*. 1989). Thus, larvae are at high risk by oxidation (Sargent *et al*. 1999), for the high quantity of n-3 HUFA in the tissue and diet, and exogenous antioxidant must be add in marine larvae culture. Sargent *et al*. (1997) suggested that the higher HUFA requirement of marine fish larvae must be combined with natural antioxidants, particularly α-tocopherol (α-T) since α-T is degraded to protect HUFA against peroxidation (Woodall *et al*., 1964; Watanabe *et al*., 1970). Moreover increased dietary polyunsaturated fatty acid (PUFA) is associated with increasing of α-T in carp (Watanabe *et al*., 1981; Schwarz *et al*., 1988).

A dietary requirement of α-T has been demonstrated in a number studies including 30 to 50 mg/kg diet for channel catfish (*Ictalurus punctatus*) (Murai and Andrews, 1974; Wilson *et al*., 1984), 99 mg/kg diet for Mrigal (*Cirrhinus mrigala*) fry (Paul *et al*., 2004), 120 mg/kg diet for Atlantic salmon (*Salmo salar*) (Hamre and Lie, 1995a), 136 mg/kg diet for sea bream larvae (González *et al*., 1995), 200 to 300 mg/kg diet for common carp (*Cyprinus curpio*) (Watanabe *et al*., 1977), and 250 mg/kg diet for sea bream broodstock (Fernández-Palacios *et al*., 1998) and 1200 mg/kg diet for adult sea bream (Ortuño *et al*., 2000).

The dietary importance of vitamin C (AA) has been demonstrated also in several studies (Kolkovski *et al*., 2000, Ruff *et al*., 2001, Ortuño *et al*., 2003, Mulero *et al*., 1998). Fish larvae are particularly sensitive to AA deficiency (Dabrowski *et al*., 1996) and their requirements seem to be larger than juveniles and adults (Kolkovski *et al*., 2000). Moreover larval dietary AA improves survival, growth, skeleton development, stress resistance and immune response (Merchie *et al*. 1996, Kolkovski *et al*., 2000).

The requirement level of AA depends on fish species, size, stage of sexual maturity, growth rate (Boonyaratpalin, 1997), diet and experimental conditions, as well as body vitamin storage status such as the hepatic AA concentration (Fournier *et al.,* 2000). Studies about the requirements for marine finfish have been scarce (Boonyaratpalin *et al.,* 1992; Saroglia and Scarano, 1992; Teshima *et al.,* 1993) and based on data obtained for freshwater species as reported by the NRC (1993). Published research on the requirement of AA range from 45.3 mg AA/kg diet for optimal growth in Juvenile grouper (*Epinephelus malabaricus)* (Lin and Shiau*,* 2005) to 3000 mg/kg for gilthead seabream juveniles (150g) to increase the activity of non-specific immune responses (Ortuño *et al.,* 1999)

Ascorbic acid (AA) seems to play an important role in the metabolism of α-T (Ruff *et al*., 2001). Several studies (Cort, 1974; Packer *et al*., 1979; Niki, 1987a, b) demonstrated the ability of AA to reduce α-T radicals and thereby regenerate them to α-T ('recycling' of α-T). The beneficial of combination α-T and AA on growth and related indices of fish has been reported in juveniles of certain species (Chávez de Martínez, 1990; Roem *et al*., 1990; Thorarinsson *et al*., 1994; Chien *et al*., 1999), however its effect in marine fish larvae has not been studied.

Previous results obtained in our laboratory on gilthead sea bream showed that larvae fed microdiets containing 2.5/1.5 DHA/EPA and 3000mg/kg α-T show a good culture performance. This study aims to determine the combined effect of dietary α-T and AA in sea bream larvae.

Materials and methods

Gilthead Sea Bream (*Sparus aurata)* larvae were obtained from natural spawning from broodstocks at the GIA facilities (Grupo de Investigacion en Acuicultura, Las Palmas de Gran Canaria, Spain) where the experiment was carried out. A trial was conducted to test five microdiets in triplicates. Larvae were previously fed enriched rotifers (DHA Protein Selco, INVE, Dendermonde, Belgium), until they reached 18 days old. In this trial larvae (standard length 5.08 ± 0.54 mm, dry body weight 143 ± 4.71 μg) were randomly distributed into the experimental tanks at a density of 2000 larvae/tank and fed one of the experimental diets tested in triplicates for 14 days, at a water temperature of 21 to 22.4 ºC.

All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L/min along the feeding trial. Water get in the tank from the bottom and get out from the top. Water quality was daily tested and no deterioration was observed. Water was continuously aerated (125 ml/min) attaining 5-8 ppm dissolved O_2 and saturation ranged between 60 and 80% in all tanks. Photoperiod by fluorescent lights was kept at 12h light: 12h dark.

Five isonitrogenous and isolipidic (70.01/17.05 formulated protein/lipid) experimental microdiets (pellet size < 250 μm) containing 2.5 % DHA and 1.5 % EPA content were formulated using DHA50 and EPA50 (CRODA, East Yorkshire, England, UK) as sources of DHA and EPA respectively and the desired lipid content was completed with a non essential fatty acid source, oleic acid (Oleic acid vegetable, Merck, Darmstadt, Germany). Different ratios of α-T (Sigma-Aldrich, Madrid, Spain) and AA (ROVIMIX Stay-C-35, Roche, Paris, France) shown in Table 8.1. The protein source used (squid meal) was defatted (3 consecutive times with a chloroform: meal ratio of 3:1) to allow a complete control of the fatty acid profile of the microdiet.

Diets were analyzed for proximate and fatty acid composition of dry basis (d.b.) (Tables 8.2). Diets were manually supplied, fourteen times per day each 45 min from 9:00-19:00. Daily feed supplied was 2 and 2.5 g/tank during the first and second week of feeding, respectively.

Final survival was calculated by individually counting all the larvae alive at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish/tank at the beginning, in the middle and at the end of the trial. In the last day of the experiment an activity test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 min and subsequently allocating them in another tank supplied with clean seawater and aeration to determine survival after 24 hours. Other 20 larvae/tank were translated to 15^{o}C sea water tanks, to determine survival after 24 hours. Besides, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition after 12 hours of starvation at the end of the trial.

Table 8.1. Dietary vitamin E and C composition

vit E/C	1500/1800	1500/3600	3000/0	3000/1800	3000/3600
vitamin E mg/kg	1500	1500	3000	3000	3000
vitamin C mg/kg	1800	3600	0	1800	3600

Table 8.2. Fatty acid composition of the experimental diets for gilthead seabream (g/100 g diet dry weight)

Diet vit E/C	1500/1800	1500/3600	3000/0	3000/1800	3000/3600
Total lipid	16.75	16.77	16.85	17.07	17.80
12:0	0.153	0.151	0.174	0.154	0.183
14:0	0.079	0.082	0.089	0.085	0.089
15:1n-5	0.013	0.017	0.015	0.014	0.016
16:0ISO	0.010	0.011	0.012	0.011	0.012
16:0	1.007	1.015	1.115	1.080	1.111
16:1n-9	n.d.	n.d.	n.d.	n.d.	0.005
16:1n-7	0.040	0.041	0.047	0.042	0.044
16:2n-4	0.008	0.008	0.009	0.009	0.009
17:0	0.022	0.020	0.025	0.023	0.024
16:3n-4	0.013	0.013	0.014	0.014	0.015
16:3n-3	0.006	0.007	0.007	0.007	0.007
16:3n-1	0.006	0.006	0.007	0.006	0.007
18:0	0.301	0.299	0.325	0.318	0.327
18:1n-9n-7	7.499	7.599	8.373	7.768	8.318
18:1n-7	n.d.	0.145	n.d.	0.143	n.d.
18:1n-5	0.007	0.009	0.008	0.010	0.008
18:2n-9	n.d.	0.005	0.005	0.005	0.005
18:2n-6	2.092	2.150	2.339	2.207	2.329
18:2n-4	0.017	0.019	0.019	0.018	0.019
18:3n-6	0.023	0.023	0.025	0.023	0.025
18:3n-4	0.015	0.014	n.d.	n.d.	0.017
18:3n-3	0.142	0.147	0.159	0.149	0.159
18:4n-3	0.101	0.104	0.113	0.105	0.113
18:4n1	0.010	0.011	0.012	0.011	0.012
20:0	0.040	0.041	0.044	0.042	0.044
20:1n-9	0.173	0.177	0.188	0.179	0.189
20:2n-9	0.012	0.012	0.013	0.012	0.013
20:3n-9	0.006	0.006	0.008	0.009	0.006
20:3n-6	0.021	0.022	0.024	0.022	0.024
20:3n-3	0.020	0.017	0.022	0.021	0.022
20:4n-6	0.155	0.158	0.172	0.162	0.171
20:4n-3	0.063	0.064	0.069	0.066	0.069
20:5n-3	1.661	1.682	1.825	1.687	1.850
22:1n-11	0.033	0.034	0.033	0.032	0.037
22:4n-6	0.106	0.107	0.116	0.110	0.117
22:5n-3	0.141	0.142	0.154	0.143	0.155
22:6n-3	2.397	2.413	2.609	2.398	2.643
Saturados	1.602	1.607	1.771	1.702	1.778
Monoenoics	7.765	8.022	8.664	8.188	8.617
n-3	4.530	4.576	4.959	4.577	5.019
n-6	2.397	2.460	2.675	2.524	2.665
n-9	7.689	7.799	8.587	7.973	8.537
n-3HUFA	4.281	4.319	4.680	4.315	4.740
AA/EPA	0.093	0.094	0.094	0.096	0.093
EPA/DHA	0.693	0.697	0.699	0.703	0.700

Oleic a./DHA	3.129	3.149	3.209	3.239	3.147
Oleic a./n-3HUFA	1.848	1.855	1.888	1.901	1.851
n-3/n-6	1.890	1.860	1.854	1.813	1.883

*n.d. not detected.

Moisture (A.O.A.C., 1995), crude protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Neutral and polar fractions of the larval total lipid were separated by adsorption chromatography on silica cartridges (Sep-pak; Waters S.A., Massachussets, USA) using 30 ml chloroform and 20 ml chloroform/methanol (49: 1, v/v) as solvent for neutral lipid, then 30 ml methanol for polar fractions according to Juaneda and Rocquelin (1985). Fatty acid methyl esters were obtained by transmethylation of polar lipids as described by Christie (1982) separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

Data were treated using one-way ANOVA and means were compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results

From the beginning of the feeding trial all diets were well accepted by fish larvae. Among all treatments, larvae fed 3000/0 diet showed the lowest survival rate, being significantly lower than larvae fed 3000/1800 diet (Fig. 8.1).

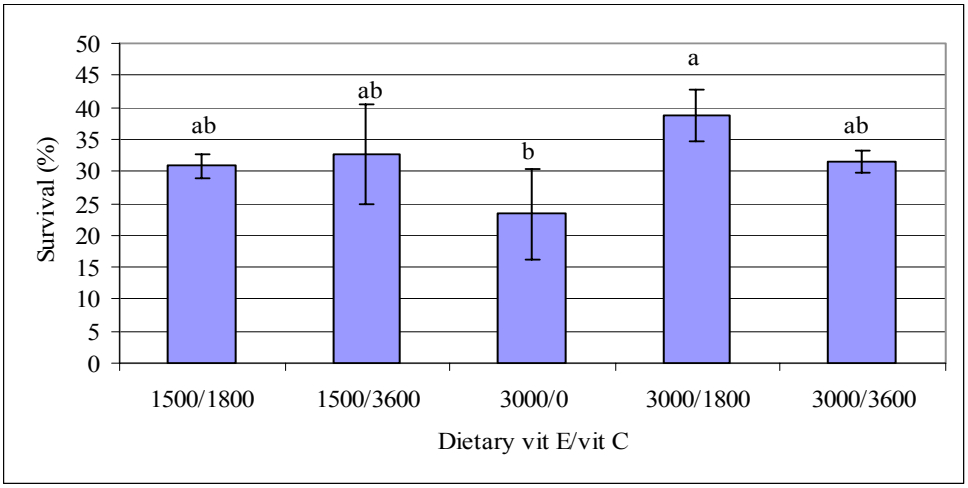

Figure 8.1. Survival rate of fish fed the experimental diets containing several vit E and vit C (n=3; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

After only one week feeding, larvae fed 3000/1800 diet was significantly bigger in total length than larvae fed 1500/1800 and 1500/3600 diets (Fig. 8.2). Moreover, in the whole body weight larvae fed with highest level of α-T and highest level AA (3000/3600) was bigger than larvae fed low α-T diets (1500/1800 and 1500/3600) and un-supplement AA diet (Fig. 8.3). In larvae fed the high level of vitamin E, the elevation of vitamin C highly correlated with dry body weight (Fig. 8.4 r^2=0.99). After two weeks of feeding, increasing levels of AA from 1800 to 3600 mg in presence of 1500 mg α-T, and the increasing of AA from 0 to 1800 or 3600 mg in presence of 3000 mg α-T respectively significantly improved the final total length, whole body dry weight, SGR and final biomass (Fig. 8.2, 8.3, 8.5, 8.6)

After 24 h of activity test no significant differences were showed among treatments in larval survival after air exposure (Fig. 8.7) while in exposure to low water temperature (15 ºC) survival of larval fed with 1500/3600 diet was significantly higher (Fig. 8.8).

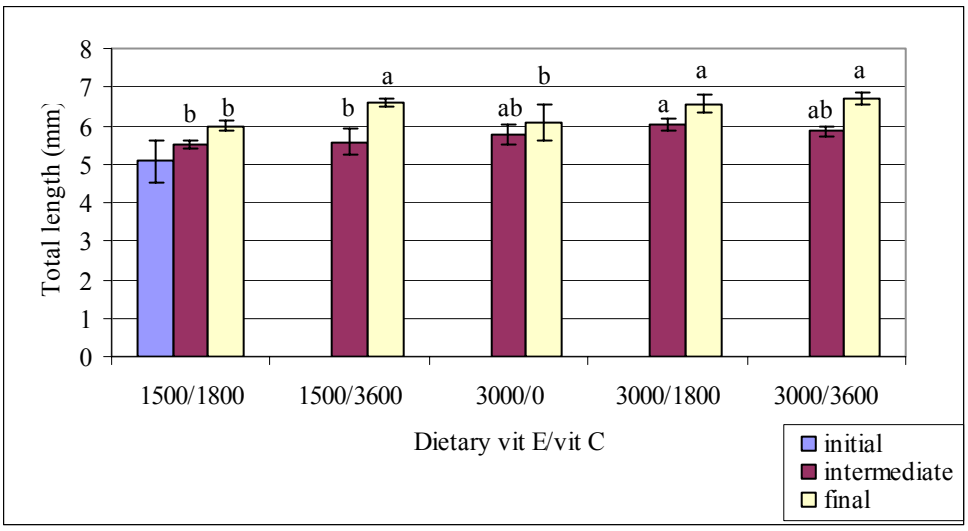

Figure 8.2. Initial, intermediate and final total length of fish fed the different experimental diets (n=90; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

Fatty acid compositions of diets (table 8.2) were similar with the level of DHA around of 2.5 and EPA around of 1.7 mg/100g diet.

In comparison to the initial larvae, fatty acid composition of larval polar lipids at the end of the experiment showed a reduction in saturated fatty acids, particularly 12:0, 14:0,15:0, 16:0, 17:0 and 18:0, and in polyunsaturated fatty acids, such as 22:5n-3, ARA, EPA and DPA, were higher in 22:6n-3 (Table 8.3).

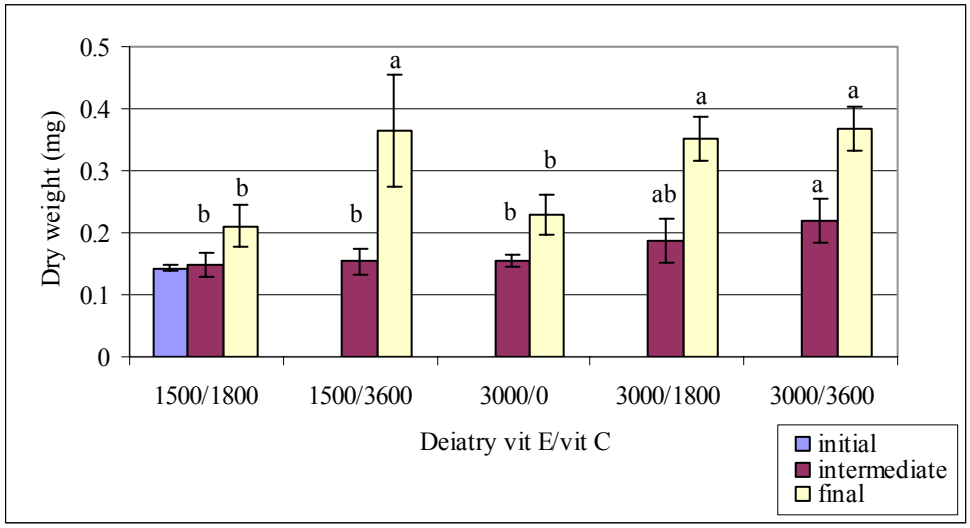

Figure 8.3. Initial, intermediate and final whole dry weight of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences ($P<0.05$) among larvae fed different diets).

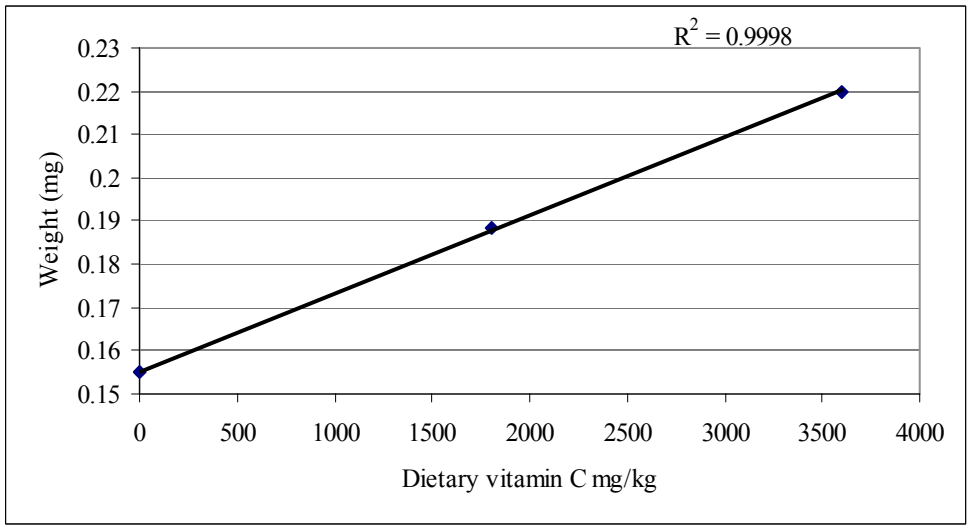

Figure 8.4. Correlation between the dietary vitamin C levels (in 3000mg/kg vit E diets) and intermediate whole body weight of fish fed the different experimental diets (n=3).

Larvae fed low α-T showed a reduction in monoenoic (4.35 % reduction) and n-9 (5.33 % reduction) fatty acids compared with those larvae fed with high α-T, while showed a increasing in DHA (7.81 % increase), n-3 (4.86 % increase) and n-3 HUFA (6.32 % increase). The different fatty acids composition of polar total lipids from whole larval body was showed in Table 8.3.

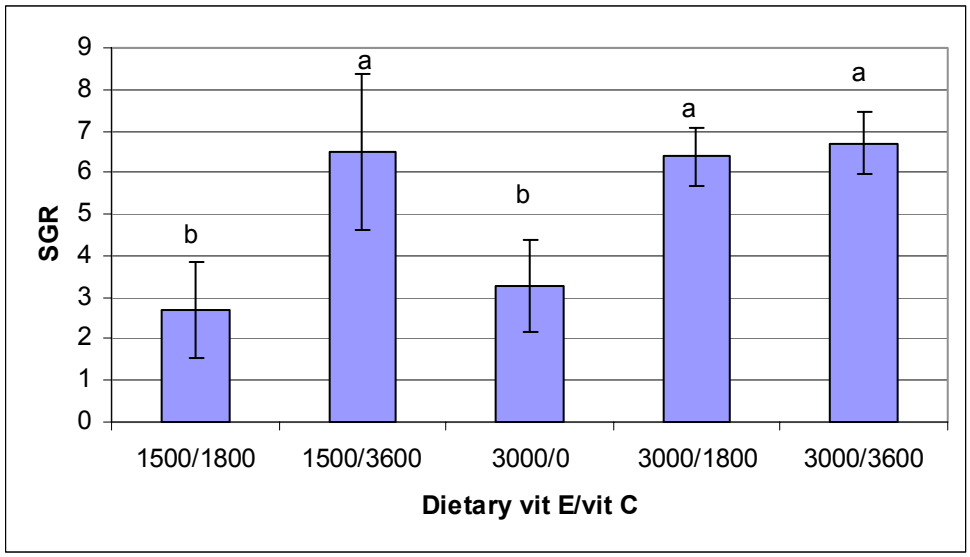

Figure 8.5. Specific growth rate of fish fed the different experimental diets (n=9; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

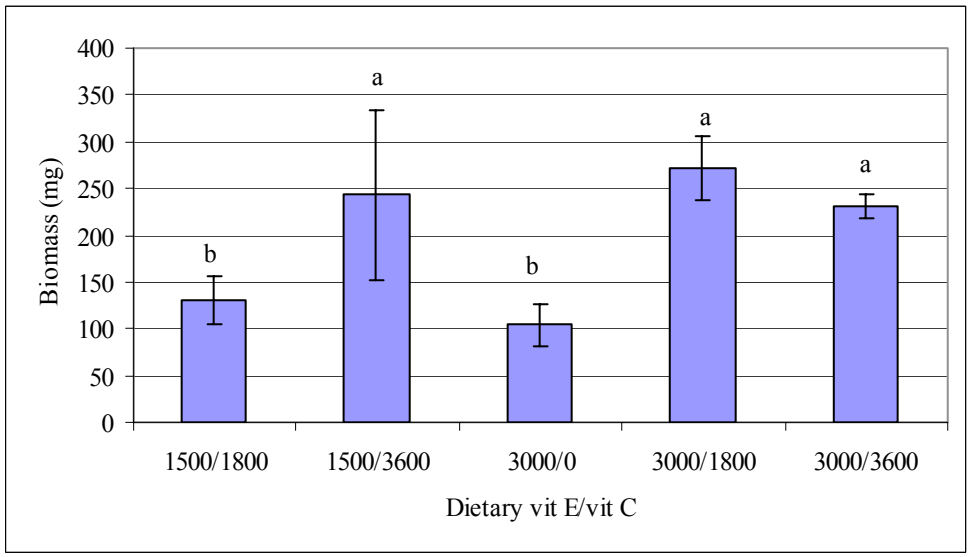

Figure 8.6. Final biomass of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

Discussion

Several studies attempted to verify the interaction between AA and α-T in animal species (Igarashi *et al*., 1991; Liu and Lee, 1998) and humans (Jacob *et al*., 1996; Hamilton *et al*., 2000). α-T acts as a lipid-soluble antioxidant protecting biological membranes and lipoproteins against oxidation, and it has been demonstrated to be an essential dietary nutrient for all fish studied (NRC, 1993).

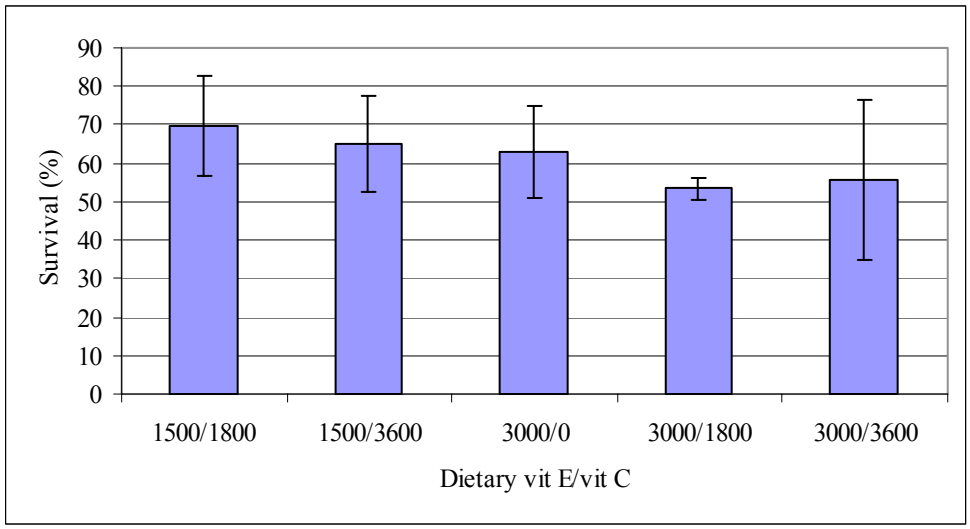

Figure 8.7. Survival after 24h of air activity test of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

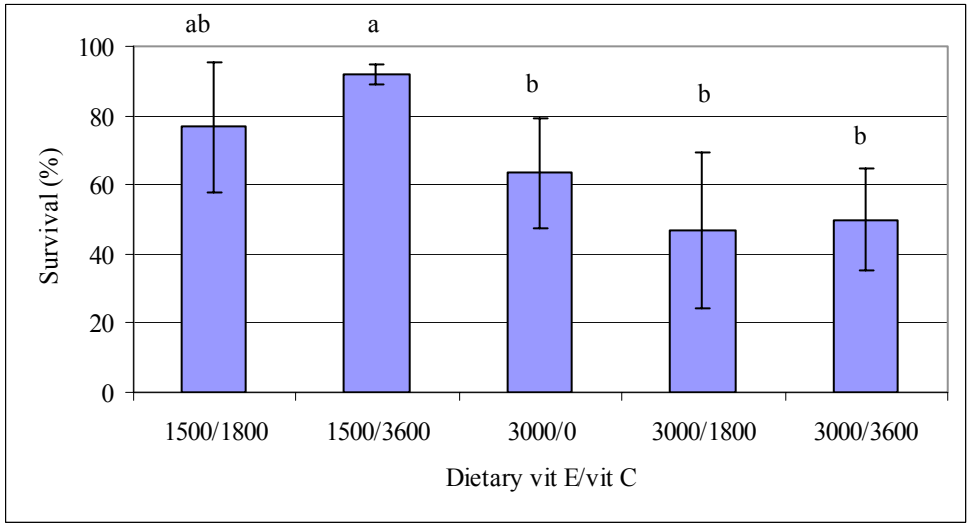

Figure 8.8. Survival after 24h of low temperature activity test of fish fed the different experimental diets (n=3; different letters for a given date denote significant differences (P<0.05) among larvae fed different diets).

In fish, AA was early recognized as a required nutrient (Kitamura *et al*., 1965) and it is considered to be an essential component in diets for teleost fish. Moreover, fish are particularly sensitive to a correct level of AA because they are unable to synthesise it *de novo* (Dabrowski, 1990) and larvae are particularly sensitive to AA deficiency (Dabrowski *et al*. 1996).

Packer *et al*. (1979) demonstrated that AA spares α-T by regenerating it from α-T radicals. Hamre *et al*. (1997) report that excess dietary AA, above the requirement, protects Atlantic salmon against α-T deficiency. Both α-T and AA are integral in the

Table 8.3. Fatty acid composition of polar lipid of the larval gilthead seabream (% total fatty acid)

Diet vit E/C	Initial	1500/1800	1500/3600	3000/0	3000/1800	3000/3600
12:0	0.169	0.118±0.15	0.088±0.07	0.126±0.06	0.060±0.03	0.062±0.02
14:0	0.863	0.652±0.16	0.524±0.04	0.592±0.12	0.589±0.12	0.495±0.04
14:1n-7	0.071	0.124±0.08	0.109±0.11	0.033±0.01	0.085±0.09	0.099±0.09
14:1n-5	n.d.	0.034±0.00	0.034±0.00	n.d.	0.043±0.00	0.109±0.11
15:0	0.469	0.261±0.13	0.266±0.15	0.222±0.08	0.250±0.14	0.286±0.19
15:1n-5	0.579	0.335±0.21	0.316±0.18	0.449±0.06	0.330±0.17	0.314±0.19
16:0ISO	0.205	0.184±0.13	0.185±0.14	0.108±0.03	0.180±0.15	0.193±0.17
Me16:0	0.487	0.384±0.06	0.353±0.00	0.336±0.04	0.389±0.01	0.378±0.00
16:0	22.612	20.435±0.93	20.757±0.67	19.122±2.09	20.698±1.14	20.699±0.49
16:1n-9	0.693	0.761±0.03	0.783±0.02	0.682±0.16	0.754±0.01	0.735±0.04
16:1n-7	4.584	1.629±0.27	1.334±0.10	1.698±0.32	1.346±0.16	1.327±0.04
16:1n-5	1.002	0.370±0.24	0.366±0.25	0.623±0.24	0.333±0.22	0.353±0.25
16:2n-6	0.511	0.628±0.28	0.290±0.11	0.556±0.43	0.406±0.09	0.516±0.41
16:2n-4	1.182	0.663±0.32	0.751±0.33	0.953±0.06	0.746±0.33	0.671±0.35
17:0	0.792	0.614±0.25	0.618±0.28	0.509±0.03	0.614±0.28	0.606±0.26
16:3n-4	n.d.	0.530±0.00	0.475±0.00	n.d.	0.471±0.00	0.476±0.00
16:3n-3	1.399	0.675±0.53	0.639±0.50	1.042±0.15	0.719±0.57	0.741±0.60
16:4n-3	n.d.	0.904±0.00	0.938±0.00	n.d.	0.948±0.00	0.988±0.00
16:3n-1	0.415	0.914±0.05	0.913±0.01	0.741±0.23	0.894±0.03	0.924±0.05
16:4n-1	n.d.	0.189±0.00	0.194±0.00	n.d.	n.d.	0.193±0.00
16:3n-4	0.270	0.199±0.03	0.193±0.00	0.210±0.01	0.201±0.01	0.208±0.00
18:0	10.632	9.133±0.58	9.111±0.29	9.462±0.35	9.236±0.30	9.250±0.34
18:1n-9	12.045	19.249±1.71	19.874±1.23	18.400±0.30	18.552±0.40	18.730±0.38
18:1n-7	5.406	2.854±0.06	2.859±0.12	2.721±0.89	2.659±0.05	2.755±0.07
18:1n-5	0.279	0.125±0.02	0.129±0.02	0.151±0.04	0.114±0.03	0.107±0.00
18:2n-9	1.174	0.311±0.01	0.295±0.05	0.346±0.06	0.263±0.04	0.291±0.02
18:2n-6	3.661	4.818±0.74	4.811±0.47	2.981±2.34	4.358±0.10	4.470±0.18
18:2n-4	0.124	0.065±0.05	0.082±0.01	2.614±3.63	0.066±0.02	0.046±0.00
18:3n-6	0.307	0.311±0.01	0.315±0.01	0.334±0.01	0.308±0.00	0.313±0.00
18:3n-4	0.082	0.071±0.00	0.024±0.00	0.076±0.00	0.045±0.02	0.024±0.00
18:3n-3	0.367	0.482±0.47	0.314±0.26	0.323±0.24	0.177±0.08	0.136±0.02
18:3n-1	n.d.	0.091±0.04	0.095±0.00	0.173±0.13	0.103±0.00	0.092±0.01
18:4n-3	0.078	0.121±0.08	0.065±0.03	0.045±0.01	0.069±0.05	0.058±0.02
18:4n-1	0.306	0.145±0.02	0.139±0.01	0.148±0.01	0.179±0.06	0.149±0.01
20:0	0.164	0.252±0.01	0.256±0.01	0.253±0.01	0.269±0.00	0.263±0.01
20:1n-9	0.776	0.767±0.07	0.765±0.05	0.780±0.11	0.734±0.02	0.756±0.03
20:1n-7	0.394	0.164±0.00	0.162±0.01	0.216±0.06	0.149±0.01	0.167±0.01
20:2n-9	0.200	0.050±0.00	0.045±0.00	0.075±0.05	0.044±0.00	0.046±0.00
20:2n-6	0.345	0.206±0.16	0.195±0.17	0.372±0.08	0.327±0.02	0.337±0.00
20:3n-9	0.130	0.179±0.22	0.353±0.00	n.d.	0.310±0.00	0.177±0.22
20:3n-6	0.366	0.176±0.02	0.159±0.01	0.193±0.03	0.150±0.03	0.153±0.01
20:4n-6	2.719	2.178±0.09	2.092±0.06	2.121±0.22	2.149±0.23	2.163±0.12
20:3n-3	n.d.	0.066±0.00	0.065±0.00	n.d.	0.064±0.00	0.062±0.00
20:4n-3	0.336	0.163±0.04	0.135±0.01	0.162±0.05	0.134±0.01	0.126±0.01
20:5n-3	5.718	4.356±0.48	4.282±0.24	4.616±0.53	4.153±0.34	4.197±0.15
22:1n-11	0.098	0.078±0.02	0.080±0.02	0.064±0.04	0.086±0.01	0.070±0.02

22:4n-6	0.551	0.626±0.35	0.617±0.34	0.851±0.06	0.627±0.35	0.649±0.37
22:5n-6	0.087	0.432±0.58	0.445±0.59	0.018±0.01	0.452±0.60	0.861±0.00
22:5n-3	1.984	1.236±0.17	1.126±0.11	1.337±0.22	1.185±0.11	1.165±0.03
22:6n-3	15.446	22.993±2.59	23.110±2.69	24.672±0.92	25.060±1.01	24.826±0.43
Saturated	36.394	32.032±2.4	32.158±1.65	30.731±2.82	32.284±2.15	32.232±1.53
Monoenoics	25.929	26.455±2.71	26.777±2.13	25.816±2.23	25.143±1.17	25.414±1.13
n-3	25.328	30.998±4.37	30.674±3.85	32.197±2.12	32.509±2.15	32.302±1.27
n-6	8.548	9.376±2.23	8.925±1.78	7.426±3.2	8.777±1.42	9.464±1.1
n-9	15.019	21.317±2.04	22.115±1.36	20.282±0.68	20.658±0.47	20.734±0.69
n-3HUFA	23.484	28.814±3.28	28.718±3.05	30.787±1.73	30.595±1.46	30.378±0.62
AA/EPA	0.476	0.5±0.19	0.489±0.25	0.459±0.42	0.518±0.69	0.515±0.78
EPA/DHA	0.370	0.189±0.18	0.185±0.09	0.187±0.57	0.166±0.33	0.169±0.35
Oleic a./DHA	0.780	0.837±0.66	0.86±0.46	0.746±0.32	0.74±0.39	0.754±0.88
Oleic a./n-3HUFA	0.513	0.668±0.52	0.692±0.4	0.598±0.17	0.606±0.27	0.617±0.61
n-3/n-6	2.963	3.306±1.96	3.437±2.17	4.336±0.66	3.704±1.52	3.413±1.15

*n.d. not detected.

intracellular defence mechanism used to protect bone cells from free radicals (Xu *et al*., 1995).

In the present study, survival of larvae fed 3000/1800 diet was higher than those fed un-supplemented AA diet (3000/0 diet) indicating the importance of supplement AA in diet for gilthead seabream larvae at level 1800mg α- T diet. But further AA level did not lead to better survival rate. In yellow perch, (*Perca flavescens)* growth and survival were significantly higher in fish fed a diet contain AA without α-T or diet contain both vitamins than those fish fed with diet which did not contained these vitamins (Lee and Dabrowski 2004). Addition of AA to larval diets improved survival, growth performance, skeleton development, stress resistance and immune response (Merchie *et al*. 1996).

After one week feeding, the larvae fed high α- T level and basal AA level was significantly higher than those larvae fed low α- T diets in total length. The whole body weight of larva fed high level of α- T and AA was significantly higher than larvae fed un-supplement AA and low α-T diets, moreover there was a relationship between the elevation of dietary AA in the 3000mg α- T treatments and intermediate dry whole body weight (r^2= 0.9998). This data suggest that the interaction of both vitamins appears after a short time of feeding. These is in agreement with Shiau and Hsu, (2002) who suggest that the high supplementation level (3 x requied for growth) of AA could spare α-T in diets for hybrid tilapia.

After two weeks of feeding larvae fed un-supplement AA (3000/0) and 1500/1800 diets were significantly lower in total length, whole body weight, SGR and biomass than those larvae fed high AA levels diets (1500/3600, 3000/3600) or high α- T with basal AA (3000/1800). These results showed the beneficial effect of increasing of AA either from 1800 to 3600 mg in presence of 1500 mg α- T, or from 0 to 1800 mg AA in presence of 3000 mg α- T. In high α- T treatment the elevation of AA from 0 to 1800 increased significantly the growth parameters but increasing from 1800 to 3600 mg AA increased only slightly those parameters. However in the 1500 α- T treatments elevation of AA from 1800 to 3600 mg increased significantly the growth parameters suggesting that with low α- T dietary level the AA elevation (from 1800 to 3600) may spare α- T resulting in higher significant growth, while this signification was not observed in high α- T dietary level. Also elevation of α-T from 1500 to 3000 mg in presence of 1800 mg AA significantly improved the growth and biomass. The un-supplement AA with high α- T level showed poor growth results. The high level dietary vitamins used in this experiment account the rapid growth rate suggesting that larvae have high vitamin requirements.

Golden shiner (*Notemigonus crysoleucas)* fed with four AA level (from 23 to 222 mg/kg) and with or without α- T (8 diets) during 14 weeks showed that survival rate of fish fed α- tocopherol-unsupplemented diets was lower, and after 10 feeding week these fish showed darkened skin fish, which was reduced with the elevation of dietary AA (Chen *et al*., 2004). Besides (Chen *et al*., 2004), survival after exposure to stressful water temperature (36-37 ºC) was lower in fish fed α- T-unsupplemented diet.

Dietary AA prevented the appearance of α-T -deficiency signs in Atlantic salmon in a dose-dependent manner (Hamre *et al*., 1997). Moreover hepatic α-T concentration decreased with increasing dietary AA in hybrid striped bass (Sealey and Gatlin, 2002). In seabream Ortuño *et al*. (2003) suggest that the short-term dietary administration of high doses of AA and E may reduce stress effect. In this study the survival after the temperature (15 ºC) activity test, the larvae fed 1500/3600 diet was significantly higher than those fed high α- T level with different AA level diets.

The result suggest that at this stage of development larval gilthead seabream requires about 1800 mg/kg AA for dietary vit E contents of about 3000 mg/kg, whereas lower dietary vitamin E level down to 1500mg/kg required an increase of AA up to 3600mg/kg.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Ai, Q.H., Mai, K.S., Li, H.T., Zhang, C.X., Zhang, L., Duan, Q.Y., Tan, B.P., Xu, W., Ma, H.M., Zhang, W.B. and Liufu, Z.G, 2004. Effects of dietary protein to energy ratios on growth and body composition of juvenile Japanese seabass, *Lateolabrax japonicus*. Aquaculture 230, 509– 516.

Alexis, M.N., Karanikolas, K.K. and Richards, R.H., 1997. Pathological findings owing to the lack of ascorbic acid in cultured gilthead bream (*Sparus aurata* L.). Aquaculture, 151, 209–218.

Boonyaratpalin, 1997. Nutrient requirements of marine food fish cultured in Southeast Asia. Aquaculture, 151, 283-313.

Boonyaratpalin, M., Unprasert, N., Kosutharak, P., Sothana, W. and Chumsungnem, S., 1989a. Effect of different levels of vitamin C added in diet on growth, feed efficiency and survival rate of seabass. Technical Paper No. 6. National Institute of Coastal Aquaculture, Department of Fisheries, Thailand, 21 pp. (in Thai).

Boonyaratpalin, M., Unprasert, N. and Buranapanidgit, J., 1989b. Optimal supplementary vitamin C level in seabass fingerling diet. In: M. Takeda and T. Watanabe (Editors), The Current Status of Fish Nutrition in Aquaculture. Tokyo University of Fisheries, Tokyo, Japan, pp. 149-157.

Boonyaratpalin, M., Boonyaratpalin, S. and Supamattaya, K., 1992. Ascorbyl-phosphate-Mg as a dietary vitamin C source for seabass (*Lares calcarifer*). Presented at the 3rd Asian Fisheries Forum, 26-30 October 1992, Singapore.

Chavez De Martinez, M.C., 1990. Vitamin C requirement of the Mexican native cichlid *Ciclasoma urophtalmus* (Gunther). Aquaculture, 86, 409–416.

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K. and Lee, K.J., 2004. Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). Aquaculture, 242, 553–569.

Chien, L.T., Hwang, D.F. and Jeng, S.S., 1999. Effect of thermal stress on dietary requirement of vitamin C in Thornfish *Terapon jarbua*. Fish. Sci., 65, 731–735.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Cort, W.M., 1974. Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid, and their mode of action. Journal of the American Oil Chemists Society, 51, 321–325.

Gouillou-Coustans, M.F., Bergot, P. and Kaushik, S.J., 1998. Dietary ascorbic acid needs of common carp (*Cyprinus carpio*) larvae. Aquaculture, 161, 453–461.

Dabrowski, K., 1990. Absorption of ascorbic acid and ascorbic sulfate and ascorbate metabolism in stomachless fish, common carp. J. Comp. Physiol., B 160, 549–561.

Dabrowski, K., Moreau, R., El-Saidy, D. and Ebeling, J., 1996. Ontogenetic sensitivity of channel catfish to ascorbic acid deficiency. J. Aquat. Anim. Health, 8, 22-27.

Fernández-Palacios, H., Izquierdo, M.S., Gonzalez, M., Robaina, L. and Valencia, A., 1998. Combined effect of dietary α-tocopherol and n-3 HUFA on egg quality of gilthead seabream broodstock (*Sparus aurata*). Aquaculture, 161, 475–476.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

Fournier, V., Gouillou-Coustans, M.F. and Kaushik, S.J., 2000. Hepatic ascorbic acid saturation is the most stringent response criterion for determining the vitamin C

requirement of juvenile European sea bass (*Dicentrarchus labrax*). *Journal of Nutrition,* 130, 617–620.

Gaylord, T.B., Rawles, S.D. and Gatlin III, D.M., 1998. Re-evaluation of Vitamin E supplementation of practical diets for channel catfish, *Ictalurus punctatus* production. Aquac. Nutr., 4, 109–114.

González, M.M., Izquierdo, M.S., Salhi, M., Hernández- Cruz, C.M. and Fernández-Palacios, H., 1995. Dietary vitamin E for *Sparus aurata* larvae. Europ. Aqua. Soc. Spec. Publ., 24, 239–242.

Hamilton, I.M.J., Gilmore, W.S., Benzie, I.F., Mulholland, C.W. and Strain, J.J., 2000. Interaction between vitamins C and E in human subjects. Br. J. Nutr., 84, 261–267.

Hamre, K. and Lie, Ø., 1995a. Minimum requirement of vitamin E for Atlantic salmon, *Salmo salar*, at first feeding. Aquacult. Res., 26, 175–184.

Hamre, K. and Lie, Ø., 1995b. Alpha-tocopherol levels in different organs of Atlantic salmon (*Salmo salar* L.) effect of smoltification, dietary levels of n-3 polyunsaturated fatty acids and vitamin E. Comp. Biochem. Physiol., 111A, 547–554.

Hamre, K., Waagbb, R., Berge, R. and Lie, a., 1997. Vitamin C and E interact in juvenile Atlantic salmon (*Salmo salar* L.). Free Radic. Biol. Med., 22, 137– 149.

Igarashi, O., Yonekawa, Y. and Fujiyama-Fujihara, Y., 1991. Synergistic action of vitamin E and vitamin C in vivo using a new mutant of Wistar-strain rats, ODS, unable to synthesize vitamin C. J. Nutr. Sci. Vitaminol., 37, 359– 369.

Izquierdo, M. and Fernández-Palacios, H., 1997. Nutritional requirements of marine fish larvae and broodstock. Cah. Options Mediterr., 22, 243–264.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Jacob, R.A., Kutnink, M.A., Csallany, S., Daroszewska, M. and Burton, G.W., 1996. Vitamin C nutriture has little short-term effect on vitamin E concentrations in healthy women. J. Nutr., 126, 2268– 2277.

Juaneda, P. and Rocquelin, G., 1985. Rapid and convenient separation of phospholipids and non-phosphorous lipids from rat heart using silica cartridges. Lipids, 20, 40–41.

Kawatsu, H., 1969. Studies on the anemia of fish-III. An example of macrocytic anemia found in brook trout, *Salvelinus fontinalis*. Bulletin of the Freshwater Research Laboratory, 19, 161–167.

Kitamura, S., Ohara, S. and Suwa, T., 1965. Studies on vitamin requirements of rainbow trout, *Salmo gairdneri* I. On ascorbic acid. Bull. Jpn. Soc. Sci. Fish, 31, 818-827.

Kolkovski, S., Czesny, S., Yackey, C., Moreau, R., Cihla, F., Mahan, D. and Dabrowski, K., 2000. The effect of vitamins C and E in (n-3) highly unsaturated fatty acids-enriched *Artemia nauplii* on growth, survival, and stress resistance of fresh water walleye *Stizostedion vitreum* larvae. Aquac. Nutr., 6, 199–206.

Lee, K.J. and Dabrowski, k., 2004. Long-term effects and interactions of dietary vitamins C and E on growth and reproduction of yellow perch, *Perca flavescens*. Aquaculture, 230, 377–389.

Lin, M-F. and Shiau, S-Y. 2005. Dietary l-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, Epinephelus malabaricus. Aquaculture, 244, 215– 221.

Liu, J.-F. and Lee, Y.-W., 1998. Vitamin C supplementation restores the impaired vitamin E status of guinea pigs fed oxidized frying oil. J. Nutr., 128, 116– 122.

Mahajan, C. and Agrawal, N.K., 1980. Nutritional requirement of ascorbic acid by Indian major carp, *Cirrhina mrigala*, during early growth. Aquaculture, 19, 37–48.

Merchie, G., Lavens, P., Storch, V., Ubel, U., De Nelis, H., Leenheer, A. andSorgeloos, P., 1996. Influence of dietary vitamin C dosage on turbot *Scophthalmus maximus* and European sea bass *Dicentrarchus labrax* nursery stages. Comp. Biochem. Physiol., 114, 123-133.

Merchie, G., Lavens, P. and Sorgeloos, P., 1997. Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture, 155, 165–181.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L. and Vergara, J. M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiology and Biochemistry , 18, 399–407.

Montero, D., Tort, L., Robaina, L., Vergara, J.M. and Izquierdo, M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish and Shellfish Immunology, 11, 473–490.

Mulero, V., Esteban, M. A. and Mesenguer, J., 1998. Effects of in vitro addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes. Veterinary Immunology and Immunopathology, 66, 185–199.

Murai, T. and Andrews, J.W., 1974. Interaction of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 104, 1416–1431.

National Research Council (NRC), 1993. Nutrient Requirements of Fish. National Academy Press, Washington, D.C. (114 pp.).

Niki, E., 1987a. Antioxidants in relation to lipid peroxidation. Chemistry and Physics of Lipids, 44, 227–253.

Niki, E., 1987b. Interaction of ascorbate and α-tocopherol. Ann. N.Y. Acad. Sci., 498, 186–199.

Ortuno, J., Esteban, M.A. and Meseguer, J., 1999. Effects of high dietary intake of vitamin C on non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 9, 429– 443.

Ortuño, J., Esteban, A. and Meseguer, J., 2000. High dietary intake of α-tocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 10, 293–307.

Ortuño, J., Esteban, M.A. and Meseguer, J., 2003. The effect of dietary intake of vitamins C and E on the stress response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 14, 145–156.

Packer, J.E., Slater, T.F. and Wilson, R.L., 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature, 278, 737–738.

Paul, B.N., Sarkar, S.N. and Mohanty., 2004. Dietary vitamin E requirement of mrigal, *Cirrhinus mrigala* fry. Aquaculture, 242, 529-536.

Putnam, M.E. and Comben, M., 1987. Vitamin E. Veterinary Record, 121, 541-545.

Roem, A.J., Kohler, C.C. and Stickney, R.R., 1990. Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. Aquaculture, 87, 155–164.

Ruff, N., P. Lavens, P., Huo, J.Z., Sorgeloos, P., Nelis, h.J. and DE Leenheer, A., 2001. Antioxidant effect of dietary tocopherol and ascorbic acid on growth and survival of *Litopenaeus vannamei* Postlarvae. Aquaculture International, 9, 115–126.

Sakai, T., Murata H., Endo, M., Yamauchi, K., Tabata, N. and Fukudome, M., 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola aquiqueradiata*. Agricultural and Biological Chemistry, 53, 1739–1740.

Sargent, J.R., McEvoy, L.A. and Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEnvoy, L., Tocher, D. and Estevez A., 1999. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177,191-199.

Saroglia, M. and Scarano, G., 1992. Experimental induction of ascorbic deficiency in sea bass in intensive aquaculture. Bull. Eur. Ass. Fish Pathol., 12, 97–99.

Schwarz, F.J., Kirchgessuner, M., Steinhart, H. and Runge, G., 1988. Influence of different fats with varying additions of α-tocopherol acetate on growth and body composition of carp (*Cyprinus carpio* L.). Aquaculture, 69, 57–67.

Sealey, W.M. and Gatlin III, D.M., 2002. Dietary vitamin C and vitamin E interact to influence growth and tissue composition of juvenile hybrid striped bass (*Morone chrysops XM. Saxatilis*) but have limited effects on immune responses. J. Nutr., 132, 748– 755.

Shiau, S.Y. and Hsu, C.Y., 2002. Vitamin E sparing effect by dietary vitamin C in juvenile hybrid tilapia, *Oreochromis niloticus x O. aureus*. Aquaculture, 210, 335–342.

Teshima, S.I., Kanazawa, A., Koshio, S. and Itoh, S., 1993. L-Ascorbyl-2-phosphate-Mg as vitamin C source for the Japanese flounder *(Paralichthys oliuaceus*). In: S.J. Kaushik and P. Luquet (Editors), Fish Nutrition in Practice. Coll. Les. Colloq., No. 61. INRA, Paris, pp. 157-166.

Thorarinsson, R., Landolt, M.L., Elliott, D.G., Pascho, R.J. and Hardy, R.W., 1994. Effect of dietary vitamin E and selenium on growth, survival and the prevalence of *Renibacterium salmoninarum* infection in chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture, 121, 343–358.

Tocher, D.R., Mourente, G., van der Eecken, A., Evjemo, J.O., Díaz, E., Bell, J.G., Geurden, I., Lavens, P. and Olsen, Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquac. Nutr., 8, 195–207.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz E., Wille, M., Bell J.G. and Olsen, Y., 2003. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International, 11, 195–216.

Wang, X.J., Kim, K.W., Bai, S.C., Huh, M.D. and Cho, B.Y., 2003. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). Aquaculture, 215, 21– 36.

Watanabe, T., Takashima, F., Ogino, C. and Hibiya T., 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi, 36, 623–630.

Watanabe, T., Takeuchi, T., Matsui, M., Ogino, C. and Kawabata, T., 1977. Effect of α-tocopherol deficiency on carp: VII. The relationship between dietary levels of linoleate and α-tocopherol requirement. Bull. Jpn. Soc. Sci. Fish., 43, 935– 946.

Watanabe, T., Takeuchi, T. and Wade, M., 1981. Dietary lipid levels and α-tocopherol requirement of carp. Bulletin of the Japanese Society of Scientific Fisheries, 47(12), 1585–1590.

Wilson, R.P., Bowser, P.R. and Poe, W.E., 1984. Dietary vitamin E requirement of fingerling channel catfish. J. Nutr., 114, 2053– 2058.

Woodall, A.N., Ashley, L.M., Halver, J.E., Scott, H.S. and Van de Veen, J., 1964. Nutrition of salmonid fishes: XIII. The α-tocopherol requirement of chinook salmon. J. Nutr., 84, 125– 135.

Xu, H., Watkins, B.A. and Seifert, M.F., 1995. Vitamin E stimulates trabecular bone formation and alters epiphyseal cartilage morphometry. Calcif. Tissue Int., 57, 293–300.

CHAPTER 9

GENERAL CONCLUSION

Along the studies in this thesis the following general conclusions can be drawn in a succinct form:

1. Gilthead seabream larval survival is markedly associated to dietary EPA and DHA levels, whereas DHA seems to be more important for growth success. Optimum EPA/DHA dietary ratios over 1 in microdiets for gilthead seabream had negative effects on growth.
2. When DHA dietary levels cover the requirements for this fatty acid, increase of both EPA and ARA seems to be more important to enhance growth and survival than the solely elevation of one of them. For a better larval performance EPA/ARA ratios in microdiets for gilthead seabream should be kept lower than 8 and higher than 2, being the optimum dietary ratio close to 4.
3. In both gilthead sea bream and European seabass dietary ARA was more efficiently incorporated into larval tissues than EPA. Increased dietary EPA or ARA contents respectively reduced the incorporation of ARA or EPA into larval lipids, denoting their competition as substrates for different enzymes.
4. When sea bream and seabass are fed high DHA levels during larval development, both species seem to have a requirement for at least 1.2% ARA at EPA/ARA ratios close to 4 to show good growth performance, as well as high survival and stress resistance.
5. DHA is neccesary for optimum European sea bass growth, however, high dietary levels of this fatty acid have a negative impact on seabass survival, particularly when dietary antioxidants such as vitamin E are insufficient.
6. In sea bass, dietary vitamin E increase up to 3000 mg/kg has a marked positive effect on fish growth and stress resistance. Besides, and depending upon dietary polyunsaturated fatty acids contents, increase in vitamin E enhances survival.

7. In seabream, increase in dietary vitamin E up to 3000 mg/kg in microdiets markedly improves larval growth and is positively correlated to survival depending upon dietary polyunsaturated fatty acids, protecting them from oxidation. However, further elevation of vit E up to 6000 mg/kg markedly reduced EPA suggesting the pro-oxidant effect of vit E excess.
8. Vitamin C deficiency markedly reduced survival in larval gilthead seabream who showed to require about 1800 mg/kg AA for dietary vit E contents of about 3000mg/kg. A clear interrelation was found between both vitamins, the reduction in dietary vitamin E level down to 1500mg/kg increasing AA requirements up to 3600mg/kg.

CAPÍTULO 10

RESUMEN AMPLIO

LISTA DE ABREVIATURAS

AA: Ácido Ascórbico

AGE: Ácidos Grasos Esenciales

ARA: Ácido Araquidónico

DHA: Ácido Docosahexaenoico

EPA: Ácido Eicosapentaenoico

HUFA: Ácidos Grasos Altamente Insaturados

n-3 HUFA: Ácidos Grasos Altamente Insaturados con 20 o más átomos de carbono y 3 o más dobles enlaces

PE: Fosfatidiletanolamina

PI: Fosfatidilinositol

PL: Fosfolípidos

PUFA: Ácidos Grasos Poliinsaturados

Vit: Vitamina

α-T: α-Tocoferol

10.1 _ Introducción general

10.1.1 - Importancia de la nutrición lípidica de peces para el desarrollo de la acuicultura

La acuicultura es uno de los sectores con mayor crecimiento en la producción animal mundial, con una tasa superior al 5% anual en la última década (Izquierdo *et al*., 2008). En la acuicultura, el éxito del cultivo de cualquier especie de peces marinos está limitado por la cantidad y la calidad de la producción en masa de sus alevines (Izquierdo y Fernández - Palacios, 1997).

Por lo tanto, para cubrir las crecientes demandas de la producción de semillas, es necesario mejorar la calidad nutritiva de sus larvas, lo cual todavía constituye una de las principales limitaciones para el desarrollo del cultivo de especies de peces marinos (Watanabe *et al*., 1983; Yúfera y Pascual, 1984; Sargent *et al*. 1997; Izquierdo *et al*., 2000).

Aunque la producción Mediterránea del cultivo de peces marinos se ha incrementado en varias especies, la dorada *Sparus aurata* continúa siendo la especie mas cultivada (Izquierdo, 2005), con una producción anual de alevines que supera los 120 millones/ año.

La demanda de alevines de buena calidad está aumentando a un ritmo del 10% anual, pero el éxito de la producción de los juveniles se ve muy afectado por la eficacia de la primera alimentación y la calidad nutricional de la dieta de arranque (Kolkovski *et al*., 1993; Sargent *et al*., 1997; Izquierdo *et al*., 2000). En general, la dorada y la lubina europea (*Dicentrarchus labrax*) son las especies más importantes de peces marinos criados en la región mediterránea y han caracterizado el desarrollo de la acuicultura marina en esta región en las últimas tres décadas (FAO, 1999). Además, en la producción, de ambas especies, se prevee una mayor expansión (Basurco y Abellán, 1999). Sin embargo, a pesar de que el engorde de estas especies está bien controlado, el conocimiento de sus necesidades nutricionales, en comparación con otras especies como salmónidos y carpas, sigue siendo incompleto (NRC, 1993). Por lo tanto, para obtener un mejor crecimiento y una mayor tasa de supervivencia, es esencial una dieta que responda a las necesidades nutricionales de las larvas, tanto cualitativas como cuantitativas (Kolkovski *et al*., 1993; Sargent *et al*., 1997). Además, incluso antes de que comience el desarrollo larvario, los huevos de los peces deben contener todos los

nutrientes que cubran los requerimientos adecuados para el desarrollo del embrión (Izquierdo y Fernández - Palacios, 1997).

En los últimos años, las investigaciones han prestado gran atención a la importancia de los lípidos dietéticos para larvas de peces marinos (Izquierdo *et al*., 2003), puesto que son esenciales para el crecimiento y el desarrollo de los mismos (Watanabe, 1982; Sargent *et al*., 1999a). Como reflejo de esta importancia, la dieta para dorada y lubina se ha convertido en una dieta altamente energética (25% de lípidos) en comparación con la década anterior (12% de lípidos) (Izquierdo *et al*., 2003).

Los lípidos dietéticos proporcionan una fuente rica de energía y fosfolípidos y son fundamentales para la estructura de las biomembranas. Los lípidos dietéticos también sirven como vehículos para la absorción de otros nutrientes, como las vitaminas liposolubles A, D, E, y K, y pigmentos naturales o sintéticos. Los lípidos son componentes de las hormonas y precursores para la síntesis de diversos metabolitos funcionales, como las prostaglandinas y otros eicosonoides. Además, los lípidos dietéticos son reconocidos como uno de los factores nutricionales más importantes que afectan el crecimiento y la supervivencia de las larvas (Watanabe *et al*., 1983), porque constituyen materiales esenciales para la formación normal de célula, las membranas de los tejidos y el desarrollo de órganos (Izquierdo *et al*., 1998, 2003; Pousaò *et al*., 2003). Sin embargo, la utilización de los lípidos dietéticos por las larvas puede verse afectada directa o indirectamente por varios cambios morfológicos y fisiológicos que ocurren durante el desarrollo larvario. En los últimos años ha habido más interés en todos estos aspectos nutricionales de los lípidos en las larvas de peces, debido a la importancia de utilización de los lípidos dieteticos para el óptimo crecimiento y supervivencia larvaria (Izquierdo *et al*., 2000).

10.1.2 - Los ácidos grasos esenciales

Los ácidos grasos esenciales (AGE) son componentes fundamentales de los lípidos, que no pueden ser bio-sintetizados en cantidades suficientes por los peces. Watanabe (1982) sugirió que las especies de peces marinos tienen una capacidad limitada para convertir ácidos grasos de 18C en ácidos grasos poliinsaturados con cadena más larga, en contraste con la mayoría de vertebrados. Probablemente todos los vertebrados tienen un requerimiento en ácidos grasos de la serie n - 3 y n - 6 en la dieta

en cantidades suficientes para cubrir sus requerimientos, dependiendo de la capacidad de síntesis de cada especie a partir de los precursores de 18 C por la acción de las enzimas elongasas y desaturasas (Teles, 2000). Particularmente, es probable que todos los peces requieran dietas que contengan ácidos grasos de la serie n-3 (PUFA): como el ácido docosahexaenoico (DHA, 22:6n - 3) y el ácido eicosapentaenoico (EPA, 20:5n-3) o de la serie n-6 como el ácido araquidónico (ARA, 20:4n-6), puesto que juegan unas funciones muy importantes en estas especies, pero son sintetizados en cantidades muy pequeñas (Izquierdo *et al*., 2005).

Considerando que en los peces de agua dulce parecen tener mayores actividades la Δ6 y Δ5 desaturasas, y elongasas para producir ARA, EPA y DHA, si sus precursores los ácidos linoléico (18:2n-6) y linolénico (18:3n-3) están presentes en la dieta, esta actividad enzimática es inexistente o muy limitada en peces marinos y como consecuencia, los ácidos grasos de cadena larga tienen que estar incluidos en la dieta y se consideran esenciales (Sargent *et al*., 1995; Izquierdo, 1996). Hace años, el gen de la Δ6 desaturasa fue descodificado en pez cebra (*Danio rerio*) (Hastings *et al*., 2001). Este tipo de genes también se ha encontrado en especies de peces marinos como la dorada (Seiliez *et al*., 2003) aunque su expresión está inhibida. Más recientemente (Izquierdo *et al*., 2008) se ha demostrado que los lípidos dietéticos son capaces de regular la expresión de la Δ6-desaturasa de la larva en dorada, aunque la capacidad de esta especie para sintetizar DHA no es suficiente para cubrir los requerimientos en este ácido graso.

Los AGE (ácidos grasos esenciales) juegan unos papeles fisiológicos importantes en los peces como precursores de eicosanoides y otros sustancias biológicamente muy activas (Bell *et al*., 1986). Por esa razón, durante mucho tiempo su estudio ha sido tratado por muchos grupos de investigación y los requerimientos en ambos EPA y DHA (n-3 HUFA ácidos grasos altamente insaturados con 20 o más átomos de carbono y 3 o más dobles enlaces), se han determinado en varias especies de peces en las diferentes etapas de desarrollo (Watanabe *et al*., 1978a, b 1982; Wilson, 1991; Wantanabe, 1993; Mourente *et al*., 1993; Rodrıguez *et al*., 1993 ; Watanabe, 1993; Rodrıguez, 1994; Salhi *et al*., 1994; Izquierdo, 1996; Watanabe y Kiron, 1994; Izquierdo, 1989; 1996; Sargent *et al*., 1999a; Izquierdo *et al*., 2000, 2005).

El requerimiento en estos ácidos grasos se puso de manifiesto por la mortalidad elevada y el bajo crecimiento de las larvas después de 10 -15 días de la alimentación con una dieta carente en estos AGE (Izquierdo *et al*., 1989; Sargent *et al*., 1997; Izquierdo, 2005) y por la reducción de la calidad de los huevos después de 14 días de

alimentecion de los reproductores con una dieta carente en AGE (Harel *et al*., 1994; Izquierdo *et al*., 2001a).

La reducción en el crecimiento se ha observado en larvas de peces marinos alimentados con rotíferos, *Artemia* o microdieta deficientes en AGE, en rodaballo (*Scophthalmus Maximus)* (Gatesoupe & Le Milinaire, 1985), el pargo rojo (*Pagrus major)* (Izquierdo *et al*., 1989a, 1989b) y la dorada (Rodríguez *et al*., 1993, 1994; Salhi *et al*., 1994).

Niveles inadecuados de n-3 HUFA en la dieta ocasionan la reducción de la supervivencia en el pargo rojo (Izquierdo *et al*., 1989a, 1989b), dorada (Rodríguez *et al*, 1993, 1994; Salhi *et al*., 1994) y fletán (*Hippoglossus hippoglossus)* (Holmefjord y Olsen, 1991).

La mala pigmentación fue relacionada con niveles bajos de n-3 HUFA, especialmente DHA, en larvas de rodaballo (Rainuzzo *et al*., 1994).

Los niveles dietéticos inadecuados en AGE también causaran una pobre alimentación y actividad natatoria (Izquierdo, 1996) y alteraron el comportamiento de las larvas de peces (Benítez *et al*., 2007). Además, alimentos deficientes en AGE retrasan la aparición de la respuesta a estímulos visuales, de acuerdo con la reducción del contenido de DHA en el cerebro y los ojos de estas larvas y sugiere un retraso en el desarrollo funcional del cerebro y la visión (Benítez *et al*. 2007). Estos estudios demuestran que la reducción del contenido de EPA y DHA disminuye el diámetro del ojo de la larva de dorada (Izquierdo *et al*., 2000; Roo *et al*., enviado). Este hecho, junto con la mayor densidad de conos fotorreceptores implica una mejora significativa en capacidad visual de la larva (Roo *et al*., enviado).

La reducción en la natación y actividad alimenticia en larvas deficientes en AGE (Izquierdo *et al*., 1989; Rodríguez *et al*., 1993; 1994) es reconocida por las larvas flotando en la superficie del agua, lo que también indica alteraciones en el funcionamiento de la vejiga natatoria (Koven, 1991). Así, los n-3 HUFA han sido reconocidos como un factor limitante importante que define el valor nutritivo de la dieta larvaria de especies marinas, siendo indispensables durante las primeras etapas de vida (Watanabe y Kiron, 1994; Izquierdo, 1996; Sargent *et al*., 1999a).

Los factores ambientales afectan, tanto cualitativa como cuantitativamente, el requerimiento en AGE, incluidos la temperatura (Olsen y Skjervold, 1995), la salinidad (Borlongan y Benítez, 1992; Dantagnan *et al*., 2007) y la luz (Ota y Yamada, 1971).

Asumiendo que las larvas crecen más rápido que los juveniles o adultos, la necesidad larvaria en n-3 HUFA es superior en las primeras fases del desarrollo que en los juveniles (Izquierdo *et al*., 1989a). Por ejemplo, la elevación de los n-3 HUFA en la dieta para juveniles hasta un 1% mejora el crecimiento y la eficiencia alimentaría en dorada, pero la elevación de este nivel resultó en un detrimento de estos parámetros.

Por ello, 1% n - 3 HUFA es el requerimiento recomendado para juveniles de dorada (Ibeas *et al*., 1996). Sin embargo, Rodríguez *et al*. (1998) mostraron que la elevación de n - 3 HUFA de 0,9 a 1,5% en rotiferos *Brachionus plicatilis* (peso seco) mejora el crecimiento en la primera alimentación de larvas de dorada.

10.1.3 Importancia específica de DHA, EPA y ARA

El DHA es necesario para la reproducción, el crecimiento y la supervivencia de los peces (Izquierdo, 2005). Su incorporación en la membrana celular regula la integridad y función de la misma (Izquierdo, 2005). Además, este ácido graso es un componente importante de los fosfoglicéridos, en particular de la fosfatidiletanolamina y fosfatidilcolina en larvas de peces. El DHA es específicamente retenido durante el ayuno o en peces alimentados con bajos niveles de AGE, debido a la menor oxidación celular que otros ácidos grasos (Madsen *et al*., 1999). DHA es más esencial que el EPA como AGE en larvas de peces marinos (Watanabe *et al*., 1989; Watanabe, 1993) ocasionando un mejor crecimiento y supervivencia en las larvas (Izquierdo, 1996). El requerimiento mínimo recomendado de DHA dietético para larvas de dorada es 0,8% (Rodríguez *et al*., 1998; Salhi *et al*., 1999) y para juveniles es 0,6% (Montero *et al*., 1996).

Los peces alimentados con DHA cómo el principal n-3 HUFA mostraron un mayor crecimiento y una mayor supervivencia después del test de actividad que las larvas alimentadas con EPA como principal n-3 HUFA (Rodríguez *et al*., 1997).

El EPA es otro AGE importante para el crecimiento de larvas de peces marinos (Watanabe *et al*., 1989), siendo uno de los principales precursores de las prostaglandinas en peces marinos (Ganga *et al*., 2005). Además mejora la actividad de la lipasa digestiva no-específica en larvas de dorada (Izquierdo *et al*., 2000) y la incorporación de DHA en los fosfolípidos (PL) de larvas (Izquierdo *et al*., 2000, 2001b). EPA es considerado como un componente principal de los lípidos polares y su

presencia contribuye también a regular la integridad y la función de la membrana (Izquierdo, 2005).

Desde que se ha conocido que el ARA es un precursor importante de eicosanoides, en peces también se ha prestado más atención a este ácido graso (Henderson y Sargent, 1985; Henderson *et al*., 1985; Bell *et al*., 1994; Sargent *et al*., 1994).

Los eicosanoides constituyen un grupo de compuestos altamente activos, de acción hormonal local que, entre otras moléculas, incluyen prostaglandinas, tromboxanos, y leucotrienos, que participan en la regulación de varios procesos fisiológicos. Además, se considera que las interacciones competitivas entre el EPA y ARA son importantes para el equilibrio adecuado en la síntesis de eicosanoides (Izquierdo, 1996).

Numerosos estudios han mostrado que ARA dietético mejora el crecimiento de peces (Castell *et al*., 1994; Bessonart *et al*., 1999), especialmente cuando se ofrece junto con los niveles adecuados de otros ácidos grasos esenciales como EPA y DHA (Bessonart *et al*., 1999). Koven *et al*. (2001) demostraron que el ARA dietético suplementado a larvas de dorada de 3 a 19 días después de eclosión mejora notablemente la supervivencia después de un estrés agudo.

En juveniles de rodaballo, las deficiencias de ARA en la dieta resultan en una elevada mortalidad y una obvia patología (Bell *et al*., 1985). En la lubina europea, los reproductores mostraron un elevado requerimiento dietético en ARA (Sargent *et al*., 1999a). Las larvas de dorada mostraron una retención preferencial de ARA durante el ayuno (Castell *et al*., 1994; Rodríguez *et al*., 1994; Izquierdo, 1996), que indica también la importancia de este ácido graso.

10.1.4 - La importancia de diferentes ratios de DHA, EPA y ARA

La competencia entre estos tres ácidos grasos ha sido demostrada a varios niveles fisiológicos, sugiriendo que no sólo el requerimiento específico de cada uno de estos ácidos grasos, sino también las proporciones entre ellos, deben tenerse en cuenta para determinar sus requerimientos (Izquierdo *et al*., 2001). Por ejemplo, las lipasas digestivas han mostrado diferentes afinidades por EPA o DHA (Izquierdo *et al*., 2000). De este modo, una tasa óptima EPA/DHA parece ser necesaria para obtener el mejor

crecimiento (Izquierdo y Fernández-Palacios, 1997) y pigmentación (Reitan *et al*., 1994) en las primeras etapas larvarias.

Rodríguez *et al*. (1997) demostraron que con el mismo nivel dietético de n-3 HUFA la disminución del contenido en EPA/DHA de los rotíferos a 1/1.3 mejora el crecimiento de las larvas de dorada, lo que indica que el DHA es más importante que el EPA durante las primeras etapas larvarias (Watanabe *et al*, 1989). Por otra parte, Rodríguez *et al*. (1997) han encontrado una relación significativamente negativa entre el contenido de las larvas en EPA/DHA (lípidos polares total) y el crecimiento. En larvas de rodaballo, Reitan *et al*. (1994) han demostrado que la tasa óptima de EPA/DHA es de 2. En los juveniles de dorada, el mejor crecimiento y el peor índice hepatosomático se obtuvieron cuando la tasa EPA/DHA aumentó de 1/2 a 2/1, cuando el total de n-3 HUFA se mantuvo constante en la dieta (Ibeas *et al*., 1997), sugiriendo que las tasas óptimas también pueden variar a lo largo del ciclo de vida. Sin embargo, la mayoría de los estudios ensayaron un rango estrecho de estas tasas.

Debido a la importancia del ARA y sus interrelaciones con los otros ácidos grasos esenciales, recientemente, la investigación se ha centrado en la relación entre estos tres ácidos grasos (McEvoy *et al*., 1998; Estévez *et al*., 1999; Sargent *et al*., 1999a). La relación entre el DHA, EPA y ARA dietéticos parece ser un factor crítico para el rendimiento de los reproductores y las larvas (Sargent *et al*., 1999a, b; Bell y Sargent, 2003), debido a la competencia entre ellos. Por ejemplo, hay evidencias de la competencia entre estos tres ácidos grasos (ARA, EPA y DHA) a nivel de enzimas digestivas (Iijima *et al*., 1998; Izquierdo *et al*., 2000). Por lo tanto, no sólo los valores dietéticos absolutos para cada uno de estos ácidos grasos esenciales, sino también la óptima relación dietética entre cada uno de ellos debe definirse, dado que ambos factores afectaran al menos a su incorporación en el tejido lípidico y por tanto a la fluidez y la función de la membrana, el valor energético obtenido de su beta-oxidación y la producción de compuestos metabólicamente activos (Izquierdo *et al*., 2000; Izquierdo, 2005). Así, varios estudios demuestran la importancia de considerar las cantidades relativas de DHA, EPA, y ARA (McEvoy *et al*., 1998; Bessonart *et al*., 1999; Estévez *et al*., 1999; Sargent *et al*., 1999a; Copeman *et al*., 2002; Bell y Sargent, 2003; Koven *et al*., 2003). Estévez *et al*. (1999) en larvas de rodaballo mostraron que el aumento del ARA dietéticos afecta la composición de ácidos grasos de los fosfoglicéridos cerebrales fosfatidiletanolamina (PE) y fosfatidilinositol (PI) más que el aumento del EPA dietético, mientras que la tasa EPA: ARA $\geq$ 1 en PI (fracción del

cerebro) se asocia con una buena pigmentación normal. Además, en el mismo estudio se observó que dando un nivel suficiente de DHA dietético, el nivel óptimo de EPA dietético no es una función del DHA, sino de ARA, y finalmente los niveles desequilibrados de HUFA en la dieta durante el desarrollo larvario podrían tener graves efectos sobre la formación del cerebro, la diferenciación neuroanatómica, y la acción de sustancias neuroendocrinas.

Copeman *et al*. (2002) observaron en larvas de limanda nordica (*Limanda ferruginea*) que los peces alimentados con rotíferos enriquecidos con un nivel alto de DHA (y bajo EPA y ARA) fueron significativamente más grandes y tuvieron mayor supervivencia, en comparación con las larvas alimentadas con rotíferos enriquecidos con DHA y más nivel de EPA y ARA o sólo con aceite de oliva. También se observó una mayor relación entre la tasa DHA/EPA en la dieta y el tamaño de las larvas (r^2 = 0,75, P = 0,005) o la supervivencia (r^2 = 0,86, P = 0,001). Además, la incidencia de una mala pigmentación fue mayor en las larvas alimentadas con un nivel dietético alto de DHA + AA que en todos los otros tratamientos. Por último, las larvas alimentadas con la dieta enriquecida con DHA tuvieron mayor incorporación de ARA que las alimentadas con dieta enriquecida con DHA + EPA, lo que podría indicar una interacción competitiva entre EPA y ARA para su incorporación en los (PL).

Tanto ARA como EPA son precursores de eicosanoides (Ganga *et al*., 2005), compitiendo por las mismas enzimas, por lo que su tasa influye en la producción de eicosanoides en diferentes tejidos (Sargent *et al*., 1995, 1999b; Ganga *et al*., 2005,). McEvoy *et al*. (1998) demostraron que una tasa alta EPA/AA dietético mejora la pigmentación en presencia de altos niveles de DHA en larvas de halibut atlántico. Por ejemplo, Bessonart *et al*. (1999) mostraron que al aumentar los niveles de ARA dietético hasta un 1% mejora el crecimiento de las larvas de dorada (DHA/EPA/ARA 1.4/0.8/1). Sargent *et al*. (1999b) sugieren que es necesario una proporción DHA/EPA/ARA 10:5:1 para larvas de peces marinos mientras la proporción óptima sería especifica de cada especie. Castell *et al*. (2001), sugirieron la tasa de 40:5:4 para larvas de eglefino (*Melanogrammus aeglefinus*). Bessonart *et al*. (1999) estudiaron el efecto de diferentes niveles de ARA para larvas de dorada sin cambiar la tasa de EPA y DHA, sugiriendo que la proporción de DHA/EPA/ARA óptima es de 1.4/0.8/1. Por todo ello, una parte del presente estudio fue realizada para investigar con más detalle el efecto de diferentes tasas dietéticas de DHA/EPA/ARA en un rango amplio en larvas de dorada y lubina alimentadas con microdietas.

10.1.5 - Importancia de las vitaminas antioxidantes en la nutrición lípidica

Los ácidos grasos altamente insaturados son muy propensos a la peroxidación. La peroxidación lipídica, es altamente dañina para las biomembranas celulares (Kanazawa, 1991, 1993) y subcelulares, como de las mitocondrias, causando varias condiciones patológicas en los peces (Kawatsu, 1969; Watanabe *et al*., 1970; Murai y Andrews, 1974; Sakai *et al*., 1989). La oxidación de los ácidos grasos altamente insaturados produce compuestos como hidroxiperóxidos, hidróxidos de ácidos grasos, aldehídos e hidrocarbonos que están implicados en varias condiciones patológicas en peces (Kawatsu, 1969; Watanabe *et al*., De 1970; Murai y Andrews, 1974; Sakai *et al*., 1989).

Los peces tienen un sistema endógeno de defensa antioxidante (Filho *et al*., 1993), las enzimas captadoras de radicales libres jugando un papel importante en la protección fisiológica antioxidante (Blazer, 1982). Estas enzimas, la catalasa y la superóxido dismutasa (SOD), actúan sobre el peróxido de hidrógeno (H_2O_2) y el superóxido (O_2^-), respectivamente, mientras que la glutatión peroxidasa (GPX), captura H_2O_2 e hidroperóxidos (Winston y Di Giulio, 1991; Halliwell y Gutteridge, 1996). Existe una variación en las actividades de defensa antioxidante enzimática en diferentes órganos de peces de agua dulce y marinos (Wdzieczak *et al*., 1982; Lemaire *et al*., 1993), dependiendo del comportamiento alimentario (Radi y Markovics, 1988), del período alimentario (Mourente *et al*., 2002), las condiciones ecológicas (Winston y Di Giulio, 1991) y el contenido de antioxidantes dietéticos. Las vitaminas E y C tienen una gran importancia como componentes exógenos en la dieta de peces de acuicultura, y actúan como enzimas de defensa antioxidante en la reducción de los productos de la peroxidación lipídica y los radicales libres (Machlin, 1984). Mourente *et al*. (2002) mostraron una reducción de los productos de peroxidación en el hígado de la dorada alimentada por dieta suplementada con aceite oxidado y vitamina E en comparación con los alimentados con dietas que carecen de esta vitamina.

Debido al alto riesgo de peroxidación de los ácidos grasos poliinsaturados, los requerimientos elevados en HUFA en las primeras etapas larvarias que conducen a altos niveles dietéticos de estos ácidos grasos, implican niveles elevados de compuestos antioxidantes, incluyendo vitaminas, con el fin de promover el máximo beneficio de estos nutrientes y evitar los problemas de oxidación lipídica que pueden causar

patologías, enfermedades y mortalidad posterior en particular en larvas de peces marinos.

10.1.6 - Vitamina E

La vitamina E es una de las vitaminas liposolubles que se absorben del tubo digestivo en asociación con las moléculas de grasa y pueden ser almacenadas en las reservas de grasa en el cuerpo. La vitamina E es un componente estructural de las membranas celulares (Comben y Putnam, 1987), funciona como un antioxidante liposoluble (Sargent *et al*., 1997) y mantiene la calidad del filete, la inmunidad, la resistencia normal de los glóbulos rojos a la hemolisis, y la permeabilidad de las capilares y el músculo cardíaco (Halver, 2002). La importancia de la vitamina E en la ruptura de la cadena del proceso antioxidante, juega un papel importante en los procesos biológicos (Burton *et al*., 1982; Burton y Ingold, 1989; Sies y Murphy, 1991). Numerosos estudios han demostrado que la vitamina E es esencial para distintas especies de peces (Watanabe *et al*., 1970; Murai y Andrews, 1974; González *et al*., 1995) y afecta a una gran rango de parámetros.

Varios estudios indican el papel predominante de la vitamina E en la defensa antioxidante en muchas especies de peces mejorando la estabilidad de los tejidos lípidicos a la oxidación, en trucha (*Oncorhynchus mykiss) (*Frigg *et al*., 1990), salmón del Atlántico (*Salmo salar)* (Waagbo *et al*., 1993), rodaballo (Stéphan *et al*., 1995) y lubina (Messager *et al*., 1992). La fragilidad eritrocitaria es uno de los parámetros importantes que se utiliza para determinar el estado de la vitamina E en peces (Halver, 1995). La vitamina E aumenta también la actividad no-específica del sistema inmunológico en la dorada (Montero *et al*., 1998). Además, la vitamina E y los n-3 HUFA dietéticos tienen un efecto sinérgico sobre la respuesta inmune no-específica y la resistencia a las enfermedades en la platija japonesa (*Paralichthys olivaceus*, Wang *et al*., 2006). Bajo estrés agudo y crónico, juveniles de dorada alimentados con una dieta carente de vitamina E mostraron una reducción en el crecimiento y la supervivencia (Montero *et al*., 2001), así como una menor resistencia al estrés.

Tocher *et al*. (2003) también han demostrado que el suplemento con vitamina E mejora el crecimiento en la dorada alimentada con dieta suplementada con aceite oxidado, y reduce los productos de la peroxidación lipídica en dorada y rodaballo. Estos autores sugirieron que el sistema antioxidante está influenciado por la especie, etapa de

desarrollo y de la temperatura de cultivo. Por último, el aumento de vitamina E en la dieta mejora la viabilidad de huevos y el porcentaje de huevos anormales en la dorada (Fernández-Palacios *et al*., 1998).

Los requerimientos dietéticos en la vitamina E se han estudiado en una serie de peces (NRC, 1993) que incluyen 120 mg / kg dieta (Hamre y Lie, 1995a) para el salmón atlántico, de 30 a 50 mg / kg dieta para el bagre de canal *(Ictalurus punctatus*, Murai y Andrews, 1974; Wilson *et al*., 1984) y de 200 a 300 mg / kg dieta para la carpa común (*Cyprinus carpio,* Watanabe *et al*., 1977), 1200 mg / kg (Ortuño *et al*., 2000) para la dorada; 99 mg / kg para alevines de Mrigal (*Cirrhinus mrigala* ,Paul *et al*., 2004). El nivel de 250 mg de α-tocoferol / kg dieta es suficiente para cumplir los requerimientos para el éxito de la reproducción (Fernández-Palacios *et al*., 1998).

En larvas, el mayor requerimiento en HUFA debe ser también asociado con mayores niveles dietéticos de vitamina E (Sargent *et al*., 1997) como se sugiere en la carpa (Watanabe *et al*., 1981; Schwarz *et al*., 1988) y salmón del Atlántico (Hamre y Lie, 1995b). Las larvas de dorada han mostrado un creciente contenido de vitamina E desde la incubación hasta el día 10, mientras que desde esta edad y hasta el día 20, se observó una disminución de la vitamina E (Izquierdo y Fernández-Palacios, 1997). Esta reducción fue paralela a la reducción en el contenido de los ácidos grasos poliinsaturados en larvas a lo largo del desarrollo larvario (Izquierdo, 1988), lo que sugiere una estrecha relación entre ambos nutrientes (Woodall *et al*., 1964; Watanabe *et al*., 1970; Izquierdo y Fernández-Palacios, 1997).

En larvas de dorada González *et al*. (1995) han probado un rango de 22,27-780 mg a-tocoferol / kg microdietas y encontraron que la elevación dietética de la vitamina E hasta un máximo de 136 mg / kg mejora el crecimiento y la supervivencia, mientras que al superar este nivel se reduce considerablemente la supervivencia de las larvas.

10.1.7 - Vitamina C

La vitamina C (AA) es una vitamina muy importante en peces (Kitamura *et al*., 1965; Mulero *et al*., 1998; Kolkovski *et al*., 2000; Ruff *et al*., 2001; Ortuño *et al*., 2003;). Las larvas de peces son particularmente sensibles a la deficiencia en vitamina C (Dabrowski *et al*., 1996). Su rápida tasa de crecimiento sugiere que las larvas tienen mayores requerimientos de vitamina que los juveniles y adultos (Dabrowski *et al*., 1988; Dabrowski, 1990; Kolkovski *et al*., 2000). La adición de la AA en dietas para

larvas mejora la supervivencia, crecimiento, desarrollo esqueleto, la resistencia al estrés y la respuesta inmune (Al-Amoudi *et al*., 1992; Roberts *et al*., 1995; Merchie *et al*., 1996; Montero *et al*., 1999; Kolkovski *et al*., 2000; Anbarasu y Chandran, de 2001; Ortuno *et al*., 2001; Ai *et al*., De 2004; Lin y Shiau, 2005).

Debido a la baja estabilidad del ácido ascórbico, hasta el 50% de la vitamina se puede perder en el agua en los primeros 10 s después de la alimentación, aproximadamente 50% es destruida durante la extrusión y el 30% durante la granulación (Lim y Lovell, 1978). Las formas más estables de la vitamina C, así como suplementos con niveles más altos se incluyen en la dieta de peces.

El nivel de los requerimientos en AA depende de la especie, del tamaño, la etapa de la madurez sexual, la tasa de crecimiento (Boonyaratpalin, 1997), la dieta, las condiciones experimentales y también podría estar basada en la condición de la vitamina almacenada en el cuerpo, como la concentración hepática de AA (Fournier, *et al*., 2000). El ácido ascórbico suministrado en la dieta es crucial para el desarrollo larvario de peces, puesto que la mayoría de las especies no son capaces de sintetizar esta vitamina (Gouillou-Coustans *et al*., 1998). Los requerimientos en (AA) parecen disminuir con el aumento del tamaño del pez (Boonyaratpalin *et al*., 1989b). El suplemento dietético inadecuado del AA resulta en un número de síntomas de deficiencia como la deformación de la columna vertebral, alteración de la formación de colágeno, hemorragias internas, retraso en el crecimiento e inmuno depresión (Halver *et al*., 1969; Al-Amoudi *et al*., 1992; Gouillou-Coustans *et al*., 1998). Cuando se alimenta con dietas deficientes en vitamina C se obtiene un bajo apetito en los peces, crecimiento deficiente, coloración oscura, erosión en las aletas, patología en los arcos branquiales; hemorragias branquiales y pérdida del equilibrio (Dabrowski *et al*., 1988; Boonyaratpalin, 1997). En los peces loro (*Oplegnathus fasciatus*) que fueron alimentados con la dieta sin AA se encontró una mortalidad total en la séptima semana de alimentación (Wang *et al*., 2003).

Los estudios de los requerimientos en AA necesarios para peces marinos son escasos (Boonyaratpalin *et al*., 1992; Saroglia Scarano y 1992; NRC 1993; Teshima *et al*., 1993) y principalmente basados en especies de agua dulce (NRC 1993). Varios estudios sugirieron que un requerimiento en AA de 200 mg / kg en la lubina europea para el crecimiento normal y la saturación hepática (Saroglia y Scarano, 1992); hasta 1600 µg / g d.w. *Artemia* en larvas del bagre africano (*Clarias gariepinus*) (Merchie *et al*., 1997), mientras 1400 µg / g d.w. *Artemia* es suficiente para la óptima concentración

de AA en el tejido y el desarrollo larvario (Merchie *et al*., 1995). Para la dorada de 150 g el aumento de AA de 500 a 3000 mg / kg aumenta la actividad de las respuestas del sistema inmune no especifico de dorada (Ortuño *et al*., 1999), mientras en la dorada de 9g el aumento de AA de hasta 200 mg / kg mejora significativamente la eficiencia proteíca (Henrique *et al*., 1998), y para alevines de 0,5 g alimentados con dieta sin AA se observó una elevada mortalidad en comparación con dietas suplementadas con AA de 50 a 3200 mg / kg (Alexis *et al*., 1997). Para juveniles de lubina europea, 121 mg de AA / kg parece ser el nivel adecuado (Fournier *et al*., 2000). Para juveniles de Chancharro coreano (*Sebastes schlegeli)* 103 mg de AA/ kg en la dieta parece ser suficiente para cubrir el requerimiento; para larvas de carpa común el nivel 45 mg AA/ kg dieta mejorar el crecimiento, mientras más de 270 mg/kg dieta aumenta la concentración corporal de vitamina C (Gouillou-Coustans *et al*., 1998); para juveniles de grouper *Epinephelus malabaricus* 45,3 mg AA/kg dieta es suficiente para un crecimiento óptimo, mientras que se necesita 6 veces mas para mejorar la respuesta inmunitaria no-específica y mantener la supervivencia de los peces infectados con bacterias (Lin *et al*., 2005). Para la lubina japonesa se ha estimado el nivel de 53,5 mg AA/ kg para mejorar el crecimiento, 93,4 mg AA/ kg basado en la concentración del hígado y 207,2 mg AA/ kg basado en la concentración de los músculos (Ai *et al*., 2004); para peces de pez loro (*Oplegnathus fasciatus*) se estimó 118 ± 12 mg AA / kg para un crecimiento máximo (Wang *et al*., 2003). Para alevines de lubina asiática (*Lates calcarifer)* cultivadas en agua dulce durante 10 semanas, se ha estimado 1000 mg/kg o superior como el nivel óptimo para el mejor crecimiento (Boonyaratpalin *et al*., 1989a). Para larvas de mrigal (*Cirrhina mrigala*) el nivel óptimo de los requerimientos se encuentran entre 650-700 mg AA / kg, basado en el aumento de peso, la mortalidad o del comportamiento y criterios morfológicos (Mahajan y Agrawal 1980).

10.1.8 - Relación vitamina E y C

El ácido ascórbico, juega un papel sinérgico con la vitamina E en el mantenimiento de la actividad de la glutation peroxidasa y la superóxido dismutasa (Le Grusse y Watier, 1993). La vitamina C y la vitamina E funcionan como antioxidantes biológicos, para proteger, a las macromoléculas celulares (ADN, proteínas, lípidos) y otras moléculas antioxidantes, de la oxidación incontrolada por radicales libres durante el metabolismo normal o bajo condiciones oxidativas tales como infecciones, estrés o

contaminación (Chen *et al*., 2004). El beneficio de la combinación de vitaminas E y C en el crecimiento y en los parámetros relacionados con las peces se ha estudiado en pocas especies de peces (Martínez de Chávez , 1990; Roem *et al*., 1990; Thorarinsson *et al*., 1994; Chien *et al*., 1999). Por lo tanto, debido a su potencial de interacción, los requerimientos dietéticos de las vitaminas C y E han sido considerados a veces conjuntamente (Moreau *et al*., 1999; Dabrowski y Lee, 2003).

El ácido ascórbico parece jugar un papel importante en el metabolismo de α-tocoferol. Varios estudios (Cort, 1974; Packer *et al*., 1979; Niki, 1987a, b) demostraron la capacidad de AA para reducir los radicales α-tocopheroxyl y de este modo regenerar el α-tocoferol. Así, la concentración hepática de vitamina E se protege con el aumento dietético de la vitamina C (Sealey y Gatlin, 2002). Sin embargo, un nivel constante de la vitamina C y niveles altos de vitamina E reducen la vitamina A hepática en la trucha arco iris (Furones *et al*., 1992). Estos resultados contradictorios podrían estar relacionados con un exceso en el nivel de una o ambas vitaminas y denota la importancia de determinar específicamente sus necesidades teniendo en cuenta los niveles de cada uno de estos nutrientes en la dieta. Además, sus requerimientos para las larvas de peces marinos son en gran medida desconocidos.

10.1.9 - Objetivos

Considerando tanto la importancia de la cantidad como de las proporciones de DHA, EPA y ARA y teniendo en cuanta el papel que juegan la vitamina E y C, como los antioxidantes dietéticos más importantes de estos ácidos grasos, el presente estudio tiene como objetivo general determinar los valores y las relaciones óptimas de DHA/EPA/ARA y de Vitamina E y C en larvas de peces marinos, en concreto para larvas de dorada y lubina alimentadas con microdietas. Para alcanzar este objetivo general, se definieron una serie de objetivos concretos:

1- Determinar el efecto de un amplio rango de relaciones de EPA / DHA en dieta sobre el desarrollo larvario, con el fin de entender mejor la importancia de estos ácidos grasos y las consecuencias de su implementación en la dieta.
2- Determinar el valor nutritivo del ARA, en microdietas en larvas de peces marinos, y la relación con los niveles de EPA / ARA dietético, determinando sus efectos sobre la supervivencia, el crecimiento y la resistencia al estrés.

3- Contribuir al conocimiento actual sobre la incorporación de ácidos grasos esenciales dietéticos en la composición lipídica de las larvas, en relación con sus valores relativos en dieta, principalmente EPA / DHA y EPA / ARA y sus consecuencias sobre la producción de larvas de peces.
4- Entender mejor la relación entre las necesidades larvarias en ácidos grasos esenciales y su relación con los niveles de vitamina E, determinando los efectos dietéticos de vitamina E en la cría larvaria de dorada y lubina.
5- Dilucidar el efecto de protección o daños asociados al uso dietetico de altas dosis de vitamina E, estudiando su efecto sobre las composiciones de ácidos grasos en los lípidos larvarios, asi como sus consecuencias en el crecimiento y la supervivencia.
6- Determinar si el aumento del contenido de vitamina E, en las dietas de larvas, debe estar asociado a un incremento en el contenido dietético de vitamina C y evaluar la importancia nutritiva de la Vitamina C en el rendimiento y la producción de larvas de peces.

Table. 10.1. Relación óptima de EPA/DHA, DHA/EPA, DHA/EPA/ARA y ARA para las diferentes especies de peces

Especie	**Ingrediente**	**El tamaño del pez**	**Parámetro Probado**	**Requisito dietético**	**Autor**
Sparus aurata	EPA/DHA	Juveniles	mejor crecimiento y el más bajo índice hepatosomático	2/1	Ibeas *et al*., 1997
Melanogrammus aeglefinus	DHA/EPA/ARA	Larva	optima	40:5:4	Castell *et al*., 2001
Limanda ferruginea	DHA/EPA	Larva	aumento en el crecimiento y la supervivencia	8:1	Copeman *et al*., 2002
Paralichthys oliuaceus	EPA/DHA	Larva	éxito en la pigmentación dorsal	menos de 4	McEvoy *et al*., 1998
Scophthalmus maximus	EPA/DHA	Larva	optima	sobre 2	Reitan *et al*.,1994
Sparus aurata	EPA/DHA	Larva	mejora el crecimiento	1/1.3	Rodríguez *et al*.,1997
Sparus aurata	EPA/DHA	Larva	mejora el crecimiento	1/1.5	Rodríguez, 1994
Marine fish	DHA/EPA/ARA	Larva	optima	10:5:1	Sargent *et al*.,1999b
Paralichthys dentatus	ARA	Larva	mayor crecimiento, supervivencia y tolerancia al estrés	mas de 6%	Willey *et al*., 2001

Table 10.2. Óptimo de vitamina E para las diferentes especies de peces

Especie	**Ingrediente**	**El tamaño del pez**	**Parámetro Probado**	**Requisito dietético**	**Autor**
Sebastes schlegeli	Vitamina E	Juveniles	buen crecimiento	mas de 45 mg/kg	Bai and Lee, 1998
Ictalurus punctatus	Vitamina E	-------	requerimiento	50 mg/kg	Murai and Andrews, 1974
Cirrhina mrigala	Vitamina E	alevines	mejora el crecimiento	99 mg/kg	Paul *et al*., 2004
Epinephelus malabaricus	vitaimina E	Juveniles	crecimiento	≥100 mg/kg	Lin and Shiau, 2005a
Salmo salar	Vitamina E	Larva	requerimiento	120mg/kg	Hamre and Lie, 1995a
Labeo rohita	Vitamina E	Alevines	crecimiento	hasta 131.91 mg/kg	Sau *et al*., 2004
Sparus aurata	Vitamina E	Larva	mejor crecimiento y supervivencia	136 mg/kg	González *et al*., 1995
Cyprinus carpio	Vitamina E	----------	----------	300 mg/kg	Watanabe *et al*., 1977
Latris lineata	Vitamina E	Larva	mejora el crecimiento	mas 437 mg/kg	Brown *et al*., 2005
Sparus aurata	Vitamina E	Juveniles	mejor crecimiento y supervivencia	hasta 1000 mg/kg	Tocher *et al*., 2002
Sparus aurata	Vitamina E	Alevines	sistema immuno no-específica	1200 mg/kg	Ortuño *et al*., 2000
Sparus aurata	Vitamina E	Reproductores	requerimiento	250 mg/kg	Izquierdo *et al*, 2001a

Tabla 10.3. Óptimo de vitamina C para las diferentes especies de peces

Especie	**Ingrediente**	**El tamaño del pez**	**Parámetro Probado**	**Requisito dietético**	**Autor**
Cyprinus carpio	Vitamina C	Larva	mejora el crecimiento	45 mg/kg	Gouillou-Coustans *et al.,* 1998
Epinephelus malabaricus	Vitamina C	Juveniles	mejora el crecimiento	45.3 mg AA/kg	Lin and Shiau, 2005a
Sebastes schlegeli	Vitamina C	Juveniles	mejora el crecimiento	103 mg AA/kg	Montero *et al*., 1998
Oplegnathus fasciatus	Vitamina C		optima crecimiento	118 ±12 mg AA/kg	Wang *et al.,* 2003
Dicentrarchus labrax	Vitamina C	----------	crecimiento normal	121mg/kg	Fournier *et al*., 2000
Sparus aurata	Vitamina C	Alevines	mejora el Razón de Eficiencia Proteica	200 mg/kg	Henrique *et al.,* 1998
Dicentrarchus labrax	Vitamina C	---------	crecimiento normal	200 mg	Saroglia and Scarano, 1992
Cyprinus carpio	Vitamina C	Larva	concentración máxima de vitamina C in el cuerpo	270 mg/kg	Gouillou-Coustans *et al.,* 1998
Epinephelus malabaricus	Vitamina C	Juveniles	mejorar la respuesta inmunitaria no-específica y promover la supervivencia de los peces infectados con bacterias.	271.8 mg AA/kg	Lin and Shiau, 2005a
Lutes calcarifer	Vitamina C	Alevines	requerimiento	500mg/kg	Boonyaratpalin *et al.,* 1989b
Cirrhina mrigala	Vitamina C	Larva	basado en el aumento de peso, la mortalidad o de comportamiento y criterios morfológicos	650–700 mg AA/kg	Mahajan and Agrawal, 1980
Lutes calcarifer	Vitamina C	Alevines	requerimiento	1000mg/kg	Boonyaratpalin *et al.,* 1989a

Clarias gariepinus	Vitamina C	Larva	mejora el crecimiento	1600 mg/kg d.w. in *Artemia*	Merchie *et al.,* 1997
Scophtalmus maximus	Vitamina C	Juveniles	mejorar la respuesta inmunitaria no-específica	2000mg/kg	Robers *et al*., 1995
Sparus aurata	Vitamina C	Juveniles	aumenta la actividad de la respuesta inmunitaria no-específica	aumentar de 500 a 3000 mg / kg	Ortuño *et al.,* 1999
Sparus aurata	Vitamina C	Juveniles	mejora el Sistema de inmunitaria	2.9g/kg	Cuesta *et al.,* 2002

10.2. Materiales y métodos

10.2.1.Peces

10.2.1.1. Dorada

Las larvas de dorada (*Sparus aurata*) fueron obtenidas de puestas naturales de reproductores existentes en las instalaciones del GIA (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, España) en donde se llevaron a cabo los experimentos de la presente tesis. En los experimentos de dorada, las larvas fueron previamente alimentadas con rotíferos (*Brachionus plicatilis*) enriquecidos con Selco (DHA de Proteínas Selco, INVE, Dendermonde, Bélgica) hasta que llegaron a 18 días de edad. Al día 18 las larvas fueron distribuidas al azar en los tanques experimentales y fueron alimentadas con una de las dietas experimentales ensayadas por triplicado.

10.2.1.2. Lubina

Las larvas de Lubina (*Dicentrarchus labrax*) fueron obtenidas de la puesta natural de la empresa Ecloserie Marina de Gravelines (Gravelines, Francia). Las larvas fueron previamente alimentadas con rotíferos (*Brachionus plicatilis*) enriquecido por Selco (DHA de Proteínas Selco, INVE, Dendermonde, Bélgica) seguido de la microdieta comercial (Microdieta Comercial, Skretting, Noruega) hasta que llegaron a 18 y 35 días de edad.

10.2.2. Condiciones experimentales

Todos los experimentos y análisis del laboratorio presentados en la tesis se llevaron a cabo en las instalaciones de la GIA (ICCM & IUSA), Islas Canarias, España, entre 2005 y 2007.

Los tanques fueron provistos con agua de mar (alrededor de 37 ppm de salinidad) filtrada por malla de 50 μm de luz. La intensidad de la luz se mantuvo a 1700 lux (Lux digital Tester YF-1065, Powertech, Australia Occidental, Australia). La temperatura y el oxígeno se midieron diariamente utilizando el aparato Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, EE.UU.). Los tanques se limpiaron diariamente de manera manual entre las 18:00 y las 20:00, con una manguera por un sistema de sifón.

Los tanques (170 l, cilíndricos de fibra de vidrio, con color gris claro, Fig. 1) fueron suministrados con agua de mar filtrada y previamente almacenada en un tanque

de 500 l para la desgasificación. Los tanques fueron sometidos a una circulación abierta del agua que fluye a diferentes tasas y que fueron incrementadas a lo largo de los ensayos alimentarios. La calidad del agua fue comprobada diariamente y no se observó deterioro. El agua era continuamente aireada (125ml/min). La temperatura del agua y el oxígeno disuelto se registraron diariamente a las 15:00 y estuvo entre 5-8 ppm y la saturación osciló entre el 60 y el 80% en todos los tanques experimentales. El fotoperíodo se mantuvo en 12h luz: 12h oscuridad mediante luces fluorescentes.

10.2.3. Dietas y alimentación

10.2.3.1. Formulación de las dietas

Varias microdietas experimentales (tamaño <250 μm), isoproteicas e isolipídicas (16,8-20.56 y 70.01-71.1 proteínas y lípidos respectivamente) con diferentes tasas de DHA, EPA, ARA, vitamina E ó vitamina C, fueron formuladas en los diferentes experimentos usando los aceites EPA50 y DHA50, en forma de triglicéridos como fuente de EPA y DHA (CRODA, East Yorkshire, Inglaterra, Reino Unido), DHA45, EPA45 y ARA44 (Polaris, Pleuven, Francia) como una fuente de DHA, EPA and ARA, y la vitamina E (Sigma-Aldrich, Madrid, España) y la vitamina C (ROVIMIX Estancia - C-35, Roche, París, Francia). El contenido de lípidos deseado se completó con una fuente de ácidos grasos no esenciales (ácido oleico, Merck, Darmstadt, Alemania). Se utilizó harina de calamar (Riber & Son, Bergen, Noruega) como fuente proteica, la cual fue desengrasada (3 veces consecutivas con una tasa cloroformo: harina de 3:1) para permitir un mejor control del perfil de ácidos grasos de la misma. Los perfiles de los ácidos grasos de la harina de calamar desengrasada (2,6% del contenido en lípidos), DHA50, DHA45, EPA50, EPA 45 y ARA44 se muestran en la Tabla 1.

10.2.3.2. Preparación de microdietas

Las microdietas fueron preparadas de la forma siguiente: la harina de calamar fue cuidadosamente mezclada con los otros ingredientes hidrosolubles (atractantes, minerales y vitaminas hidrosolubles como se muestra en la Tabla 2, Sigma-Aldrich, Madrid, España), en un mortero. A parte, los aceites y las vitaminas liposolubles fueron mezclados para obtener una mezcla homogénea, que fue luego añadida a la mezcla de polvo. A continuación, se disolvió la gelatina en agua caliente y cuando su temperatura fue inferior a 35 º C se añadió al resto de los ingredientes previamente mezclados. La

pasta formada se moldea en cuerdas (Severin, Suderm, Alemania) se seca en una estufa (Ako, Barcelona, España) a 38 º C durante 24 h. Por último, se pulveriza (Braun, Kronberg, Alemania) y se tamiza (Filtra, Barcelona, España) para obtener el tamaño deseado de las partículas. Se realizó el análisis proximal y la composición en ácidos grasos de las dietas en peso seco y cada dieta se ensayó por triplicado.

10.2.4. Alimentación de las larvas

Las dietas fueron suministradas manualmente, catorce veces al día cada 45 minutos de 9:00 a 19:00 horas. En el experimento realizado con larvas de dorada, que fueron más jóvenes que las larvas de lubina, los rotíferos fueron alimentados con levadura de pan durante al menos 7 días para evitar cualquier contenido de n-3 HUFA. Se añadieron a la alimentación dos veces al día (a las 12:00 y a las 16:00 h), en una proporción fija de 2 ind/ml al inicio del experimento, que fue progresivamente reducido hasta 1 ind/ml al final del experimento.

Para garantizar la disponibilidad del alimento su suministro diario se mantuvo en el 2 y 2,5 g / tanque durante la primera y segunda semana de la alimentación, respectivamente. Después de alimentar, las larvas se observaron bajo el microscopio binocular para determinar la aceptación de la comida. Se observaron si había diferencias en el consumo del alimento a lo largo de las diferentes dietas experimentales y la aceptación de la dieta se determinó por el cálculo del porcentaje de ocupación de microdieta en el intestino por análisis de imagen en fotografías del digestivo de las larvas. Para este tipo de estudio, se tomaron 30 larvas por tanque, se observó su cavidad abdominal en una lupa binocular (Leica Wild M3Z, Optotek, California, EE.UU.) y se midió la zona del intestino ocupado por el material digerido en microfotografías a una magnificación de 25 μm, utilizando Image Pro Plus ® (Media Cibernética inc., Silver Springs, MD, EE.UU.) con un sistema semiautomático de análisis de imagen.

10.2.5. Muestreo

Al inicio, en el medio y al final de cada experimento se tomaron muestras de larvas, las cuales fueron se mantuvieron en ayuno durante la noche, para determinar la longitud total y el peso corporal. Al final del experimento, las larvas vivas fueron muestreadas para la prueba de actividad. Por último, para analizar la composición bioquímica, el resto de las larvas de cada tanque, después del ayuno de 12 h, se

recogieron, se lavaron con agua destilada, muestrearon y se guardaron a -80 º C en un ambiente sin aire, las muestras fueron etiquetadas individualmente en bolsas de plástico hasta sus análisis.

10.2.6. La prueba de actividad y la supervivencia

Antes del final del experimento, la prueba de actividad fue llevada a cabo, sacando 20 larvas / tanque del agua en una red durante 1 min y posteriormente, se colocaron en un recipiente que contenía agua de mar con aireación, para determinar la supervivencia después de 24 horas. Otra prueba de actividad se realizó con 20 larvas / tanque que se trasladaron a tanques de agua de mar a 15 ºC, para determinar la supervivencia después de 24 horas. La supervivencia final se calculó individualmente contando todas las larvas vivas al principio y al final del experimento.

10.2.7. Evaluación del crecimiento

El crecimiento fue determinado midiendo el peso seco corporal y la longitud de las larvas en inanición. El peso corporal se determinó por 4-3 repeticiones de 5-10 larvas en inanición lavadas con agua destilada y secadas en un porta objetos de vidrio en una estufa a 110 º C hasta peso constante, durante aproximadamente 24 h, seguido de períodos de 1 hora. La longitud total o estándar de 20-30 larvas anestesiadas de cada tanque se midió en un proyector de perfil (V-12A Nikon, Nikon Co, Tokio, Japón).

La tasa de crecimiento específica (SGR) se determinó según la ecuación:

SGR = ((ln peso corporal al final) - (ln peso corporal al inicial)) x 100 / t

Donde t = días del período la alimentación.

10.2.8. Análisis bioquímicos

Todas las muestras de larvas y de las dietas fueron sacadas del congelador -80 ºC, descongeladas y homogeneizadas en el interior de las mismas bolsas de las muestras para evitar la evaporación de agua.

10.2.8.1. La humedad

El contenido de humedad se determinó por secado térmico hasta un peso constante en horno a 110 º C, con un primer período de secado de 24 horas, seguido por períodos de 1 h hasta llegar a un peso constante. El peso de la muestra se registró antes del secado y después de cada período de secado, después de la refrigeración en un desecador para pesar siempre a la misma temperatura. La humedad se expresó como un porcentaje del peso de acuerdo a los Métodos Oficiales de Análisis AOAC, (1995), mediante la siguiente ecuación:

$$\text{Contenido de humedad \%} = (100\,(B - A) - (C - A)) / (B - A)$$

Dónde:

A = peso del matraz vacío

B = peso del matraz + muestra húmeda

C = peso seco de la muestra + matraz

10.2.8.2. Proteínas

El análisis de la proteína fue hecho de acuerdo con el método Kjeldahl (A.O.A.C., 1995), que mide el contenido de nitrógeno total en la muestra, y la conversión de esta cifra a un valor de la proteína cruda total multiplicando por el factor empírico de 6,25.

10.2.8.3. Total de lípidos

Los lípidos se extrajeron según el método de Folch *et al*. (1957). Se pesó una cantidad de muestra de las dietas y las larvas enteras (0,1-0,2g), se homogenizó en 10 ml de una mezcla de cloroformo: metanol (2:1 v: v) en ultra turrax durante 5 min. Luego los lípidos fueron separados por centrifugación durante 5 min (2000 rpm), la fase inferior de cloroformo que contiene los lípidos fue cuidadosamente aspirado y evaporada para obtener el peso de los lípidos.

10.2.8.4. La separación de los lípidos polares

Las fracciones de lípidos neutros y polares de los lípidos totales de las larvas fueron separados por cromatografía de adsorción en cartuchos de sílice (Sep-pak; Water S.A., Massachusetts, EE.UU.), utilizando 30 ml de cloroformo y 20 ml de cloroformo / metanol (49: 1, v / v) como disolvente de los lípidos neutros, seguido por un lavado de 30 ml de metanol para obtener las fracciones polares según el método de Juaneda y Rocquelin (1985).

10.2.8.5. Preparación y cuantificación de ésteres metílicos de ácidos grasos

Los lípidos polares o totales se disolvieron en tolueno y los ésteres metílicos de los ácidos grasos fueron obtenidos por transmetilación con 1% de ácido sulfúrico en metanol (Christie, 1982). La reacción se llevó a cabo en la oscuridad durante 16h a 50 º C, en atmósfera de nitrógeno. Después, los ésteres metílicos de los ácidos grasos se extrajeron con hexano: éter dietílico (1:1 v / v) y purificados por cromatografía de adsorción en cartuchos NH2 Sep-pack (Water S.A., Massachusetts, EE.UU.) (Christie, 1982). Los ésteres metílicos de ácidos grasos fueron separados por GLC (GC-14A, Shimadzu, Tokio, Japón) en una columna capilar de sílice (Supercolvax-10) (longitud 30 m, diámetro interior 0,32 mm; Supelco, Bellefonte, EE.UU.) utilizando helio como gas portador. La temperatura de la columna fue de 180 º C durante los primeros 10 minutos, aumentando a 215 º C a razón de 2,5 º C / min y luego se fijo en 215 º C durante 10 min, según las condiciones descritas por Izquierdo *et al*. (1990). Los ésteres metílicos de ácidos grasos se han cuantificado con FID e identificados por comparación con estándares externos y aceites de pescado bien caracterizados (EPA 28, Nippai, Ltd Tokio, Japón).

10.2.9. Análisis estadístico

La media y las desviaciones estándar se calcularon para cada parámetro medido. Todos los datos fueron pasados a una por prueba de normalidad y la transformación se hizo cuando los datos no eran normales. Las diferencias entre grupos se determinaron mediante ANOVA de una vía y las medias se compararon mediante la prueba de

Duncan (P <0,05) usando el programa SPSS (SPSS 11,5 para Windows; SPSS Inc, Chicago, IL, EE.UU.). Para el análisis de dos-vías para el crecimiento (peso, longitud, SGR, de la biomasa), la normalidad y la homogeneidad de varianza se verificó, y se utilizó el siguiente modelo lineal general

$$Y_{ijk} = \mu^* + F_i + H_j + (FH)_{ij} + \varepsilon_{ijk}$$

Donde Y_{ijk} es el valor medio de tanque, μ es la media de la población, F_i es el efecto fijo de un factor (e.g. vitamina), H_j es el efecto fijo de otro factor (e.g. PUFA), $(FH)_{ij}$ la interacción entre los efectos fijos, y ε_{ijk} es el error residual.

Tabla 10.4. Principales ácidos grasos dietéticos de las principales fuentes lípidicas utilizadas en los experimentos

	DHA 50	EPA 50	DHA 45	EPA 45	ARA 44	Harina de calamar desengrasado
14:0	0,031	0,042	0,361	1,196	0,428	1,685
14:1n-5	0,202	0,245	n.d.	n.d.	n.d.	0,036
14:1n-7	0,003	0,011	n.d.	0,053	n.d.	0,026
15:0	0,004	0,009	0,042	0,159	0,133	n.d.
15:1n-5	0,005	n.d.	n.d.	n.d.	n.d.	0,036
16:0iso	0,008	0,016	0,047	0,030	n.d.	0,108
16:0	1,214	0,362	3,226	5,151	8,250	23,808
16:1n-9	0,000	n.d.	n.d.	n.d.	n.d.	0,02
16:1n-7	0,472	0,427	1,099	2,000	0,375	0,517
Me 16:0	0,010	0,011	n.d	n.d.	n.d.	0,097
16:1n-5	0,020	n.d.	n.d.	0,048	n.d.	0,163
16:2n-6	n.d.*	0,054	0,095	0,122	n.d.	0,06
16:2n-4	0,158	0,494	n.d.	0,041	n.d.	0,485
17:0	0,121	n.d.	0,218	0,407	0,228	0,056
16:3n-4	0,097	0,212	0,326	0,375	0,038	0,072
16:3n-1	0,060	0,145	0,254	0,455	n.d.	0,116
16:4n-3	n.d.	0,002	0,133	0,112	n.d.	0,381
16:4n-1	n.d.	0,326	0,251	0,380	n.d.	0,034
18:0	2,316	0,271	5,749	2,652	6,929	3,639
18:1n-9	4,921	1,812	10,061	6,417	13,652	1,735
18:1n-7	0,870	0,555	4,149	1,329	0,779	0,941
18:1n-5	0,043	n.d.	0,109	0,068	n.d.	0,238
18:2n-9	n.d.	0,445	0,292	0,045	0,063	n.d.
18:2n-6	0,588	2,159	1,213	0,853	9,069	0,241
18:2n-4	0,074	0,679	0,580	0,122	n.d.	n.d.
18:3n-6	0,342	0,734	0,447	0,279	n.d.	n.d.
18:3n-4	0,038	0,439	n.d.	n.d.	0,238	n.d.
18:3n-3	0,204	1,670	0,769	0,313	2,568	n.d.
18:3n-1	n.d.	0,317	3,506	0,873	n.d.	n.d.
18:4n-3	0,312	10,441	0,429	0,055	n.d.	0,066
18:4n-1	0,001	1,007	n.d.	0,021	1,014	0,002
20:0	0,727	0,417	1,175	0,447	n.d.	0,004
20:1n9+n7	3,985	0,395	3,361	2,650	0,724	2,804
20:1n-5	n.d.	n.d.	0,371	0,242	n.d.	n.d.
20:2n-9	0,071	0,410	0,721	0,060	n.d.	n.d.
20:2n-6	0,543	0,128	0,256	0,293	n.d.	0,197
20:3n-9 +7	0,216	0,720	0,522	0,072	0,454	n.d.
20:3n-6	n.d.	n.d.	0,033	0,078	4,041	n.d.
20:4n-6	2,456	3,494	2,094	1,531	50,354	0,493
20:3n-3	0,443	0,114	0,178	0,179	n.d.	0,344
20:4n-3	0,916	2,486	2,245	0,661	n.d.	0,062
20:5n-3	12,662	46,483	43,232	15,582	0,113	4,315
22:1n-11	2,298	0,313	0,355	2,369	n.d.	0,041
22:1n-9	1,208	0,737	0,352	0,501	n.d.	0,106
22:4n-6	2,843	0,490	0,257	1,590	n.d.	0,126
22:5n-6	0,190	0,000	0,088	0,278	n.d.	n.d.
22:5n-3	5,527	3,209	2,057	3,609	0,124	n.d.
22:6n-3	53,803	17,721	9,345	46,304	0,426	9,397

*n.d.=not detected.

Tabla 10.5. Mezcla de atractantes, minerales y vitaminas

Atractantes		mg/100g MD
	Inosina-5-monofosfato	500
	Betaína	660
	L-Serina	170
	L-Tirosina	170
	L-Fenilalanina	250
	DL-Alanina	500
	L-Aspartato sodico	330
	L-Valina	250
	Glicina	170
	Total	3000,000
VITAMINAS		
Vitaminas hidrosolubles		mg/100g MD
	Cianocobalmina	0,030
	Astaxantina	5,000
	Acido Fólico	5,440
	Piridoxina-HCl	17,280
	Tiamina-HCl	21,770
	Riboflavina	72,530
	Pantotenato de calcio	101,590
	Acido p-aminobenzoico	145,000
	Ascorbil- polifosfato(V.C)	180,000
	Acido nicotínico	290,160
	Inositol	1450,900
	Sub Total	2289,700
Vitaminas Liposolubles		mg/100g MD
	Retinol acetato	0,180
	Ergocalciferol	3,650
	Manadiona	17,280
	α-Tocoferol acetato	150,000
	Sub total	171,110
Al final de la mezcla		
	Cloruro de colina	2965,800
	Total	5426,610
Minerales		mg/100g MD
	NaCl	215,133
	MgSO4.7H2O	677,545
	NaH2PO4.H2O	381,453
	K2HPO4	758,949
	Ca(H2PO4).2H2O	671,610
	FeC6H5O7	146,884
	C3H5O3.1/2Ca	1617,210
	Al2(SO4)3.6H2O	0,693
	ZnSO4.7H2O	14,837
	CuSO4.5H2O	1,247
	MnSO4.H2O	2,998
	KI	0,742
	CoSO4.7H2O	10,706
	Total	4500,007

Fig. 10.1. Tanques usados para el cultivo larvario durante los experimentos.

Fig. 10.2. Microdieta formulada y preparada para los experimentos larvarios.

10.3 Resultados y discusión

10.3.1 EFECTO DE DIFERENTES PROPORCIONES DE EPA/DHA DIETÉTICO EN LA INCORPORACIÓN DE ÁCIDOS GRASOS ESENCIALES EN LARVAS DE DORADA (*Sparus aurata*)

Se observo una correlación significativa entre los contenidos dietéticos de EPA y las tasas de la supervivencia, los valores más altos se obtuvieron en las larvas alimentadas con las dietas 4/1 y 4/3 (25,67 % y 27,59 % respectivamente). Además de con el nivel de EPA, la supervivencia larvaria también se incrementó con los niveles de DHA. Por tanto, el aumento de niveles dietéticos de n-3 HUFA se asoció con una mayor supervivencia larvaria. La resistencia larvaria frente a un estrés de manipulación fue mayor con la dieta que tiene un mayor contenido EPA / DHA y EPA / ARA. El crecimiento larvario también mejoró con el aumento del nivel de n-3 HUFA hasta el 8% en dieta, así como con el nivel de DHA en dieta, hasta un 6% de DHA. Los incrementos del nivel de EPA / DHA en dieta a un valor superior al 1 o el de EPA / ARA a un valor superior al 10, disminuyo notablemente el crecimiento, siendo el mejor crecimiento para los valores de 7-8 EPA / ARA. La longitud máxima total larvaria fue para aquellas larvas alimentadas con dietas que contienen un valor de (EPA + DHA + ARA) * (DHA / EPA) / ARA cercana a 50. El contenido en lípidos, del cuerpo entero de las larvas, y la composición de ácidos grasos de la dieta se relacionan con el contenido de EPA, DHA y n-3 HUFA. El incremento de la proporción EPA / DHA a un valor superior a 2, disminuyó notablemente la incorporación de DHA en la composición lipídica de las larvas, reflejando el efecto competitivo del EPA sobre el DHA. Esta reducción en el contenido de DHA en las larvas se asoció con una menor supervivencia larvaria, salvo cuando el nivel de EPA alcanzó 4%. En conclusión, podemos decir que la supervivencia de larvas está significativamente asociada a los niveles de EPA, DHA y n-3 HUFA en la dieta, mientras que en el crecimiento influye mas el contenido en DHA. Además, las proporciones de EPA / DHA y EPA / ARA en la dieta no deben superar niveles superiores a 1 y 8, respectivamente, con el fin de evitar efectos negativos sobre el crecimiento.

10.3.2 IMPORTANCIA DE LOS NIVELES DIETÉTICOS RELATIVOS DE ARA Y EPA EN EL RENDIMIENTO DE LA CRÍA LARVARIA DE LA DORADA (*Sparus aurata*)

A pesar que el aumento de ARA o EPA dietético por sí solo no mejoró significativamente la supervivencia, el aumento de los ácidos grasos mejora de forma significativa el crecimiento y la supervivencia, lo que indica que la relación óptima entre (EPA / DHA) en la dieta debe de estar cerca de 4:1. El ARA dietético fue incorporado en los tejidos larvarios de manera más eficiente que el EPA. El aumento del contenido en EPA o ARA en dieta reduce, respectivamente,la incorporación del ARA o EPA en el componente lípidico de las larvas, lo que sugiere que estos ácidos grasos compiten entre si como substratos de diferentes enzimas. El posible efecto negativo de un incremento en el nivel de ARA en dieta, así como su competición con la EPA para la síntesis de fosfolípidos, necesita nuevos estudios en larvas de peces marinos.

10.3.3 IMPORTANCIA DE LOS NIVELES DIETETICOS DE ÁCIDO ARAQUIDÓNICO EN LA SUPERVIVENCIA, EL CRECIMIENTO Y RESISTENCIA AL ESTRÉS EN LARVAS DE LUBINA (*Dicentrarchus labrax*) ALIMENTADAS CON ALTOS NIVELES DIETÉTICOS DE ÁCIDO EICOSAPENTAENOICO Y DOCOSAHEXAENOICO

El incremento del ácido araquidónico en dieta hasta el 1,2 % mostró una correlación positiva con la supervivencia de larvas y una mejora significativa en las tasas específicas de crecimiento, tanto en peso corporal como en longitud total. El ácido araquidónico en dieta fue incorporado de manera eficiente en los lípidos de las larvas, aunque se incorporan a las larvas en niveles ligeramente inferiores (0.53-0.92%) a los que se encontraban en la dieta. El aumento de la acumulación de ácido araquidónico en dieta no afectó la incorporación del ácido docosahexaenoico o eicosapentaenoico en los lípidos larvarios. Se observo una correlación positiva entre el nivel de ácido

araquidónico en la dieta y la supervivencia frente al estrés de manejo, indicando la importancia de este ácido graso en la respuesta al estrés en larvas de lubina, que estaría relacionado con el papel de este ácido graso en la regulación de ACTH inducida por la liberación de cortisol por células interrenales. Los resultados indican la importancia del ácido araquidónico para las larvas de lubina, pero es necesario incrementar los niveles dietéticos del ácido araquidónico empleados en este experimento para determinar si existe un efecto negativo de este ácido graso en la lubina, de acuerdo con lo demostrado para otras especies.

10.3.4 EFECTO DE LA VITAMINA E Y DIFERENTES NIVELES DE DHA EN EL CRECIMIENTO, LA SUPERVIVENCIA Y LA RESISTENCIA AL ESTRÉS EN LARVAS LA LUBINA (*Dicentrarchus labrax*).

El aumento del DHA en la dieta hasta un 5 % de peso seco disminuyo significativamente la supervivencia de larvas. El incremento de los niveles de la vit E (de 1500 a 3000 mg / kg) con unos niveles de DHA fijos en la dieta mejoró significativamente la resistencia al estrés de las larvas. Además, la vitamina E en la dieta mejoró significativamente el crecimiento de las larvas. Se observo un efecto positivo asociado a la elevación de la vitamina E y del DHA en la dieta (de 1 a 3 %), aunque un aumento del DHA en dieta por encima de estos niveles disminuyo significativamente la longitud total. Del mismo modo, el aumento de DHA y vitamina E mejoró significativamente el peso corporal de la larva.

10.3.5 MEJORA EN EL CRECIMIENTO DE LARVAS DE DORADA (*Sparus aurata*) POR INCLUSIÓN DE VITAMINA E EN LA DIETA Y CON RELACIÓN A DOS NIVELES DIFERENTES DE ÁCIDOS GRASOS ESENCIALES.

El aumento en la dieta de los niveles de vitamina E hasta 3000 mg / kg mejoró notablemente el crecimiento de larvas, sobre todo cuando los niveles de PUFA en la dieta fueron más bajos, lo cual indica la importancia de la actividad protectora

(antioxidante) de la vitamina E sobre los ácidos grasos esenciales, cuando estos se encuentran en pequeñas cantidades. A mayores niveles dietéticos de PUFAs, el aumento de la vitamina E de 500 a 3000 mg / kg mejoró el rendimiento global de larvas en términos de crecimiento y de supervivencia. El incremento de PUFAs en el contenido de los lípidos polares larvarios, a pasar de que eran idénticos en las dietas denota el efecto anti-oxidativo de la vitamina E. Sin embargo, el aumento de la vitamina E de 3000 a 6000 mg / kg no sólo no ha mejorado el rendimiento de larvas, sino que también redujo el contenido de PUFAs en los lípidos polares larvarios, lo que sugiere el efecto pro-oxidante del exceso de la vitamina E. En resumen, los resultados del presente estudio sugieren que la elevación de vitamina E en la dieta hasta 3000 mg / kg con 2.5/1.5 DHA / EPA produce un mejor rendimiento en las larvas de dorada que el aumento de los niveles dietéticos de DHA / EPA a 5/2.5, lo que denota la importancia de la vitamina E en la dieta para el crecimiento de larvas y su interrelación con los niveles dietéticos de PUFAs.

10.3.6 EFECTO COMBINADO DE VITAMINA C Y VITAMINA E EN MICRODIETAS PARA DORADA (*Sparus aurata*)

La supervivencia de las larvas alimentadas con una dieta 3000 / 0 (vit E/vit C) fue significativamente más baja que la de las larvas alimentadas con la dieta 3000/1800. Después de dos semanas de alimentación, el aumento de los niveles de AA de 1800 a 3600 mg en presencia de 1500 mg α-T, y el aumento de AA de 0 a 1800 o a 3600 mg en presencia de 3000 mg α-T, respectivamente, mejoró significativamente la longitud total final, el peso seco total, la tasa de crecimiento especifica y la biomasa final. En la exposición al estrés, a una temperatura de agua (15 º C), las larvas alimentadas con dieta 1500/3600 mostraron una supervivencia significativamente mayor que los alimentados con un alto nivel de α-T con diferentes niveles de AA en la dieta. En conclusión, el aumento de AA de 1800 a 3600 mg / kg en presencia de 1500 mg / kg de vitamina E y el aumento de la vitamina C (de 0 a 1800 o 3600 mg / kg) en dietas que tienen alto nivel de vitamina E (3000 mg / kg) mejora de forma significativa el crecimiento indicando la importancia de los diferentes ratios de vitamina E y C.

10.4 Bibliografía

Ai, Q.H., Mai, K.S., Li, H.T., Zhang, C.X., Zhang, L., Duan, Q.Y., Tan, B.P., Xu, W., Ma, H.M., Zhang, W.B. and Liufu, Z.G., 2004. Effects of dietary protein to energy ratios on growth and body composition of juvenile Japanese seabass, *Lateolabrax japonicus*. Aquaculture 230, 509– 516.

Al-Amoudi, M.M., El-Nakkadi, A.M.N. and El-Nouman, B.M., 1992. Evaluation of optimum dietary requirement of vitamin C for the growth of *Oreochromis spilurus* fingerlings in water from the Red Sea. Aquaculture, 105, 165–173.

Alexis, M.N., Karanikolas, K.K. and Richards, R.H., 1997. Pathological findings owing to the lack of ascorbic acid in cultured gilthead bream (*Sparus aurata* L.). Aquaculture, 151, 209–218.

Anbarasu, K. and Chandran, M.R., 2001. Effects of ascorbic acid on the immune response of the catfish, *Mystus gulio* (Hamilton), to different bacterins of *Ameromonas hydrophila*. Fish Shellfish Immunol., 11, 347–355.

A.O.A.C., 1995. Official Methods of Analysis of the Association Analytical Chemist. U.S.A., p. 1018.

Bai, S.C. and Lee, K.J., 1998. Different levels of dietary DL-α-tocopheryl acetate affect the vitamin E status of juvenile Korean rockfish, *Sebastes schlegeli*. Aquaculture, 161, 405–414.

Basurco, B. and Abellán, E., 1999. Finfish species diversification in the context of Mediterranean marine fish farming development. In: Marine Finfish Diversification: Current Situation and Prospects in Mediterranean Aquaculture. Options Mediterranéennes (eds. E. Abellan and B. Basurco), FAO, serie B: Etudes et Recherches, no. 24, pp. 9–25.

Bell, J.G. and Sargent, J.R., 2003. Arachidonic acid in aquaculture feeds: current status and future opportunities. Aquaculture, 218, 491– 499.

Bell, J.G., Henderson, R.J., Pirie, B.J.S. and Sargent, J.R., 1985. Effect of dietary polyunsaturated fatty acid deficiencies on mortality, growth and gill structure in the turbot *Scophthalmus maximus*. J. Fish Biol., 26, 181–191.

Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of supplementation with 20:3n-6, 20:4n-6 and 20:5n-3 on the production of the prostaglandins E and F of the 1, 2 and 3 series in turbot *Scophthalmus maximus* L. brain astroglial cells in primary culture. Biochim. Biophys. Acta., 1211, 335–342.

Bell, M.V., Henderson, R.J. and Sargent, J.R., 1986. The role of polyunsaturated fatty acids in Fish. Comp. Biochem. Physiol., 83B, 711-719.

Benítez-Santana, T., Masuda, R., Juárez Carrillo, E., Ganuza, E., Valencia, A., Hernández-Cruz, C.M. and Izquierdo, M.S., 2007. Dietary n-3 HUFA deficiency induces a reduced visual response in gilthead seabream *Sparus aurata* larvae. Aquaculture, 264, 408–417.

Bessonart, M., Izquierdo, M.S., Salhi, M., Hernández-Cruz, C.M., González, M.M. and Fernández-Palacios, H., 1999. Effect of dietary arachidonic acid levels on growth and survival of gilthead seabream (*Sparus aurata* L.) larvae. Aquaculture, 179, 265-275.

Blazer, V. S., 1982. The ffect of marginal deficiencies of ascorbic acid and α-tocopherol on the natural resistance and immune response of rainbow trout (*Salmo gairdneri*). Ph.D. dissertation. University of Rhode Island.

Boonyaratpalin, 1997. Nutrient requirements of marine food fish cultured in Southeast Asia. Aquaculture, 151, 283-313.

Boonyaratpalin, M., Unprasert, N., Kosutharak, P., Sothana, W. and Chumsungnem, S., 1989a. Effect of different levels of vitamin C added in diet on growth, feed

efficiency and survival rate of seabass. Technical Paper No. 6. National Institute of Coastal Aquaculture, Department of Fisheries, Thailand, 21 pp. (in Thai).

Boonyaratpalin, M., Unprasert, N. and Buranapanidgit, J., 1989b. Optimal supplementary vitamin C level in seabass fingerling diet. In: M. Takeda and T. Watanabe (Editors), The Current Status of Fish Nutrition in Aquaculture. Tokyo University of Fisheries, Tokyo, Japan, pp. 149-157.

Boonyaratpalin, M., Boonyaratpalin, S. and Supamattaya, K., 1992. Ascorbyl-phosphate-Mg as a dietary vitamin C source for seabass (*Lares calcarifer*). Presented at the 3rd Asian Fisheries Forum, 26-30 October 1992, Singapore.

Borlongan, I.G. and Benitez, L.V., 1992. Lipid and fatty acid composition of milkfish (*Chanos chanos* Forksskal) growth in freach water and seawater. Aquaculture, 104, 79-89.

Brown, M.T., Dunstan, G.A., Nichols, P.D., Battaglene, S.C., Morehead, D.T., Anna L. and Overweter, A.L., 2005. Effects of a-tocopherol supplementation of rotifers on the growth of striped trumpeter *Latris lineata* larvae. Aquaculture, 246, 367–378.

Burton, G.W. and Ingold, K.U., 1989. Vitamin E as an in vitro and in vivo antioxidant. Ann. NY Acad. Sci., 570, 7 –22.

Burton, G.W., Joyce, A. and Ingold, K.U., 1982. First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. Lancet 2, 327.

Castell, J.D., Bell, J.G., Tocher, D.R. and Sargent, J.R., 1994. Effects of purified diets containing different combinations of arachidonic and docosahexaenoic acid on survival, growth and fatty acid composition of juvenile turbot (*Scophthalmus maximus*). Aquaculture, 128, 315– 333.

Castell, J.D., Blair, T., Neil, S., Howes, K., Mercer, S., Reid, J., Young-Lai, W., Gullison, B., Dhert, P. and Sorgeloos, P., 2001. The effect of different HUFA

enrichment emulsions on the nutritional value of rotifers (*Brachionus plicatilis*) to larval haddock (*Melanogrammus aeglefinus*). In: Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloos, P. (Eds.), Larvi 01 Fish and Shellfish Larviculture Symposium European Aquaculture Society, Special Publication, vol. 30. Oostende, Belgium, pp. 111 –114.

Chávez De Martínez, M.C., 1990. Vitamin C requirement of the Mexican native cichlid *Ciclasoma urophtalmus* (Gunther). Aquaculture, 86, 409-416.

Chen, R., Lochmann, R., Goodwin, A., Praveen, K., Dabrowski, K. and Lee, K.J., 2004. Effects of dietary vitamins C and E on alternative complement activity, hematology, tissue composition, vitamin concentrations and response to heat stress in juvenile golden shiner (*Notemigonus crysoleucas*). Aquaculture, 242, 553–569.

Christie, W.W., 1982. Lipid Analysis. Pergamon Press, Oxford.

Chien, L.T., Hwang, D.F. and Jeng, S.S., 1999. Effect of thermal stress on dietary requirement of vitamin C in Thornfish *Terapon jarbua*. Fish. Sci., 65, 731–735.

Copeman, L.A., Parrish C.C., Brown, J.A. and Harel, M., 2002. Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (*Limanda ferruginea*): a live food enrichment experiment. Aquaculture, 210, 285–304.

Cort, W.M., 1974. Antioxidant activity of tocopherols, ascorbyl palmitate, and ascorbic acid, and their mode of action. Journal of the American Oil Chemists Society, 51, 321–325.

Cuesta, A., Esteban, M.A., Meseguer, J., 2002. Natural cytotoxic activity in seabream (*Sparus aurata* L.) and its modulation by vitamin C. Fish Shellfish Immunol. 13, 97–109.

Dabrowski, K., 1990. Absorption of ascorbic acid and ascorbic sulfate and ascorbate metabolism in stomachless fish, common carp. J. Comp. Physiol., B 160, 549–561.

Dabrowski, K., Hinterleitner, C., Sturmbauer, C., El-Fiky, N. and Wieser, W., 1988. Do carp larvae require vitamin C. Aquaculture, 72: 295-306.

Dabrowski, K., Moreau, R., El-Saidy, D. and Ebeling, J., 1996. Ontogenetic sensitivity of channel catfish to ascorbic acid deficiency. J. Aquat. Anim. Health, 8, 22-27.

Dantagnan, H., Bórquez, A.S., Valdebenito, I.N., Salgado, I.A., Serrano, E.A. and Izquierdo M.S., 2007. Lipid and fatty acid composition during embryo and larval development of puye Galaxias maculatus Jenyns, 1842, obtained from estuarine, freshwaterand cultured populations. J. Fish Biol., 70, 770–781.

Estévez, A., McEvoy, L.A., Bell, J.G. and Sargent, J.R., 1999. Growth, survival, lipid composition and pigmentation of turbot larvae fed live-prey enriched in arachidonic (ARA) and eicosapentaenoic (EPA) acids. Aquaculture, 180, 321–343.

FAO, 1999, Manual on Hatchery Production of Sea bass and Gilthead Sea bream. pp 5.

Fernández-Palacios, H., Izquierdo, M.S., Gonzalez, M., Robaina, L. and Valencia, A., 1998. Combined effect of dietary a-tocopherol and n-3 HUFA on egg quality of gilthead seabream broodstock (*Sparus aurata*). Aquaculture, 161, 475–476.

Filho, D.W., Giulivi, C. and Boveris, A., 1993. Antioxidative defences in marine fish: Teleosts. Comp. Biochem. Physiol., 106, 409– 413.

Folch, J., Lees, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem., 226, 497-509.

Fournier, V., Gouillou-Coustans, M.F. and Kaushik, S.J., 2000. Hepatic ascorbic acid saturation is the most stringent response criterion for determining the vitamin C requirement of juvenile European sea bass (*Dicentrarchus labrax*). *Journal of Nutrition,* 130, 617–620.

Frigg, M., Prabucki, A.L. and Ruhdel, E.U., 1990. Effect of dietary vitamin E levels on oxidative stability of trout fillets. Aquaculture, 84, 145-158.

Furones, M.D., Alderman, D.J., Bucke, D., Fletcher, T.C., Knox, D. and White, A., 1992. Dietary vitamin E and the response of rainbow trout, *Oncorhynchus mykiss* (Walbaum), to infection with Yersinia ruckeri. J. Fish Biol., 41, 1037– 1041.

Ganga, R., Bell, J.G., Montero, D., Robaina, L., Caballero, M.J. and Izquierdo, M.S., 2005. Effect of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin production in gilthead seabream (*Sparus aurata*). Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 142, 410–418.

Ganga, R., Tort. L., Acerote, L., Montero, D. and Izquierdo, M.S., 2006. Modulation of ACTH-induced cortisol release by polyunsaturated fatty acids in interrenal cells from gilthead seabream (*Sparus aurata*). Journal of Endocrinology, 190, 39–45.

Gatesoupe, F.J. and Le Milinaire, C., 1985. Adaptation de la qualite limentaire des filtreurs-proies aux besoins nutritifs des larvae de poissons marins. Coll. Fr.-japon Oceanogr., Marseille, 16-21 Sept., 8, 51-63.

González, M.M., Izquierdo, M.S., Salhi, M., Hernández- Cruz, C.M. and Fernández-Palacios, H., 1995. Dietary vitamin E for *Sparus aurata* larvae. Europ. Aqua. Soc. Spec. Publ., 24, 239–242.

Gouillou-Coustans, M.F., Bergot, P. and Kaushik, S.J., 1998. Dietary ascorbic acid needs of common carp (*Cyprinus carpio*) larvae. Aquaculture, 161, 453–461.

Halliwell, B. and Gutteridge, J.M.C., 1996. Lipid peroxidation: a radical chain reaction. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Free Radicals in Biology and Medicine. Clarendon Press, Oxford, pp. 188– 266.

Halver, J.E., 1995. Vitamin requirement study techniques. J. Appl. Ichthyol., 11, 215–224.

Halver, J.E., 2002. The vitamins. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, San Diego, CA, pp. 61–141.

Halver, J.E., Ashley, L.M. and Smith, R.E., 1969. Ascorbic acid requirements of coho salmon and rainbow trout. Trans. Am. Fish. Soc., 98, 762–772.

Hamre, K. and Lie, O., 1995a. Minimum requirement of vitamin E for Atlantic salmon (*Salmo salar* L) at first feeding. Aquac. Res., 26, 175–184.

Hamre, K. and Lie, O., 1995b. Alpha-tocopherol levels in different organs of Atlantic salmon (*Salmo salar* L.) effect of smoltification, dietary levels of n-3 polyunsaturated fatty acids and vitamin E. Comp. Biochem. Physiol., 111A, 547–554.

Harel, M., Tandler, A. and Kissil, G.W., 1994. The kinetics of nutrient incorporation into body tissues of gilthead seabream (*Sparus aurata*) females and the subsequent effects on egg composition and egg quality. Br. J. Nutr., 72, 45– 58.

Hastings, N., Agaba, M., Tocher, D.R., Leaver; M.J., Sargent, J.R. and Teale, A.J., 2001. A vertebrate fatty acid desaturase with delta5 and delta6 activity. Proceedings of the National Academy of Science, USA. 98, 14304-14309.

Henderson, R.J., Sargent, J.R., 1985. Fatty acid metabolism in fish. In: Cowey, C.B., Mackie, A.M., Bell, J.G. (Eds.), Nutrition and Feeding in Fish. Academic Press, London, pp. 349–364.

Henderson, R.J., Bell, M.V. and Sargent, J.R., 1985. The conversion of polyunsaturated fatty acids to prostaglandins by tissue homogenates of the turbot, *Scophthalmus maximus* (L.). J. Exp. Mar. Biol., Ecol. 85, 93–99.

Henrique, M.M.F., Gomes, E.F., Gouillou-Coustans, M.F., Oliva-Teles, A. and Davies, S.J., 1998. Influence of supplementation of practical diets with vitamin C on growth and response to hypoxic stress of seabream, *Sparus aurata*. Aquaculture, 161, 415–426.

Holmefjord, I. and Olsen, Y., 1991. An intensive approach to Atlantic halibut fry production. In: *Larví'9l-Fish & Crustacean Larvículture Symposium* (P. Lavens, P. Sorgeloos, E. Jaspers and F. Ollevier eds.). European Aquaculture Society, Special publication No. 15, Gent, Belgium. 1991, pp, 331-334.

Ibeas, C., Cejas, J., Gbmez, T., Jerez, S. and Lorenzo, A., 1996. Influence of dietary n-3 HUFA levels on juvenile gilthead seabream (*Spans aurata*) growth and tissue fatty acid composition. Aquaculture, 142, 221-235.

Ibeas C., Cejas J.R., Fores R., Badía P., Gómez T. and Lorenzo Hernández A., 1997. Influence of eicosapentaenoic to docosahexaenoic acid ratio (EPA/DHA) of dietary lipids on growth and fatty acid composition of gilthead seabream *Sparus aurata* juveniles. Aquaculture, 150, 91-102.

Iijima, N., Tanaka, S. and Ota, Y., 1998. Purification and characterization of bile salt-activated lipase from the hepatopancreas of red sea bream, *Pagrus major*. Fish Physiol. Biochem., 18, 59–69.

Izquierdo, M.S., 1988. Estudio de los requerimientos de ácidos grasos esenciales en larvas de peces marinos. Modificación de la composición lipídica de las presas. Thesis Doctoral, La Laguna University, Spain.

Izquierdo, M. S., 1996. Review article: essential fatty acid requirements of cultured marine fish larvae. Aquac. Nutr., 2, 183–191.

Izquierdo, M.S., 2005. Essential fatty acid requirements in Mediterranean fish species. Cah. Options Mediterr., 63, 91-102.

Izquierdo, M.S. and Fernández-Palacios, H., 1997. Nutritional requirements of marine fish larvae and broodstock. Cah. Options Mediterr., 22, 243–264.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989a. Optimal EFA levels in Artemia to meet the EFA requirements of red seabream *Pagrus major*.. In: Takeda, M., Watanabe, T. _Eds.., The Current Status of Fish Nutrition in Aquaculture. Japan Translation Centre, Tokyo, Japan, pp. 221–232.

Izquierdo, M.S., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1989b. Requirement of larval red seabream *Pagrus major* for essential fatty acids. Nippon Suisan Gakkaishi, 55, 859–867.

Izquierdo, M., Watanabe, T., Takeuchi, T., Arakawa, T. and Kitajima, C., 1990. Optimum EFA levels in Artemia to meet the EFA requirements of red seabream (*Pagrus major*). In: Takeda, M., Watanabe, T. (Eds.), The current Status of Fish Nutrition in Aquaculture. Tokyo Univ. Fisheries, Tokyo, pp. 221– 232.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. and Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem., 22, 97–107.

Izquierdo, M.S, Fernández-Palacios, H. and Tacon, A.G.J., 2001a. Effect of broodstock nutrition on reproductive performance of fish. Aquaculture, 197, 25-42.

Izquierdo, M.S., Tandler, A., Salhı´, M. and Kolkkovski, S., 2001b. Influence of dietary polar lipids quantity and quality on ingestion and assimilation of labelled fatty acids by larval gilthead seabream. Aquacult. Nutr., 6, 153– 160.

Izquierdo, M.S., Obach, A., Arantzamendi, L., Montero, D., Robaina, L. and Rosenlund, G., 2003. Dietary lipid sources for seabream and seabass: growth performance, tissue composition and flesh quality. Aquacult. Nutr., 9, 397– 407.

Izquierdo, M.S., Robaina, L., Juárez-Carrilo, E., Oliva, V., Hernández-Cruz, C.M. and Afonso, J.M., 2008. Regulation of growth, fatty acid composition and delta 6 desaturase expression by dietary lipids in gilthead seabream larvae *Sparus aurata*. Fish Physiol. Biochem., 34, 117-127.

Juaneda, P. and Rocquelin, G., 1985. Rapid and convenient separation of phospholipids and non-phosphorous lipids from rat heart using silica cartridges. Lipids, 20, 40–41.

Kanazawa, K., 1991. Hepatotoxicity caused by dietary secondary products originating from lipid peroxidation. In: Friedman, M. (Ed.), Nutritional and Toxicology Consequences of Food Processing. Plenum, New York, pp. 237–253.

Kanazawa, K., 1993. Tissue injury induced by dietary products of lipid peroxidation. In: Corongiu, F. (Ed.), Free Radicals and Antioxidants in Nutrition. Richelieu Press, London, pp. 383– 399.

Kawatsu, H., 1969. Studies on the anemia of fish-III. An example of macrocytic anemia found in brook trout, *Salvelinus fontinalis*. Bulletin of the Freshwater Fish Research Laboratory, 19, 161–167.

Kitamura, S., Ohara, S. and Suwa, T., 1965. Studies on vitamin requirements of rainbow trout, *Salmo gairdneri* I. On ascorbic acid. Bull. Jpn. Soc. Sci. Fish, 31, 818-827.

Kolkovski S., Tandler A. and Kissil G. Wm. 1993. The effect of dietary enzymes with age on protein and lipid assimilation and deposition in *Sparus aurata* larvae. In: Fish Nutrition in Practice, Les Colloques, No. 61 (ed. INRA). INRA, Paris, France.

Kolkovski, S., Czesny, S., Yackey, C., Moreau, R., Cihla, F., Mahan, D. and Dabrowski, K., 2000. The effect of vitamins C and E in (n-3) highly unsaturated fatty acids-enriched Artemia nauplii on growth, survival, and stress resistance of fresh water walleye Stizostedion vitreum larvae. Aquac. Nutr., 6, 199–206.

Koven, W.M., 1991. The combined effect of dietary n-3 highly unsaturated fatty acids and age, on growth, survival and lipid composition in larval gilthead seabream (*Sparus aurata*, Perciformes, Teleostei). Ph.D. Thesis, Hebrew University, Israel, 130 pp.

Koven, W., Barr, Y., Lutzky, S., Ben-Atia, I., Weiss, R., Harel, M., Behrens, P. and Tandler, A., 2001. The effect of dietary arachidonic acid (20:4n-6) on growth, survival and resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. Aquaculture, 193, 107– 122.

Koven, W., Van Anholt, R., Lutzky, S., Ben Atia, I., Nixon, O., Ron, B. and Tandler, A., 2003. The effect of dietary arachidonic acid on growth, survival, and cortisol levels in different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or daily salinity change. Aquaculture, 228, 307–320.

Le Grusse, J. and Watier, B.. 1993. Les vitamines. Données biochimiques, nutritionnelles et cliniques. Centre d'Etude et d'Information sur les Vitamines, Neuilly-Sur- Seine.

Lee, K.J. and Dabrowski, K., 2003. Interaction between vitamins C and E affects their tissue concentrations, growth, lipid oxidation, and deficiency symptoms in yellow perch (*Perca flavescens*). Br. J. Nutr., 89, 589–596.

Lemaire, P., Viarengo, A., Canesi, L. and Livingstone, D.R., 1993. Pro-oxidant and antioxidant processes in gas gland and other tissues of cod (Gadus morhua). J. Comp. Physiol., 163, 477– 486.

Lim, C. and Lovell, R. T. 1978. Pathology of vitamin C deficiency syndrome in channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 108, 1137–1146.

Lin, M-F. and Shiau, S-Y. 2005a. Dietary l-ascorbic acid affects growth, nonspecific immune responses and disease resistance in juvenile grouper, Epinephelus malabaricus. Aquaculture, 244, 215– 221.

Lin, Y-H. and Shiau, S-Y., 2005b. Dietary vitamin E requirement of grouper, *Epinephelus malabaricus*, at two lipid levels, and their effects on immune responses. Aquaculture, 248, 235– 244.

Liu, J., Caballero, M.J., El-Sayed, Ali.T., Izquierdo, M.S., Hernández Cruz, C.M., Valencia, A. and Fernández-Palacios, H., 2002. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream (*Sparus aurata*). Fish Science, 68, 1165–1172.

Machlin, L.J., 1984. Vitamin E. In: Machlin, L.J. (Ed.), Handbook of Vitamins. Marcel Dekker, New York, pp. 99– 145.

Madsen, L., Rustan-Arild, C., Vaagenes, H., Berge, K., Dyroy, E. and Berge, R.K., 1999. Eicosapentaenoic and docosahexaenoic acid affect mitochondrial and peroxisomal fatty acid oxidation in relation to substrate preference. Lipid, 34 (9), 951-963.

Mahajan, C. and Agrawal, N.K., 1980. Nutritional requirement of ascorbic acid by Indian major carp, *Cirrhina mrigala*, during early growth. Aquaculture, 19, 37–48.

McEvoy, L.A., Estévez, A., Bell, J.G., Shields, R.J., Gara, B. and Sargent, J.R., 1998. Influence of dietary levels of eicosapentaenoic and arachidonic acids on the pigmentation success of turbot (*Scophthalmus maximus* L.) and halibut (*Hippoglossus hippoglossus* L.). Bull. Aquacult. Assoc. Can., 4, 17– 20.

Merchie, G., Lavens, P., Storch, V., Ubel, U., De Nelis, H., Leenheer, A. and Sorgeloos, P., 1996. Influence of dietary vitamin C dosage on turbot *Scophthalmus maximus*

and European sea bass *Dicentrarchus labrax* nursery stages. Comp. Biochem. Physiol., 114, 123-133.

Merchie, G., Lavens, P. and Sorgeloos, P., 1997. Optimization of dietary vitamin C in fish and crustacean larvae: a review. Aquaculture, 155, 165–181.

Messager, J.L., Stephan, G., Quentel, C. and Baudin Laurencin, F., 1992. Effects of dietary oxidized fish oil and antioxidant deficiency on histopathology, haematology, tissue and plasma biochemistry of sea bass, *Dicentrarchus labrax*. Aquat. Living Resour., 5, 205–214.

Montero, D., Tort, L., Izquierdo, M.S., Socorro, J., Robaina, L., Vergara, J.M. and Fernández-Palacios, H., 1996. Effect of alphatocopherol and n - 3 HUFA deficient diets on blood cells, selected immune parameters and proximate body composition of gilthead seabream (*Sparus aurata*). In: Modulators of Immune Response. The Evolutionary Trail (Stolen, J.S., Fletcher, T.C., Bayne, C.J., Secombes, C.J., Zelikoff, J.L., Twerdock, L., & Anderson D.P., eds.) pp. 251–266. S.O.S. Publications, Fair Haven.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L. and Vergara, J. M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiology and Biochemistry, 18, 399–407.

Montero, D., Marrero, M., Izquierdo, M.S., Robaina, L., Vergara, J.M. and Tort, L., 1999. Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. Aquaculture, 171, 269– 278.

Montero, D., Tort, L., Robaina, L., Vergara, J.M. and Izquierdo, M.S., 2001. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. Fish and Shellfish Immunology, 11, 473–490.

Moreau, R., Dabrowski, K., Czesny, S. and Cihla, F., 1999. Vitamin C-vitamin E interaction in juvenile lake sturgeon (*Acipenser fulvescens* R.), a fish able to synthesize ascorbic acid. J. Appl. Ichthyol., 15, 250–257.

Mourente, G., Rodríguez, A., Tocher, D.R. and Sargent, J.R., 1993. Effects of dietary docosahexaenoic acid DHA; 22:6n-3.on lipid and fatty compositions and growth in gilthead sea bream *Sparus aurata* L.. larvae during first feeding. Aquaculture, 112, 79–98.

Mourente, G., Diaz-Salvago, E., Bell, J.G. and Tocher, D.R., 2002. Increased activities of hepatic anti-oxidant defence enzymes in juvenile gilthead sea bream (*Sparus aurata* L.) fed dietary oxidised oil: attenuation by dietary vitamin E. Aquaculture, 214, 343–361.

Mulero, V., Esteban, M. A. and Mesenguer, J., 1998. Effects of in vitro addition of exogenous vitamins C and E on gilthead seabream (*Sparus aurata* L.) phagocytes. Veterinary Immunology and Immunopathology, 66, 185–199.

Murai, T. and Andrews, J.W., 1974. Interaction of dietary α-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). Journal of Nutrition, 104, 1416–1431.

Niki, E., 1987a. Antioxidants in relation to lipid peroxidation. Chemistry and Physics of Lipids, 44, 227–253.

Niki, E., 1987b. Interaction of ascorbate and α-tocopherol. Ann. N.Y. Acad. Sci., 498, 186–199.

NRC, 1993. Nutritional Requirements of Fish, pp. 114.National Academic Press, Washington DC.

Olsen, Y. and Skjervold, H., 1995. Variation in content of omega 3 fatty acids in farmed Atlantic salmon, with special emphasis on effect of non dietary factor. Aquac, Int., 3, 2-35.

Ortuño, J., Esteban, M.A. and Meseguer, J., 1999. Effects of high dietary intake of vitamin C on non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 9, 429– 443.

Ortuño, J., Esteban, A. and Meseguer, J., 2000. High dietary intake of α-tocopherol acetate enhances the non-specific immune response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 10, 293–307.

Ortuño, J., Cuesta, A., Esteban, A. and Meseguer, J., 2001. Effect of oral administration of high vitamin C and E dosages on the gilthead seabream (*Sparus aurata* L.) innate immune system. Vet. Immunol. Immunopathol., 79, 167–180.

Ortuño, J., Esteban, M.A. and Meseguer, J., 2003. The effect of dietary intake of vitamins C and E on the stress response of gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol., 14, 145–156.

Ota. T. and Yamada, M.,1971. Lipid of masu salmon Onchorhynchus masou. I. Variations of the lipid content and fatty acid composition of juvenile masu salmon during the period of smolt-transformation, and the influence of light upon those variations. Bull. Fac. Fish. Hokkaido Univ., 22, 151-158.

Packer, J.E., Slater, T.F. and Wilson, R.L., 1979. Direct observation of a free radical interaction between vitamin E and vitamin C. Nature, 278, 737–738.

Paul, B.N., Sarkar, S.N. and Mohanty., 2004. Dietary vitamin E requirement of mrigal, *Cirrhinus mrigala* fry. Aquaculture, 242, 529-536.

Pousão-ferreira, p., Santos, P., Carvalho, A.P., Morais, S. and Narciso, L., 2003. Effect of an experimental microparticulate diet on the growth, survival and fatty acid profile of gilthead seabream (*Sparus aurata* L.) larvae. Aquaculture International, 11, 491–504.

Putnam, M.E. and Comben, M., 1987. Vitamin E. Veterinary Record, 121, 541-545.

Radi, A.A.R. and Markovics, B., 1988. Effects of metal ions on the antioxidant enzyme activities, proteins contents and lipid peroxidation of carp tissues. Comp. Biochem. Physiol., 90C, 69– 72.

Rainuzzo, J.R., Reitan, K.I., Jorgensen, L. and Olsen, Y., 1994. Lipid composition in turbot fed live feed cultured by emulsions of different lipid classes. Comp. Biochem. Physiol., 107A, 699–710.

Reitan, K.I., Rainuzzo, J.R. and Olsen, Y. 1994. Influence of lipid composition of live feed on growth, survival and pigmentation of turbot larvae. Aquaculture International, 2, 33-48.

Roberts, M.L., Davies, S.J. and Pulsford, A.L., 1995. The influence of ascorbic acid (vitamin C) on non-specific immunity in the turbot (*Scophthalmus maximus* L.). Fish Shellfish Immunol., 5, 27– 38.

Rodríguez, C., 1994. Estudio de los requerimientos de acidos grasos esenciales de la dorada europea *Sparus aurata* L. durante las dos primeras semanas de alimentacion. PhD Thesis, University of La Laguna, Spain.

Rodríguez, C., Pérez, J.A., Izquierdo, M.S., Mora, J., Lorenzo, A. and Fernández-Palacios, H., 1993. Essential fatty acid requirements of larval gilthead seabream, *Sparus aurata* L.. Aquacult. Fish. Manage., 24, 295–304.

Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S. and Cejas, J.R., 1994. n-3 HUFA requirement of larval gilthead seabream *Sparus aurata* when using high levels of eicosapentaenoic acid. Comp. Biochem. Physiol., 107A, 693–698.

Rodríguez, C., Pérez, J.A., Díaz, M., Izquierdo, M.S., Fernandez-Palacios, H. and Lorenzo, A., 1997. Influence of the EPA/DHA ratio in rotifers on gilthead seabream *Sparus aurata* larval development. Aquaculture, 150, 77–89.

Rodríguez, C., Pérez, J.A., Badia, P., Izquierdo, M.S., Fernández Palacios, H. and Hernández, A.L., 1998. The n-3 highly unsaturated fatty acids requirements of gilthead seabream (*Sparus aurata* L.) larvae when using an appropriate DHA/EPA ratio in the diet. Aquaculture, 169, 9–23.

Roem, A.J., Kohler, C.C. and Stickney, R.R., 1990. Vitamin E requirements of the blue tilapia, *Oreochromis aureus* (Steindachner), in relation to dietary lipid level. Aquaculture, 87, 155–164.

Ruff, N., P. Lavens, P., Huo, J.Z., Sorgeloos, P., Nelis, h.J. and DE Leenheer, A., 2001. Antioxidant effect of dietary tocopherol and ascorbic acid on growth and survival of *Litopenaeus vannamei* Postlarvae. Aquaculture International, 9, 115–126.

Sakai, T., Murata H., Endo, M., Yamauchi, K., Tabata, N. and Fukudome, M., 1989. 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola aquiqueradiata*. Agricultural and Biological Chemistry, 53, 1739–1740.

Salhi, M., Izquierdo, M.S., Hernández-Cruz, C.M., Gonzalez, M. and Fernández-Palacios, H., 1994. Effect of lipid and n-3 HUFA levels in microdiets on growth survival and fatty acid composition of larval gilthead seabream *Sparus aurata*. Aquaculture, 124, 275–282.

Salhi, M., Izquierdo, M.S., Hernández-Cruz, C.M., Bessonart, M. and Fernández-Palacios, H., 1999. Effect of different dietary polar lipid levels and different n-3 HUFA content in polar lipids on the gut and liver histological structure of seabream *Sparus aurata* larvae. Aquaculture, 179, 253–264.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1994. The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: Lahlou, B., Vitiello, P. (Eds.), Aquaculture: Fundamental and Applied Research. American Geophysical Union, pp. 103–113.

Sargent, J.R., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1995. Requirement criteria for essential fatty acids. J. Appl. Ichthyol., 11, 183– 198.

Sargent, J.R., McEvoy, L.A. and Bell, J.G., 1997. Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. Aquaculture, 155, 117-127.

Sargent, J., Bell, G., McEnvoy, L., Tocher, D. and Estevez A., 1999a. Recent developments in the essential fatty acid nutrition of fish. Aquaculture, 177,191-199.

Sargent, J.R., McEvoy, L.A., Estevez, A., Bell, J.G., Bell, M.V., Henderson, R.J. and Tocher, D.R., 1999b. Lipid nutrition of marine fish during early development: current status and future directions. Aquaculture, 179, 217–229.

Saroglia, M. and Scarano, G., 1992. Experimental induction of ascorbic deficiency in sea bass in intensive aquaculture. Bull. Eur. Ass. Fish Pathol., 12, 97–99.

Sau, S.K., Paul, B.N., Mohanta, K.N. and Mohanty, S.N., 2004. Dietary vitamin E requirement, fish performance and carcass composition of rohu, *Labeo rohita* fry. Aquculture, 240, 359–368.

Schwarz, F.J., Kirchgessuner, M., Steinhart, H. and Runge, G., 1988. Influence of different fats with varying additions of α-tocopherol acetate on growth and body composition of carp (*Cyprinus carpio* L.). Aquaculture, 69, 57–67.

Sealey, W.M. and Gatlin III, D.M., 2002. Dietary vitamin C and vitamin E interact to influence growth and tissue composition of juvenile hybrid striped bass (*Morone chrysops XM. Saxatilis*) but have limited effects on immune responses. J. Nutr., 132, 748– 755.

Seiliez, I., Panserat, S., Corraze, G., Kaushik, S. and Bergot, P., 2003. Cloning and nutritional regulation of a Δ6-desaturase-like enzyme in marine teleost gilthead

seabream (*Sparus aurata*). Comparative biochemistry and physiology, Part B: Biochemistry and Molecular Biology., 135, 449-460.

Sies, H. and Murphy, M., 1991. Role of tocopherols in the protection of biological systems against oxidative damage. J. Photochem. Photobiol., B8, 211– 224.

Stéphan, G., Guillaume, J. and Lamour, F. 1995. Lipid peroxidation in turbot (*Scophthalmus maximus*) tissue: effect of dietary vitamin E and dietary n-6 or n-3 polyunsaturated fatty acids. Aquaculture, 130, 251-268.

Teles, O.A., 2000. Recent advances in European sea bass and gilthead sea bream nutrition. Aquaculture International, 8, 477–492.

Teshima, S.I., Kanazawa, A., Koshio, S. and Itoh, S., 1993. L-Ascorbyl-2-phosphate-Mg as vitamin C source for the Japanese flounder *(Paralichthys oliuaceus*). In: S.J. Kaushik and P. Luquet (Editors), Fish Nutrition in Practice. Coll. Les. Colloq., No. 61. INRA, Paris, pp. 157-166.

Thorarinsson, R., Landolt, M.L., Elliott, D.G., Pascho, R.J. and Hardy, R.W., 1994. Effect of dietary vitamin E and selenium on growth, survival and the prevalence of *Renibacterium salmoninarum* infection in chinook salmon (*Oncorhynchus tshawytscha*). Aquaculture, 121, 343–358.

Tocher D.R., Mourente G., Van der Eecken A., Evjemo J.O., Diaz E., Bell J.G., Geurden I., Lavens P. and Olsen Y., 2002. Effects of dietary vitamin E on antioxidant defence mechanisms of juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). Aquaculture Nutrition, 8, 195–207.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz E., Wille, M., Bell J.G. and Olsen, Y., 2003. Comparative study of antioxidant defence mechanisms in marine fish fed variable levels of oxidised oil and vitamin E. Aquaculture International, 11, 195–216.

Waagbo, R., Sandnes, K., Torrissen, O. J., Sandvin, A. and Lie, é. 1993. Chemical and sensory evaluation of fillets from Atlantic salmon (*Salmo salar*) fed three levels of n-3 polyunsaturated fatty acids at two levels of vitamin E. Food Chemistry, 46, 361-366.

Wang, X.J., Kim, K.W., Bai, S.C., Huh, M.D. and Cho, B.Y., 2003. Effects of the different levels of dietary vitamin C on growth and tissue ascorbic acid changes in parrot fish (*Oplegnathus fasciatus*). Aquaculture, 215, 21– 36.

Wang Z., Mai K., Liufu Z., Ma H., Xu W., Ai Q., Zhang W., Tan B. and Wang X., 2006. Effect of high dietary intakes of vitamin E and n-3 HUFA on immune responses and resistance to Edwardsiella tarda challenge in Japanese flounder (*Paralichthys olivaceus*, Temminck and Schlegel) Aquaculture Research, 37, 681-692.

Watanabe, T., 1982. Lipid nutrition in fish. Comp. Biochem. Physiol., 73, 3-15.

Watanabe, T., 1993. Importance of docosahexanoic acid in marine larval fish. J. World Aquacult. Soc., 24, 152– 161.

Watanabe, T. and Kiron, Y., 1994. Prospects in larval fish dietetics. Aquaculture, 124, 223–251.

Watanabe, T., Takashima, F., Ogino, C. and Hibiya T., 1970. Effect of α-tocopherol on carp. Nippon Suisan Gakkaishi, 36, 623–630.

Watanabe, T., Takeuchi, T., Matsui, M., Ogino, C. and Kawabata, T., 1977. Effect of a-tocopherol deficiency on carp: VII. The relationship between dietary levels of linoleate and a-tocopherol requirement. Bull. Jpn. Soc. Sci. Fish, 43, 935– 946.

Watanabe, T., Kitajima, C., Arakawa, T., Fukusho, K. and Fujita, S., 1978a. Nutritional quality of rotifer *Brachionus plicatilis* as a living feed from the viewpoint of essential fatty acids for fish. Nippon Suisan Gakkaishi, 44, 1109– 1114.

Watanabe, T., Arakawa, T., Kitajima, C., Fukusho, K. and Fujita, S., 1978b. Nutritional quality of living feed from the viewpoint of essential fatty acids for fish. Nippon Suisan Gakkaishi, 44, 1223– 1227.

Watanabe, T., Takeuchi, T. and Wada, M., 1981. Dietary lipid levels and a-tocopherol requirement of carp. Nippon Suisan Gakkaishi, 47, 1585-1590.

Watanabe, T., Kitajima, C. and Fujita, S., 1983. Nutritional value of live organisms used in Japan for mass propagation of fish: a review. Aquaculture, 34, 115–143.

Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S. and Kitajima, C., 1989. Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acid efficacy in larval red seabream. Nippon Suisan gakkaishi, 55(9), 1635-1 640.

Wdzieczak, J., Zalesna, G., Wujec, E. and Peres, G., 1982. Comparative studies on superoxide dismutase, catalase and peroxidase levels in erythrocytes and livers of different freshwater and marine fish species. Comp. Biochem. Physiol., 73B, 361– 365.

Willey, S., Bengtson, D.A., Harel, M., 2001. Arachidonic acid requirements for larval summer flounder (*Paraalichthys dentatus*). In: Hendry, C.I., Van Stappen, G., Wille, M., Sorgeloos, P. (Eds.), Larvi 01—Fish and Shellfish Larviculture Symposium European Aquaculture Society, Special Publication, vol. 30. Oostende, Belgium, pp. 634–637.

Wilson, R.P. (ed.) 1991. Handbook of Nutrition Requirements of finfish. C.R.C. Press, Bocaraton, 196 pp.

Wilson, R.P., Bowser, P.R. and Poe, W.E., 1984. Dietary vitamin E requirement of fingerling channel catfish. J. Nutr., 114, 2053– 2058.

Winston, G.W. and Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol., 19, 137– 161.

Woodall, A.N., Ashley, L.M., Halver, J.E., Scott, H.S. and Van de Veen, J., 1964. Nutrition of salmonid fishes: XIII. The α-tocopherol requirement of chinook salmon. J. Nutr., 84, 125– 135.

Yúfera M. and Pascual E. 1984. La producción de organismos zooplanctónicos para la alimentación larvaria en acuicultura marina. Informes Técnicos Instituto de Investigación Pesquera, 119, 3–27.

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Departamento de Biología

Grupo de Investigación en Acuicultura

Instituto Canario de Ciencias Marinas
ICCM