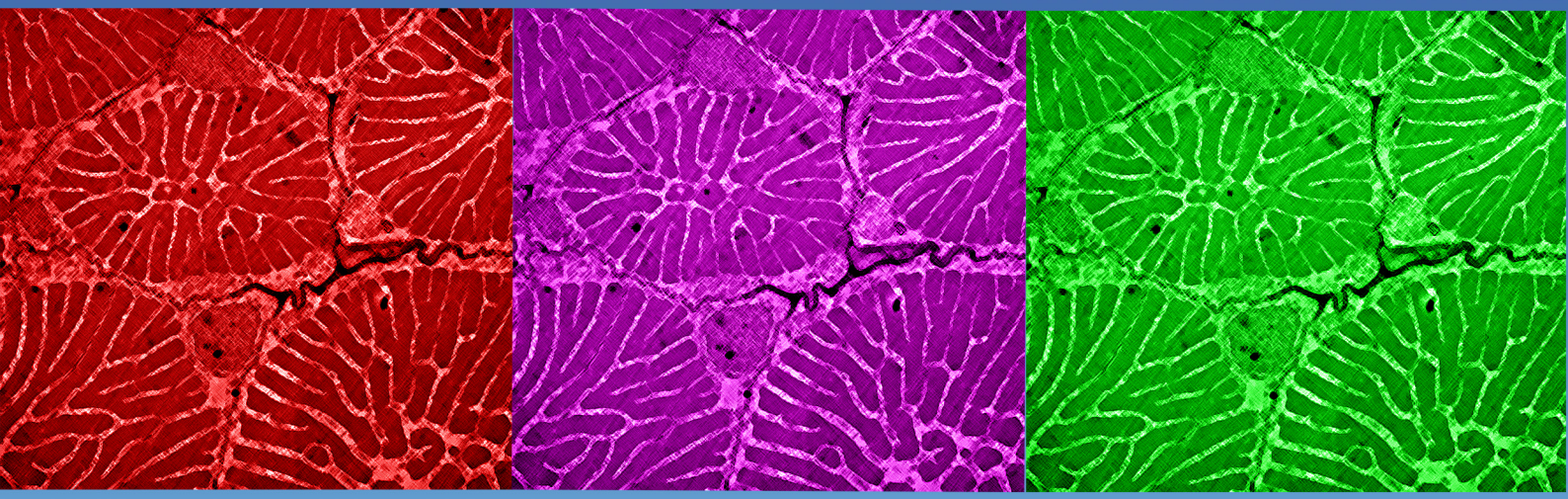
PhD Thesis

Oxidative stress in sea bass larvae (*Dicentrarchus labrax*) fed on high DHA microdiets. Involvement of several antioxidant nutrients



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Las Palmas de Gran Canaria

2012

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Instituto Universitario de Sanidad Animal
y Seguridad Alimentaria

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Oxidative stress in sea bass (*Dicentrarchus labrax*) larvae fed on high DHA microdiets. Involvement of several antioxidant nutrients

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Doctorado en Acuicultura: Producción controlada de organismos acuáticos

Grupo de investigación en Acuicultura (GIA)

Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA)

Thesis for the degree of Doctor of Phylosophy

University of Las Palmas de Gran Canaria

2012

Directors:

Prof. Mª José Caballero and Prof. Marisol Izquierdo

A mis padres

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List of Abbreviations

ALA	α-linolenic acid
ANOVA	Analysis of variance
AOE	Antioxidant enzymes
APROMAR	Asociación Empresarial de Productores de Cultivos Marinos
ATP	Adenosin triphosphate
BHT	Butylated hydroxytoluene
CAT	Catalase
Ct	Cycle threshold
cDNA	Complementary desoxiribonucleic acid
cRNA	Complementary ribonucleic acid
DEPC	Diethyl pyrocarbonate
DHA	Docosahexaenoic acid (22:6n-3)
dNTPS	Deoxynucleotide triphosphates
DPA	Docosapentaenoic acid
dph	Days post hatching
DTT	Dithiothreitol
dTTP	Deoxythymidine triphosphate
dUTP	Deoxyuridine triphosphate
DW	Dry weight
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid (20:5n-3)
EM	Electron microscope
FA	Fatty acid
FAME	Fatty acid methyl esters

FAM-6	6-carboxyfluorescein-labeled probe
FAO	Food and Agriculture Organization
FIED	Flame ionization detector
Ga	Galium
GLM	General Linear Model
HPLC	High performance liquid chromatography
ICCM	Instituto Canario de Ciencias Marinas
ICP-MS	Induced coupled plasma mass spectrophotometry
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IPTG	Isopropil-β-D-1-tiogalactopyranoside
GC	Gas chromatography
GLC	Gas liquid chromatography
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Oxidized glutathione
GSH	Reduced glutathione
HUFA	Highly unsaturated fatty acid
H&E	Haematoxilin & eosin
Laczα	Lactose z gene
LB	Lysogeny broth
LC-PUFA	Long chain polyunsaturated fatty acid
LA	Linoleic acid
MAP	Mitogen-activated protein
MDA	Malonaldehyde
M-MLVRT	Moloney murine leukemia virus
MPC	Myogenic Progenitor Cell

MRFs	myogenic regulatory factors
mRNA	Messenger ribonucleic acid
MW	Molecular weight
MyHC	Myosin heavy chain
nd	Not detected
n-3 LC-PUFA	Long chain polyunsaturated fatty acids of *n-3* series (20 or more carbon atoms)
OLA	Oleic acid
PAS	Periodic acid Schiff staining
PB	Prusian blue staining
PCR	Polymerase chain reaction
PI-3K	Phosphatidylinositol-3-OH-kinase
RNA	Ribonucleic acid
$ROO^{\cdot}$	Peroxyl radical
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Quantitative time real time PCR
SB	Sudan black staining
SD	Standard deviation
SDA	Stearidonic acid
Sc	Scandium
Se	Selenium
SEP	Selenoprotein
SEPP	Selenoprotein P
SOC	Super optimal broth with catabolite repression
SOD	Superoxide dismutase
TAE	Tris-acetate-EDTA

TBARS	Thiobarbituric reactive substances
TEM	Transmission electron microscope
ULPGC	Universidad de Las Palmas de Gran Canaria
UNG	Uracyl-N-glycosylase
UV	Ultraviolet
X-gal	Bromo-chloro-indolyl-galactopyranoside
ZN	Ziehl-Neelsen staining

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Glossary index of common and scientific names used within this thesis

Common name	Nombre común	Scientific name
African catfish	Pez gato	*Clarias gariepinus*
Atlantic salmon	Salmón del Atlántico	*Salmo salar*
Barramundi	Perca gigante	*Lates calcarifer*
Brine shrimp	Artemia	*Artemia* sp.
Brook trout	Trucha de arroyo	*Salvelinus fontinalis*
Brown sole	Acedía del Japón	*Pleuronectes herzensteini*
Cachama	Cachama	*Colossoma macropomum*
Common carp	Carpa	*Cyprinus carpio*
Channel catfish	Bagre de canal	*Ictalurus punctatus*
Common dentex	Dentón	*Dentex dentex*
Coral reef damselfish		*Acanthocromis polyacanthus*
Fat snook	Róbalo	*Centropomus parallelus*
Fine flounder	Lenguado fino	*Paralichthys adspersus*
Grouper	Mero malabárico	*Epinephelus malabaricus*
Halibut	Fletán del Atlántico	*Hippoglossus hippoglossus*
Herring	Arenque del Atlántico	*Clupea harengus*
Indian major carp	Labeo Roho	*Labeo rohita*
Japanese flounder	Falso halibut del Japón	*Paralichthys olivaceous*
Japanese parrotfish		*Calotomus japonicus*
Manchurian trout		*Brachymystax lenok*
Matrinxa	Sábalo cola roja	*Brycon cephalus*
Milkfish	Chano	*Chanos chanos*

Rainbow trout	Trucha arcoíris	*Oncorhynchus mykiss*
Red sea bream	Dorada del Japón	*Pagrus major*
Rotifer	Rotífero	*Brachionus plicatilis*
Sea bass	Dorada	*Dicentrarchus labrax*
Sea bream	Lubina	*Sparus aurata*
Senegalese sole	Lenguado senegalés	*Solea senegalensis*
Spotted wolffish	Perro pintado	*Anharhichas minor*
Stripped jack	Jurel dentón	*Pseudocaranx dentex*
Striped trumpeter		*Latris lineata*
Tilapia	Tilapia azul	*Oreochromis aureus*
Turbot	Rodaballo	*Psetta maxima*
Yellowtail	Medregal del Japón	*Seriola quinqueradiata*
Zebrafish	Pez cebra	*Danio rerio*

Acknowledgements

If I have been able to write this Thesis is thanks to the help of many people I would like to remember…

Con el permiso de mis tutoras, me gustaría empezar esta sección agradeciendo el apoyo incondicional de dos personas que siempre han confiado en mí, han sido un modelo ejemplar de esfuerzo y superación y me han animado a seguir mis sueños, porque nada hay inalcanzable. Es por esto y mucho más por lo que esta Tesis va dedicada a ellos ¡Gracias papis!

I would like to give my sincerest gratitude to my supervisors, Dr. Mª José Caballero and Dr. Marisol Izquierdo for their steady guidance during all these years. Your enthusiasm has influenced on me and been very important for this Thesis, as you have always encouraged me and listened to my imaginative theories. Thank you also for giving me the opportunity to present my work in different conferences and for bringing myself to make several stays that have enriched my scientific knowledge. Thanks for supporting and trusting on me!

My gratitude goes to Dr. Genciana Terova for opening me the doors of her lab and letting me to introduce myself into a new and unknown world. Thanks to Samuela, Elena and Simona for your patience and guidance into the gene expression analysis. I would also like to thank Dr. Gordon Bell for accepting me into his lab and giving me the chance to complete my scientific skills.

Many thanks to all the GIA members for your help during these years. I would particularly like to thank Dr. Juanma Afonso for helping me with the statistical analysis, Dr. Javier Roo for his advices on larval culture. Thanks also to Dr. Carmen Mª Hernández-Cruz for always giving a hand in samplings and for showing me the passionate world of larvae culture through university lessons…

I would also like to thank my good collegues at the GIA for some funny years. Special thanks to Tibi, Silvia, Reda, Fefy, Tati, Pedro, Mohamed, Juan, Fran, Alex, Gercende, Antonio, Davinia and Laura. Although I had my ups and downs you were always there for supporting and encouraging me and thanks to your sustain I can be right here defending my Thesis. I would also like to remember those who have gone past. Without their help this document would not be real, so thanks to Eyad and Maria, because part of you is on this Thesis.

A big thank you is due to the GIA staff, especially to the “mesocosms team”. Thanks Ruben, Desi and Enma for guiding and helping me during the running of my trials. Thanks also to Ada, Lore, Carmen, Silvia and Yurena for their technical advices and smiles. Many thanks to the “Infectious Diseases girls” Lorena, Vale, Judith and Fatima. Thanks to you and your ph-meter!

I am grateful to my colleagues from the Department of Morphology from the ULPGC for their kind help and for allowing me to use their facilities. Especial thanks goes to Alejandro and Oscar for their friendship during these years and also for all those “venting chats”.

I am grateful to the Cabildo de Gran Canaria and the Fundación Universitaria de Las Palmas for financing my research and to the Instituto Canario de Ciencias Marinas where all the experiments of the present Thesis were carried out. I would also like to thank the Instituto de Acuicultura de Torre de la Sal for kindly providing the sea bass larvae employed for this Thesis and to Croda Iberica for supplying the oils employed in diets formulation.

Many thanks to Matthew for taking the time (not much really) to review my works, but mainly for standing and calming me down when I have my fit of hysterics… Hope you can still be my diazepam in the coming years.

I would like to deeply thank my “out-door” friends. I know sometimes you do not understand me (fish can be stressed? Put larvae into a plastic bag and come to the beach!) but you have been with me since school times and have always supported me. Gracias Cris, Ele, Bea, Vane, José, Antonio, Vane, Mer, Car, Davi, Mari, Ino and Eli!

Finalmente, también me gustaría agradecer a toda mi familia la comprensión que han tenido conmigo estos años de ausencias a asaderos, sancochos, “recogidas” y otros eventos familiares. Prometo que de ahora en adelante no me pierdo uno.

Gracias a todos

Mónica

Chapter 1

General Introduction

1.1 Aquaculture production

The overexplotation of traditional fishing areas and the limitations imposed by the changes in the policy and agreements on international fishing areas caused a chronic restriction in marine products supply. According to FAO previsions, to satisfy the high demand of marine products, aquaculture is a feasible complement to wild captures. In fact, aquaculture accounted for 47.9% of total food fish supply in 2010, representing a continue increase from 42.7% in 2006 (FAO, 2011). In this sense, aquaculture remains a growing, vibrant and important sector for high-protein food and continues to be the fastest-world animal-food-producing system and to outpace population growth, with a global production that increased from 7.3 millions mT in 1980 by weight to 55.1 millions mT in 2009 (FAO, 2011). This exponential increase in production could be accomplished by the improved control of fish reproduction, the development of optimized diets and technological innovations that allowed the fast development of land- and sea-based aquaculture facilities. However, growth rates for aquaculture production are slowing, varying greatly among regions. For instance, the once leading countries in aquaculture development such as Japan, Spain or France have shown falling production in the last decades (FAO, 2011). Similarly, the average annual growth in aquaculture production in Europe and North America since 2000 has also slowed substantially to 1.7% and 1.2% respectively.

The aquaculture production in the European Union in 2008 was 1.2 millions mT (FAO, 2011), with a market value of 3.8 millions €. However, the aquaculture production rate has been decreasing since 1988 and has not been enough to fulfil the decrease in capture fisheries. Spain is the European country with a higher aquaculture production in tons (249,070 mT; 19.5%) followed by France (237,870 mT; 18.6%) and Italy (181,470 mT; 14.2%). The main marine fish species produced in southern Europe aquaculture are sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Figure 1.1),

with a production of 89.3 and 58.5 mT respectively, being the Canary Islands the main producers in Spain (32%; APROMAR, 2010). To maintain these high productions, quality juveniles must be provided by hatcheries. In 2009 there were 16 hatcheries in Spain producing 69.3 millions of sea bass and sea bream fingerlings, a reduction of 26.5% from production levels in 2008. Besides, this quantity is not enough to cover the farms demand, so importations from other European countries, such as France, Italy or Greece are necessary. It fits to mention that aquaculture in Canary Islands completely depends on sea bass fry imports due to the absence of any local commercial hatchery.

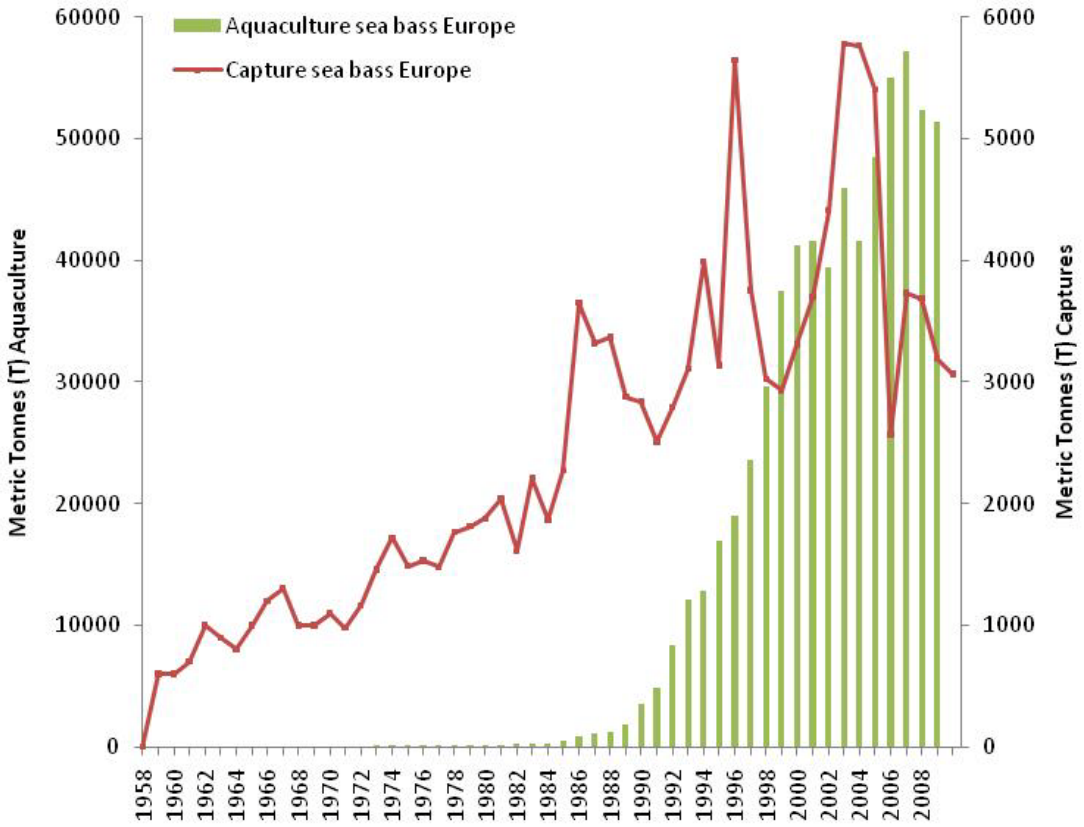

Figure 1.1 Aquaculture production *versus* captures of European sea bass in Europe from 1958 to 2009 (data obtained from FAO FISHSTAT Plus 2.30).

1.2 Rearing techniques and production considerations

The hatchery phase remains as one of the production bottlenecks in marine fish aquaculture, mainly due to the poor development, high mortality rates (over 75%) and weakness of fish larvae at hatching. Marine fish species egg hatching is produced after some days, depending on water temperature, leading to a secondary embrionary stage (Figure 1.2). Yolk reserves are mainly formed by lipoproteins and in some species,

including sea bass, by a lipid droplet (Sargent *et al.*, 2002). These components will be employed for tissues formation or to obtain energy. The embryo phase is terminated when the esophagus opens and the larval phase commences with exogenous feeding (Gatesoupe *et al*., 2001; Figure 1.2). At present, the accepted practice worldwide for the intensive larval rearing of marine fish is the feeding with live prey during the first weeks, followed by a gradual weaning to a dry diet (Fernández-Diaz and Yufera, 1997; Kolkovski *et al*., 1997). Live preys are a heterogeneous group of organisms belonging to different phytoplankton and zooplankton groups, such as crustaceans, mollusks or protozoans. Rotifers (*Brachionus* sp.) and the brine shrimp (*Artemia* sp.) are extensively used as live food for the larval stages of commercially important fish species, using the former during the first days of exogenous feeding and *Artemia* nauplii when the larvae has reached a larger size. The use of different strains of rotifers is due to the easiness of its mass production, its adequate dimensions to fit mouth size of early larvae, its planctonic habits, slow movements and the possibility to control its nutritional value for fish larvae. However, the nutritional value of this live prey varies notably according to its feed and environmental conditions such as light intensity and photoperiod, temperature or salinity. As pointed out previously, rotifers are progressively replaced by brine shrimp from 9 days post hatching (dph) onwards and, subsequently, with dry feeds of different dimensions (Barnabé, 1974). In sea bass larvae, due to their large mouth diameter, larvae can be fed with *Artemia* nauplii straight after mouth opening instead of rotifers.

Nevertheless, neither rotifer nor *Artemia* constitute the natural preys of marine fish larvae, a fact that frequently causes several problems in larval production leading to high mortalities, imposing serious constrictions to the further development of larval rearing production and controlled quality. Thus, the improvement of larval rearing, and especially larval fish nutrition, remains to be one of the principal objectives of the aquaculture industry in order to improve competitiveness.

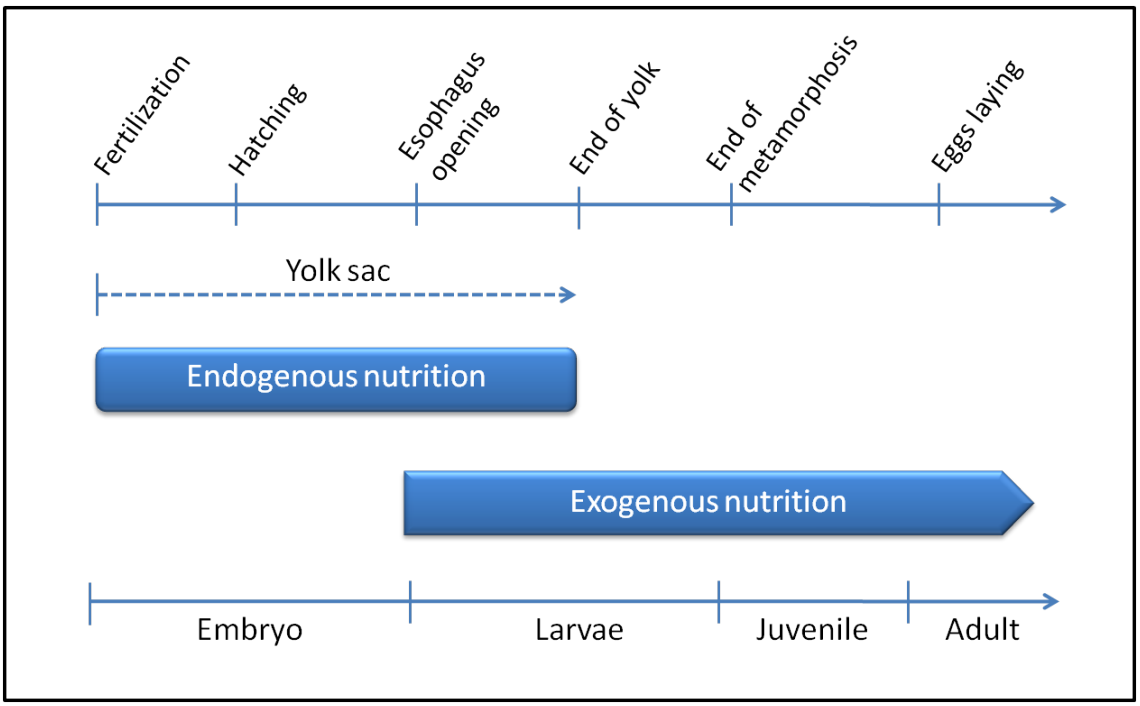

Figure 1.2 Development stages and nutrition schedule during fish life. Adapted from Gatesoupe *et al*., 2001.

1.3 Use of inert microdiets for marine fish larvae

The use of live preys in the first stages of fish culture results in some difficulties. The extra effort in terms of manpower, infrastructure, time and energy to produce rotifers and *Artemia* species represents a significant cost. In this sense, Person-Le Ruyet *et al.* (1993) calculated the cost involved with feeding live prey to sea bass larvae. The live food accounted for 79% of the production costs for juveniles up to 45 dph. In the first three months of life, live food represented 50% of the feed cost even though they represented only 1.6% of the total dry weight of the food required.

In addition, the supply and nutritional quality of live food can vary, providing a sub-optimal nutrition to the larvae (Jones *et al*., 1993; Kolkovski *et al*., 1993; Barnabé and Guissi, 1994; Roselund *et al*., 1997). Besides, from an experimental point of view, live prey enrichment with some nutrients such as phospholipids (PLs) or protein is limited since they are genetically determined (Roselund *et al*., 1997; Koven *et al*., 2001). For these reasons and for those stated before, compound diet substitution for live prey is crucial for sustaining cost effective production of high and constant quality juveniles.

However, a lower performance is commonly reported when inert diets are fed to larvae from the onset of exogenous feeding. This may be due to the composition, palatability or physical characteristics of dry feed (Person Le Ruyet *et al*., 1993) or an inability to properly digest it (Holt, 1993; Kolkovski *et al*., 1993; Walford and Lam, 1993; Zambonino-Infante and Cahu, 1994; Kolkovski, 2001). The feeding of an inert diet may also decrease water quality if not carefully controlled (Leu *et al*., 1991). Nevertheless, results can be clearly improved when inert food is combined with live prey (co-feeding) from first feeding (Fernández-Díaz and Yúfera, 1997; Kolkovski *et al*., 1997; Rosenlund *et al*., 1997; Sandel *et al*., 2010).

Microparticulated diets can be prepared in microbound, microcoated or microencapsulated. In microbound diets, the powdered ingredients are microbound with a water stable matrix such as agar, carrageenan or calcium alginate (López-Alavarado *et al*., 1994) or by a protein such as casein or zein (Person-Le Ruyet *et al*., 1993). The manufacturing process of microbound diets is the simplest and most commonly used method, as it has been suggested that due to the absence of a capsule are easily digested and attraction is increased through greater nutrient leaching (Kolkovski, 2001; Yúfera et *al*., 2003; Kolkovski, 2006). Microcoated diets are produced by coating the powdered ingredients with a glucidic (carraggeenan, alginate), proteic (gelatin, zein) or lipid binder to reduce leaching (Onal and Langdon, 2004). Microencapsulated diets are defined as microparticulate diets made by encapsulating a solution, colloid or suspension of diet ingredients within a membrane and is produced with a cross-linking agent (Yúfera *et al*., 1999). The capsule wall helps maintain the integrity of the food particle until it is consumed preventing leaching and degradation of the nutritional ingredients in the water. Microencapsulation produces regular shape and water soluble microparticles, but the microcapsules can be difficult to digest and can reduce the larvae attraction to the food particles (Yúfera *et al*., 2003; Kvåle *et al*., 2006; Kolkovski, 2008).

The production of microparticulate diets could be a convenient and economic alternative to live feed, in spite of the problems of nutrient leaching and water stability normally encountered when producing microparticles (Baskerville-Bridges and Kling, 2000; Onald and Landgon, 2000; Pousão-Ferreira *et al.*, 2003). An integrative approach needs to be taken in the development of microdiets for fish larvae, taking into account the physiology and ontogeny of the larval digestive system, feed technology (leaching, binders, feeding regime) as well as nutritional requirements. In this sense, the nutritional components of microparticulate diets for fish larvae should be determined on the basis of the requirements of the larval fish for proteins, amino acids, lipids, carbohydrates,

vitamins and minerals. Moreover, the efficient development of microparticulate diets for the fish larvae has promoted the improvement of nutritional requirement studies.

The optimum protein level for fish larvae differs, probably due to a variety of factors, such as the differences in food habits, age of larvae, water temperature, protein sources used and energy level of the diet. However, because the requirements of larval fish are still undefined, protein sources having high nutritional values are used, such as krill meal, squid meal, scallop meal or fish meal (Teshima *et al*., 1982). Knowledge about the vitamin requirements of larval fish are limited. Most studies on vitamin requirements of fish have been conducted on juveniles. Few studies exist on vitamin requirements in fish larvae, mainly because there were no efficient compound diets available for these developmental stages. On the other hand, many experiments have been conducted to determine the optimal lipid composition in diets formulated for marine fish larvae, paying particular attention to long chain polyunsaturated fatty acids (LC-PUFA) and PL requirements (Takeuchi, 1997). Lipids included in microparticulate diets come, in part, from meals incorporated in the diet as protein sources. Other lipids, such as cod liver oil, roe oil or menhaden oil are added as triglycerides and PLs come from soy lecithin (terrestrial) or marine PLs (from fish or krill). To have a better control of the lipid fraction in the diet, meals can be defatted, so that lipids will result from the addition of different oils.

1.4 Importance of lipids in marine fish larvae nutrition

Lipids are, along with proteins, the major organic constituents of fish, with carbohydrates being quantitatively less prominent. Indeed the lipid content of fish can markedly exceed the protein content, reflecting the major role of lipids (Sargent *et al*., 2002). Lipids are an important source of metabolic energy, components of biological membranes and precursors of essential metabolites (Sargent *et al*., 1989). Dietary lipids provide a rich source of energy and PLs, vital to the structure of biomembranes. Besides, 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. On the other hand, lipids are components of hormones and precursors for synthesis of various functional metabolites, such as prostaglandins. Besides, lipids are of particular importance in teleost fish larvae, which are characterized by extremely high growth rates coupled with high demands for energy and structural components. Additionally, a relationship exists between dietary lipids and skeletal formation in aquatic animals (Cahu and Zambonino-

Infante, 2003; Villeneuve *et al.*, 2005, 2006; Roo *et al*., 2009; Izquierdo *et al*., 2010; Sandel *et al*., 2010; Scolamacchia, 2010). As a reflect of this importance, for instance in marine fish species such as the sea bream and the sea bass diets have become highly energetical (~25% lipid) in comparison with a decade or so ago (~12% lipid) (Izquierdo *et al*., 2003). The importance of lipids in marine larvae nutrition has made them the focus of numerous studies. Therefore, the success of larval rearing is greatly influenced by first-feeding regimes and the nutritional quality of the diets used, with dietary lipids being recognized as one of the most important nutritional factors that affect larval growth and survival (Izquierdo *et al.*, 2000).

However, dietary lipid utilization by the larvae is directly or indirectly affected by several morphological and physiological changes occurring during larval development. For instance, although at the end of the larval lecithotrophic phase the enterocytes of sea bass larvae are functional, they are still poorly developed (Deplano *et al*., 1991; Zambonino-Infante *et al*., 1997), their size, number and expansion of organelles being increased in the following days. Therefore, throughout larval development the number of intestinal folds is also increased, the stomach is formed and its function improved. These changes in enterocytes and the digestive system imply an improvement in the digestion and absorption efficiency of the juvenile. Another important fact is that lipid levels may affect larval lipid digestion capability. Hoehne (1999) and Olsen *et al*., (2000) indicated that a high dietary lipid level might lead to lower larval digestion ability. Similarly, Kjørsvik *et al*., (1991) reported an overload of the digestive capacity in the hindgut of turbot larvae fed rotifers with a high lipid content. However, conversely to these results, Zambonino-Infante and Cahu (1999) proved an earlier maturation of enterocytes in sea bass larvae fed high lipid levels as well as better larval development.

Lipids are constituted by fatty acids (FA), being fish rich in LC-PUFA with carbon chain lengths of 20 or more carbons and 3 or more ethylenic bonds. In order to achieve a normal growth and development including reproduction, fish require three LC-PUFA: docosahexaenoic acid (DHA; 22:6*n*-3), eicosapentaenoic acid (EPA; 20:5*n*-3) and arachidonic acid (ARA; 20:4*n*-6). The essentiality of these fatty acids is corroborated as they are selectively retained throughout embryonic development (Rainuzzo *et al*., 1993; Lie, 1993) or at the expense of other fatty acids during periods of starvation (Tandler *et al*., 1989). Besides, these fatty acids are considered essential in marine fish species due to the limited activity of Δ6- and Δ5-desaturase and elongase enzymes to synthesize ARA, EPA and DHA when their precursors are included in the diet, in contrast to fresh water species (Figure 1.3). In this sense, although certain Δ6-desaturase expression has

been found in sea bream larvae, its activity was not enough to fulfil sea bream requirements for this fatty acid (Izquierdo *et al*., 2008).

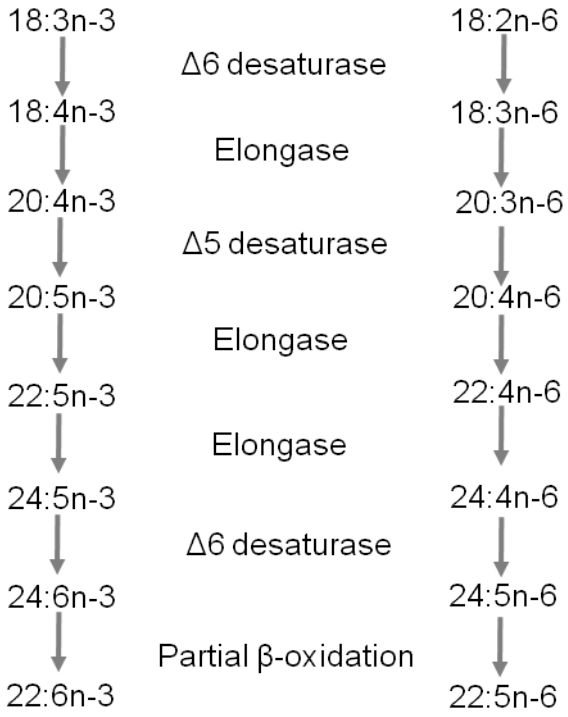

Figure 1.3 Main biosynthesis pathways of LC-PUFA from C18 precursors, α-linoleic acid (18:2*n*-6) and γ-linolenic acid (18:3*n*-3). Enzyme Δ6 desaturase acts in two steps, whereas Δ5 desaturase acts only in one (Sargent *et al*., 1995).

The biochemical, cellular and physiological functions of these three PUFAs are broadly the same in fish as in other vertebrates and fall into two categories:

(1) Role in maintaining the structural and functional integrity of cell membranes: Fish tissues have in general much higher concentrations of DHA and EPA than ARA and fish have correspondingly high dietary requirements for *n*-3 LC-PUFA. As structural components of the membrane phospholipids, essential FA (EFA) can facilitate key-intramembranal reactions and processes, where DHA is particularly important (Izquierdo and Koven, 2010)

(2) Precursors of the group of highly biologically active hormones, known as eicosanoids: Eicosanoids are a range of highly active C20 compounds formed in small or even trace amount by virtually every tissue in the body, are involved in a great variety of physiological functions and are produced in response to stressful situations. The major precursor of eicosanoids in fish is

ARA with eicosanoids formed from EPA being less biologically active than those formed from ARA (Tocher, 2003).

Thus, adequate levels of lipids, especially LC-PUFA, must be included in marine fish diets. However, this is not always feasible. As it has been pointed out previously, aquaculture is one of the most rapid developing animal production systems. To sustain such fast growth, an increased demand in fishmeal and fish oil is required. However, the use of ingredients of terrestrial vegetal origin is necessary to fullfil lipid sources demand. These ingredients lack essential fatty acids (EFA) and this restricts their use in diets for marine species. Therefore, requirements for such fatty acids must be precisely determined to predict optimal levels of inclusion. To study requirements of these fatty acids in any fish larvae species some premises must be taken into account:

- The marked chemical similarities of the three LC-PUFA lead to competitive interactions in the plethora of biochemical and physiological reactions they and their precursors and products undergo.
- Marine fish species have a limited ability to synthesize EFA from their precursors by the action of elongase and desaturase enzymes, requiring DHA, EPA and ARA to be supplied by their diets.
- EFA requirements could be affected both quantitatively and qualitatively by environmental factors such as temperature (Farkas *et al*., 1980; Olsen *et al*., 1999), salinity (Borlogan and Benítez, 1992) and light (Ota and Yamada, 1971) or the presence of other nutrients such as antioxidants (Izquierdo and Koven, 2010).
- As larvae grow faster than juveniles or adults, their necessity of *n*-3 LC-PUFA is expected to be higher than juveniles in the early stages of development (Izquierdo *et al*., 1989a).

The necessity of these fatty acids was first pointed out by the reduced growth observed in turbot (*Scophtalmus maximus*) (Gatesoupe and Le Millinaire, 1985), red sea bream (*Pagrus major*) (Izquierdo *et al*., 1989a, 1989b) and gilthead sea bream (Rodríguez *et al*., 1993, 1994; Salhi *et al*., 1994) larvae fed deficient levels of EFA. Similarly, a drop in survival was observed when larvae were fed low levels of these fatty acids in red sea bream (Izquierdo *et al*., 1989a, 1989b), sea bream (Rodríguez *et al*., 1993, 1994; Salhi *et al.*, 1994) and halibut (*Hippoglossus hippoglossus*) (Holmefjord and Olsen, 1991). On the other hand, insufficient contents of these EFA in the diet give rise to several alterations in larvae such as poor feeding and swimming activities (Izquierdo *et al*., 1989a; Rodríguez *et al*., 1993, 1994), altered fish larvae behavior (Benítez-Santana *et al*., 2007), hydrops (Yamashita, 1981), deficient swim bladder inflation

(Koven, 1991), abnormal pigmentation (Kanazawa, 1993; Rainuzzo *et al*., 1994), disaggregation of gill epithelia (Arnaiz *et al*., 1993), immune-deficiency and raised cortisol levels (Izquierdo, 1996).

Competitive interactions exist between DHA and EPA or EPA and ARA. The DHA and EPA competition results from both molecules using the same enzymes to esterify fatty acids into PL structures (Mourente *et al*., 1991; Sargent *et al.*, 1999). However, in all marine fish larvae, DHA has been demonstrated to be superior to EPA in conferring vitality to the larvae (Watanabe, 1993). Besides, although both EFA have been found to be essential to marine fish larvae, DHA is generally present at about twice the level of EPA in fish membrane PLs, being the highest levels of DHA contained in phosphatidylethanolamine (PE; Rodríguez *et al*., 1997; Copeman *et al*., 2002). All these evidences prove the essentiality of this fatty acid regarding to the other EFA.

1.5 Importance of DHA for larvae

DHA is a carboxylic acid with a 22-carbon chain and six *cis* double bonds. As stated previously, marine fish have a limited, almost negligible, capability to synthesize EFA from their precursors, linoleic and linolenic acids, and, therefore, it must be included in sufficient amounts in larval feeds. Besides, adequate DHA supply is very important in rapidly growing and developing marine fish larvae (Table 1.1). These high requirements of DHA are reflected in larval tissue composition, being incorporated in the developing visual and neural tissues (Mourente, 2003), which, at this stage, account for a higher percentage of neural tissue in their relatively small body mass.

The particular structure of DHA provides this fatty acid with many important functions in fish metabolism (Izquierdo, 2005). It is incorporated into cell membranes regulating membrane integrity and function, this fatty acid being an important component of phosphoglycerides, particularly PE and phosphatidylcholine in larvae. The essentiality of DHA for fish is corroborated by its retention in starved or low-EFA fed fish, possibly due to the lower cell oxidation rates than other fatty acids (Koven *et al.*, 1989; Madsen *et al*., 1999). Still in terms of essentiality, it seems to have a greater potential than EPA to promote growth and stress resistance in red sea bream among other species (Watanabe *et al*., 1989; Watanabe and Kiron, 1994), being its requirement more limiting for growth and survival than those for *n*-3 LC-PUFA (Izquierdo, 1996).

Table 1.1 DHA requirements for fish larvae of different marine species (% DW in rotifers, *Artemia* or microdiets)

Species	Tested parameter	Requirement	Reference
Acanthochromys poliacanthus	Growth Survival	0.5	Southgate and Kavanagh, 1999
Calotomus japonicus	Growth	1-2	Kanazawa, 1993
Centropomus parallelus	Growth Survival Swim bladder inflation	>0.6	Seiffert *et al.,* 2001
Dentex dentex	Growth Survival	2.4	Mourente *et al*., 1999a
Hippoglossus hippoglossus	Pigmentation Eye migration	2.5	Hamre and Harboe, 2008
Latris lineata	Growth Survival Behaviour	1.3	Brandsen *et al*., 2004
Pagrus major	Growth Survival Salinity tolerance Growth Survival	0.95-1.62 1.5 Rotifers or *Artemia*	Furuita *et al*., 1996a Izquierdo *et al*., 1990
Pagrus pagrus	Growth Survival	1.5	Hernández-Cruz *et al*., 1999
Paralychthis olivaceous	Growth	1	Kanazawa, 1995
Psetta maxima	Growth	0.8	Reitan *et al*., 1994
Pseudocaranx dentex	Growth	1.6-2.2	Takeuchi *et al*., 1996
Pseudopleuronectes herzensteini	Growth Survival Starvation resistance Larval development	0.6 Rotifers 1.4-2.8 *Artemia*	Satoh and Takeuchi, 2009a
Sparus aurata	Growth Survival	>3	Izquierdo, 2005
Scophtalmus maximus	Growth	3.2	Le Milinaire, 1984
Seriola dumerilii	Growth Survival	4	Izquierdo, 2005

The importance of DHA for larvae, starts prior to the hatching. Eggs contain an adequate content of DHA (Laurel *et al*., 2010) to ensure proper larval development on hatching, and this can be achieved by controlling broodstock feeding (Fernández-Palacios *et al*., 2011). Thus, the DHA, vital for early survival and development of newly hatched larvae is determined by the lipids derived directly from the dietary input of broodstock in the period preceding gonadogenesis (Kjørsvik *et al*., 1990; Sargent, 1995). However, Watanabe (1993) reported that the DHA content in marine fish larvae rapidly

decreases during the first ten days after hatching, therefore high contents of DHA must be supplied to larvae in order to maintain the adequate levels of DHA in growing larvae.

A deficiency in this fatty acid has been shown to cause alterations of the neurological system in fish, such as impaired vision at low light intensities in herring (*Clupea harengus*) (Bell *et al*., 1995) or retarded development of normal behavior in yellowtail (*Seriola quinqueradiata*) (Masuda *et al.*, 1998), striped trumpeter (*Latris lineata*) (Brandsen *et al*., 2005) and sea bream (Benítez-Santana *et al*., 2007) larvae. Besides, low dietary levels of DHA can increase the incidence of skeletal deformities in sea bass (Cahu *et al*., 2003), gilthead sea bream (Roo *et al*., 2010), red porgy (*Pagrus pagrus*) (Roo *et al*., 2009; Izquierdo *et al*., 2010) or brown sole (*Pseudopleuronectes herzensteini*) (Satoh and Takeuchi, 2009b) larvae, reduce tolerance to stressful situations in beluga (*Huso huso*) larvae (Jalali *et al*., 2008), cause malpigmentation and irregular eye migration in flatfish (McEvoy *et al*., 1998; Bell *et al*., 2003) or altered gut and liver structure in striped trumpeter larvae (Brandsen *et al*., 2005). But the main detrimental effects observed may be those related to the low culture performance and survival in different larval species (Watanabe *et al*., 1989; Furuita *et al*., 1996a, b; Copeman *et al*., 2002; Rezek *et al*., 2010).

Not only the decrease in dietary DHA can cause alterations in fish, also excessive levels of this EFA may lead to several changes, especially when the increase in *n*-3 LC-PUFA is not accompanied by adequate quantities of antioxidants. For instance, an excessive amount of PUFA accelerated osteoblasts differentiation when dietary vitamin A levels were low, causing supranumerary vertebrae in sea bass larvae (Villeneuve *et al*., 2006). Thus, to avoid adverse effects and improve performance, supplementation of antioxidants, such as vitamin E are necessary when high levels of *n*-3 LC-PUFA are incorporated into larval diets. For instance, beluga larvae fed with high LC-PUFA enriched *Artemia* showed a better performance in terms of growth and tolerance to salinity stress when a 20% of α-tocopherol was included in the enrichment media (Jalali *et al*., 2008). Similarly, Stéphan *et al*. (1995) reported that *in vivo* and *in vitro* oxidation of lipids in turbot larvae muscles is reduced when dietary α-tocopherol was supplemented in the diet. The supplementation with antioxidants is necessary as the susceptibility of a particular PUFA toward oxidation increases with an increase in the number of unsaturated sites in the lipid chain due to the vulnerability of the methylene group located between two double bonds (Nagaoka *et al*., 1990). Since DHA has five of these methylene groups, it is particularly oxidizable. Therefore, DHA will be highly at risk of suffering oxidative processes that any other PUFA.

Typically, high amounts of DHA can be found in commercial preparations used to enrich the live preys (Koven *et al*., 2001) to compensate the reduced capability of marine fish larvae to convert linolenic acid to DHA. This trend is being followed in marine fish larvae microdiets formulation, by the premise that high DHA contents will lead to higher culture performances, therefore much money is spent to include concentrated DHA oils. However, it has been shown that too high levels of this EFA could have detrimental effects on larvae (Brandsen *et al*., 2005; Villeneuve *et al*., 2005; Izquierdo *et al*., submitted) or even have no effects at all (Kraul, 1993; Seiffert *et al*., 2001), therefore high inclusion of DHA in microdiets for marine fish larvae needs a deep revision.

1.6 Reactive oxygen species: Generation, detoxification and oxidative stress

The evolution of aerobic metabolic processes such as respiration, unavoidably leads to the production of reactive oxygen species (ROS), all having a high capacity to cause oxidative damage to proteins, DNA and lipids. As it has been pointed out previously, fish and, specially, fish larvae, are highly at risk of suffering peroxidative attack to their membranes, as they contain great quantities of LC-PUFA. To counteract ROS negative effects, complex arrays of non-enzymatic and enzymatic detoxification mechanisms exist. However, when ROS production exceeds their removal by these mechanisms oxidative stress occurs and ROS may cause cellular damages and alterations on the different cellular elements.

1.6.1 Generation of ROS

Molecular oxygen, essential for aerobic organisms, has a dominant role in eukaryotes being that of terminal electron acceptance in mitochondrial respiration, where it is ultimately reduced to water during the process of oxidative phosphorilation, the major source of ATP in aerobes. Oxygen may be converted to the much more reactive ROS forms either by energy transfer or by electron transfer reactions (Figure 1.4). The former leads to the formation of singlet oxygen, whereas the latter results in the sequential reduction to superoxide, hydrogen peroxide and hydroxyl radical (Klotz, 2002).

Pathological effects of ROS depend on the free radical produced. Superoxide radical ion ($O_2^{\cdot -}$) stimulates the production of leukocyte and other cells degrading

enzymes. Can cause direct lesions to lipids, proteins and DNA and usually acts close to the place where it is produced. Hydrogen peroxide (H_2O_2) can be converted to hydroxyl radical (OH·) or hypochlorite (OCl^-), both having capacity to destroy microbes and cells. This free radical can act away from their production site. Hydroxil radical represents the most common free radical derived from oxygen and is mainly responsible of lipid, protein and DNA attack.

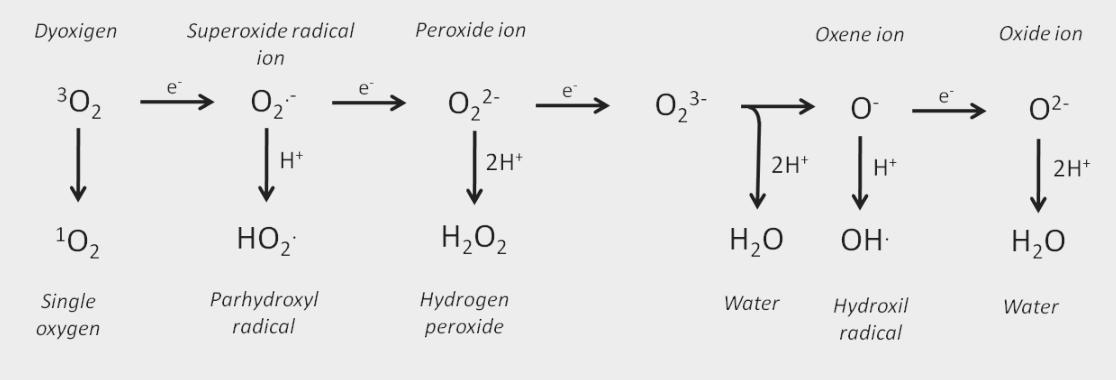

Figure 1.4 Generation of different ROS by energy transfer or sequential univalent reduction of ground state triple oxygen. Adapted from Apel and Hirt (2004).

In addition to mitochondrial electron transport, other sources of endogenous ROS production include the electron transport chains of microsomes (Winston and Cederbaum, 1983), the respiratory burst associated with phagocytosis by leukocytes (Chung and Secombes, 1988) and the activities of enzymes, such as xanthine oxidase, tryptophan dioxygenase, diamine oxidase and prostaglandin synthase (Fridovich, 1978; Halliwell, 1978).

1.6.2 ROS detoxification: antioxidant defence mechanisms

An antioxidant is any substance that, when present at low concentrations compared to those of an oxidable substrate is able to interact with free radicals to terminate the reaction (Halliwell and Gutteridge, 1990). An array of antioxidants defence mechanisms to detoxify ROS has evolved to counteract the potentially deleterious effects of activated oxygen (Yu, 1994). The antioxidant systems in living organisms may be divided in two types, one is represented by enzymes and the other one by low

molecular weight molecules. These compounds may be found in the cell plasma, mitochondria or cell membranes (Figure 1.5). Antioxidants can act at different stages in the oxidation process and some may have more than one mechanism of action. They can exert their action by different mechanisms such as breaking the chain of an initiated sequence, scavenging singlet oxygen or decreasing local O_2 concentrations.

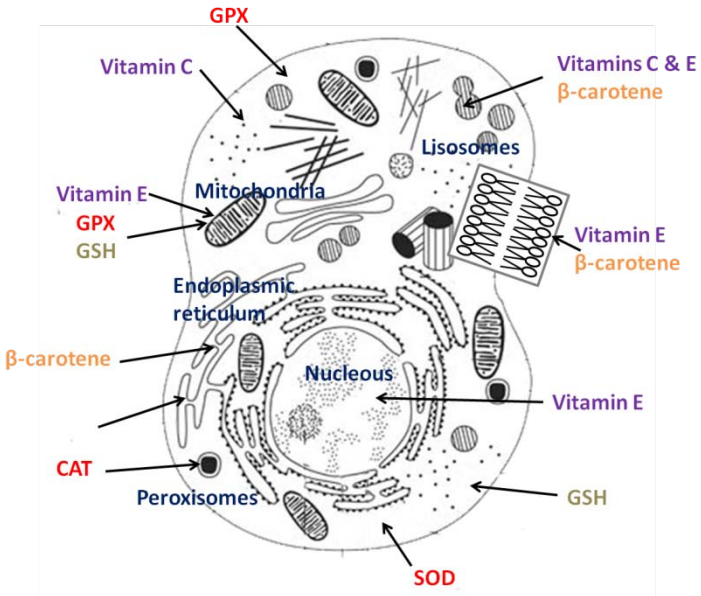

Figure 1.5 Antioxidant mechanisms within the cell. Adapted from Machlin and Bendich (1987). CAT= Catalase; SOD = Superoxide dismutase; GPX = Glutathione peroxidase; GSH = Reduced glutathione.

1.6.2.1 Enzymatic systems

Diverse antioxidant enzymes (AOE) prevent the cascade of oxidant reactions, intercepting and inactivating the reactive intermediates of oxygen. Particularly adapted enzymes such as superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GPX; EC 1.11.1.19), glutathione reductase (GR) or catalase (CAT; EC 1.11.1.6) have been detected in most fish species studied to date (Rudneva, 1997). All these enzymes are very important in antioxidant defence, although SOD plays a crucial role being the first enzyme responding to oxygen radicals and preventing the initialization of the radical chain reaction that the superoxide anion produces (McCord and Fridovich, 1969; Winston and Di Giulio, 1991). Superoxide is converted to hydrogen peroxide by the action of SOD (Figure 1.6). SOD does not easily cross biological membranes, consequently it must be detoxified in the compartment where it is generated (Fridovich 1995). This explains the presence of different SOD in eukaryotes, which are classified in two distinct groups according to metal content; manganese SOD and copper/zinc SOD.

Hydrogen peroxide is subsequently metabolized to oxygen and water by the selenium-containing enzyme GPX, which uses glutathione (GSH) as a cofactor in the reaction. GPX converts most of the hydrogen peroxide in the cytoplasm and there is also evidence that GPX plays a role in cellular signaling by mediating hydrogen peroxide concentrations (Brigelius-Flohe, 1999; Figure 1.6). There are four main GPX; cytosolic (GPX1), gastrointestinal (GPX2), plasma (GPX3) and phospholipid hydroperoxidase (GPX4). Selenium is required at the active sites of all the GPXs. Finally, GR reduces the oxidized glutathione (GSSG) into GSH.

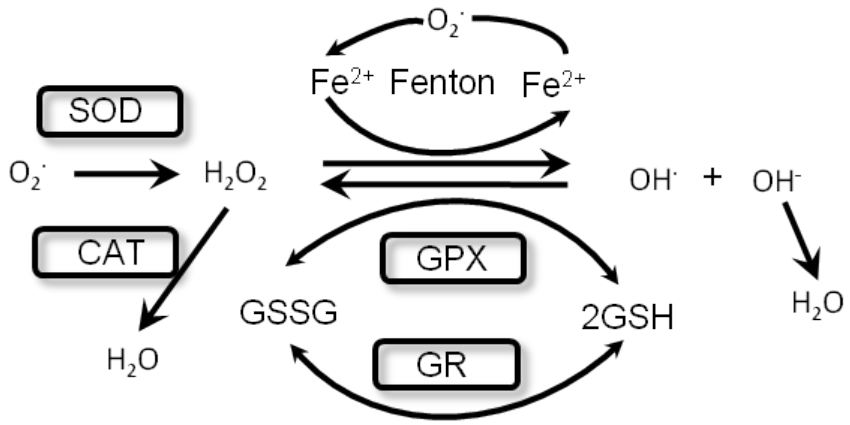

Figure 1.6 Mechanisms of AOE protection. Superoxide ($O_2^{\cdot}$) is dismutated to hydrogen peroxide (H_2O_2) by SOD. H_2O_2 is further reduced to water by the action of either GPX or CAT. The linked oxidation and reduction cycles of glutathione between the reduced (GSH) and oxidized forms (GSSG) are shown in relation to the activities of GPX and GR. H_2O_2 can generate ($OH^{\cdot}$) by reacting with metal ions such as iron in the fenton reaction

CAT is a hemoprotein with four atoms of iron per molecule and its function is to break down hydrogen peroxide to molecular oxygen and water (Figure 1.6). At sites of relatively high concentrations of hydrogen peroxide, such as peroxisomes, catalase is an important antioxidant enzyme. Hydroxil radical and peroxynitrite are among the most reactive species present in biological systems and are capable of oxidizing nucleic acids, protein, lipids and carbohydrates moieties in cell.

In several fish species it has been reported that the level of AOE rises with larval development, whereas, the level of low molecular weight antioxidants falls (Aceto *et al*., 1994; Rudneva, 1999; Zhang *et al*., 2009). This fact indicates a compensation for a lower AOE level by an increase in antioxidant molecules. In this way, increase in AOE levels could be due to the higher oxygen consumption during the initial stages of larval

development. Therefore, the AOE system seems to be induced as a response to these unfavourable conditions. For instance, CAT and GPX activities are increased from hatching to 9 dph in unfed *Dentex dentex* larvae, whereas vitamin E content decreases with age (Mourente *et al*., 1999b). CAT activity is also increased after hatching in rainbow trout (*Oncorhynchus mykiss*) (Aceto *et al*., 1994) and from embryos to 11 dph in turbot larvae (Peters and Livingstone, 1996). Similarly, in Senegal sole (*Solea senegalensis*) larvae, CAT and GPX activities tend to increase along development (Solé *et al*., 2004).

1.6.2.2 Non enzymatic scavengers

A second line of defence is established by antioxidants that can be provided only by nutritional supplements (Sen, 1995) such as vitamin E, C, carotenoids or selenium. Due to the high levels of PUFA in marine organisms, the presence of low molecular weight antioxidants, including water and lipid soluble substances, promotes the resistance of cell membrane in developing embryos against the oxidative stress.

1.6.2.2.1 Vitamin E

Vitamin E is a general term for a group of lipid soluble compounds, tocopherols and tocotrienols, that protect polyunsaturated lipids against oxidation (Table 1.2). The tocopherols and tocotrienols are all powerful antioxidants in food and edible oils, while in fish the vitamin E activity of α-tocopherol by far exceeds that of the vitamin E homologues (Hamre, 2011).

The pure tocopherols are fat-soluble oils that are capable of esterification to form crystalline compounds. In the absence of oxygen, the tocopherols are stable to heat and acids, but are rapidly oxidized in the presence of peroxides or other oxidizing agents (Dam and Sondergaard, 1964). The tocopherols are sensitive to ultraviolet light and are excellent antioxidants in the free form, whereas the tocopherols esters are poor *in vitro* antioxidants. The esters are more stable and are commonly used as dietary supplements, anticipating hydrolysis in the gut and absorption of the free alcohol to act as an active intra- and inter-cellular antioxidant.

Table 1.2 Derivatives of tocol or trienol

Tocopherols	**Chemical formula**	
α-Tocopherol	$C_{29}H_{50}O_2$	5,7,8-Trimethyltocol
β-Tocopherol	$C_{28}H_{48}O_2$	5,8-Dimethytocol
γ-Tocopherol	$C_{28}H_{48}O_2$	7,8-Dimethytocol
ζ_2-Tocopherol	$C_{28}H_{48}O_2$	5,7-Dimethytocol
η-Tocopherol	$C_{27}H_{46}O_2$	7-Methylcolol
δ-Tocopherol	$C_{27}H_{46}O_2$	8-Methylcolol
ε-Tocopherol	$C_{28}H_{42}O_2$	5,8-Dimethyltocotrienol
ζ_1-Tocopherol		5,7,8-Trimethyltocotrienol

α-tocopherol is a structural component of biological membranes (Putnam and Comben, 1987) and functions as a lipid soluble antioxidant (Sargent *et al*., 1997). In fish, as in other animals, vitamin E is also thought to affect disease resistance and health through modulation of the immune response (Waagbø, 1994, 2006; Montero et al., 1995; Verlach Trichet, 2010).

The tocopherols act as inter- and intra-cellular antioxidants to maintain homeostasis of labile metabolites in the cell and tissue plasma. α-tocopherol short-circuits the destructive propagative cycle and can intercept the peroxyl radical (ROO^-) more rapidly than PUFA. The α-tocopherol donates its phenolic hydrogen atom to the radical and converts it to a hydroperoxide product (Figure 1.7), thereby breaking the chain of reactions involved in lipid auto-oxidation. Result of this reaction is the tocopheroxyl radical, that is sufficiently stable to be unable to continue the oxidation chain and, instead, is removed from the cycle by reaction with another peroxyl radical to form inactive non radical products. Besides, the tocopheroxyl radical is resonance stabilized and reacts slowly with PUFA and could be reduced by ascorbate, thereby regenerating α-tocopherol (Figure 1.7). Therefore, unless it is regenerated, vitamin E will need to be replenished either directly through the diet or from reserves elsewhere (Burton and Traber, 1990).

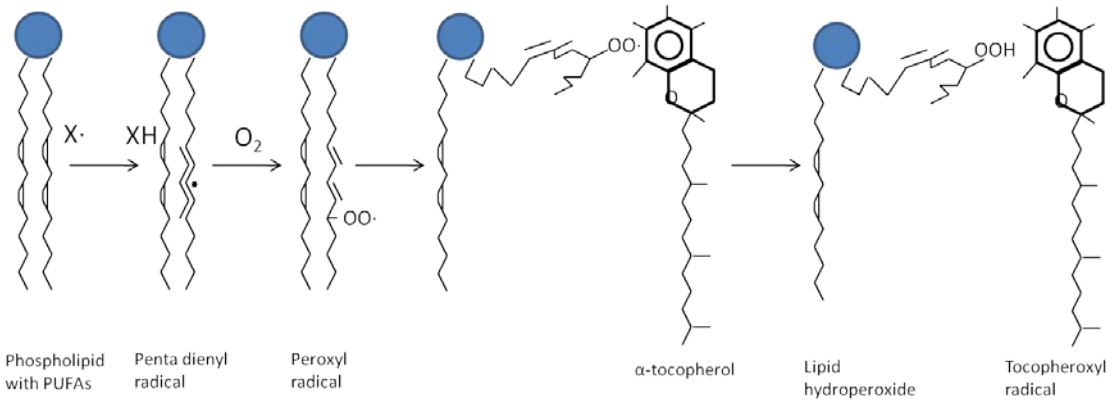

Figure 1.7 Proposed mechanism for the reaction of α-tocopherol with oxidizing lipids. The peroxyl radical group formed during lipid oxidation is polar and floats to the surface of the membrane where it can react with α-tocopherol, rendering a lipid hydroperoxide and the tocopheroxyl radical. Adapted from Hamre, 2011.

Because α-tocopherol can compete for peroxyl radicals much faster than can PUFA, a small amount of α-tocopherol is able to protect a large amount of fatty acids. In this sense, Burton *et al.* (1983) calculated that one molecule of α-tocopherol can protect approximately 1000 molecules of PUFA against oxidation. Besides, it has been reported that vitamin E supplementation improves fillet quality in rainbow trout (Frigg *et al*., 1990; Chaiyapechara *et al*., 2003, Yildiz, 2004), Atlantic salmon (*Salmo salar*) (Hamre *et al*., 1998; Scaife *et al*., 2000), turbot (*Psetta maxima*) (Ruff *et al*., 2003; 2004) and sea bass (Gatta *et al*., 2000; Pirini *et al*., 2000). α-tocopherol has been proved to protect flesh from oxidation, improve shelf life, avoid colour deterioration and prevent the appearance of rancid flavour. In fact, α-tocopherol has been proposed to be the most important factor in maintaining the post-mortem membrane stability of fish fillet (Baker, 1997).

The role of vitamin E in skeletal health has been studied extensively in terrestrial mammals but not in fish. Vitamin E is important for proper skeletal development (Xu *et al*., 1995; Jilka *et al*., 1996). Tocopherol associates with the lipid bilayer of bone cells allowing it to be the first line of defence against free radicals (Arjmandi *et al*., 2002). Endogenous and exogenous free radicals stimulate osteoclastic differentiation and inhibit osteoblastic activity (Tintut *et al*., 2002; Parhami, 2003). This may cause bone resorption and lead to improper bone formation/abnormalities. However, not many studies exist on the effect of ROS on fish bone. Lewis-McCrea and Lall (2007) described the effect of feeding juvenile Atlantic halibut with moderately oxidized oils on the development of skeletal abnormalities, finding an increase in the incidence of scoliosis along with increasing levels of oxidized oils. On the other hand, some studies exist regarding the

effect of chemical pollution on fish vertebral column (Karen *et al*., 2001; Mochida *et al*., 2008; Danion *et al*., 2011). These consequences may be quite similar to those observed when an oxidative stress is produced through the diet, as pollutants usually exert their negative effects by altering the oxidative status.

Little is known about the exact vitamin E requirements in marine fish larvae, as most of the studies have been mainly focused on adults or juvenile fish, values ranging from 25 to 120 mg kg^{-1}. It is generally believed that fish larvae have higher nutrient requirements than adults, therefore using adult values may underestimate the requirements (Table 1.3). In this sense, fish larvae may have specific growth rates in the range of 30% per day (Otterlei *et al*., 1999), which suggest that they need a nutritious food.

Table 1.3 Optimal vitamin E for different larval fish species

Species	Tested parameter	Requirement	Reference
Latris lineata	Growth	437 mg kg^{-1}	Brown *et al*., 2005
Salmo salar	Growth	120 mg kg^{-1}	Hamre and Lie, 1995a
Sparus aurata	Growth Survival	136 mg kg^{-1}	González *et al*., 1995
Sparus aurata	Growth Survival	3000 mg kg^{-1}	Atalah, 2008

It is also important to keep in mind that in larvae higher LC-PUFA requirements must be associated with higher dietary levels of vitamin E as it has been suggested in carp (*Cyprinus carpio*) (Watanabe *et al*., 1981; Schwarz *et al*., 1988), Atlantic salmon (Hamre and Lie, 1995b) and sea bream (Atalah *et al*., 2008). In this sense, Izquierdo and Fernández-Palacios (1997) observed an increase in vitamin E content from hatching up to 10 dph, whereas from there on and until day 20, content decreased in sea bream larvae. Interestingly, this reduction was parallel to the reduction in the larval PUFA contents along larval development (Izquierdo, 1988), suggesting a close relation between both nutrients.

On the other hand, formulated diets for marine fish larvae contain high levels of PUFA and pro-oxidants, for example in the form of minerals. Besides, the high surface to volume ratio of the feed particles also favors oxidative processes as fatty acids are more exposed. It is therefore important to supplement marine fish larval diets with enough amount of vitamin E.

1.6.2.2.2 Vitamin C

Ascorbic acid (vitamin C) is a water soluble vitamin considered to be an essential component in diets for teleost fish as they are unable to synthesize it or synthesize it fast enough to meet the requirements, due to the lack of the enzyme gulonolactone oxidase (Chatterjee, 1973; Dabrowski, 1990). It is a white, odorless, crystalline compound, soluble in water but insoluble in fat solvents. Ascorbic acid readily forms salts and is labile to free oxygen. Reduced ascorbic acid is very stable in acid solutions because of the preservation of the lactone ring, but in alkaline solutions hydrolysis occurs rapidly and vitamin activity is lost. Vitamin C is very heat labile and prone to atmospheric oxidation, especially in the presence of copper, iron or several other metallic catalysts. The reduced form is the most biologically active form, but several derivatives or salts may be formed with varying degrees of ascorbate activity (Woodruff, 1964; WHO, 1970).

Ascorbic acid is a cofactor in many biological processes including collagen synthesis and cellular functions related to neuromodulation, hormone and immune system. In channel catfish (*Ictalurus punctatus*) adequate vitamin C must be supplied for the formation of structural components such as bone matrices, collagen synthesis and connective tissue (Wilson and Poe, 1973). Ascorbic acid is a cofactor in the hydroxylation of proline and lysine, necessary for the conversion of procollagen to mature collagen (Barnes and Kodicek, 1972; Padh, 1991). Lower proportions of hydroxylysine and hydroxyproline are common in ascorbic acid deficient fish resulting in underhydroxylated collagen (Satoh *et al*., 1982). Besides, when dietary ascorbic acid levels are low, a decrease in alkaline phosphatase levels is observed, indicating a reduction in osteblastic activity. Therefore, vitamin C deficiency results in poor bone calcification and metabolism (Tietz *et al*., 1983; Johnston *et al*., 1994).

In this sense, skeletal malformations common in ascorbic acid deficient fish are caused by impaired biosynthesis of collagen (Halver, 2002). Decreased proline hydroxylation results in soft, brittle bones as observed in scorbutic channel catfish and turbot (Wilson and Poe, 1973; Coustans *et al*., 1990), while in Indian major carp (*Labeo rohita*) poor calcium absorption in bone, muscle, gills and skin was observed (Agrawal and Mahajan, 1980). Enlarged, slightly curved vertebrae are frequently one of the early signs of vitamin C deficiency as observed in channel catfish (Lim and Lovell, 1978). Skeletal abnormalities such as lordosis and scoliosis have been observed in scorbutic brook trout (*Salvelinus fontinalis*) (Poston, 1976), rainbow trout (Dabrowski *et al*., 1990)

and Atlantic salmon (Hardie *et al.,* 1991). In agreement, restricted dietary vitamin C contents have been found to cause cartilage reduction and damage that reflects in anomalies especially in structures that undergo chondral ossification (for instance, jaws) together with an over stimulation of the vitamin C receptor (Darias *et al*., 2009).

Since ascorbic acid is a prominent antioxidant, poor quality feeds can increase the amount of free radicals on the body thus decreasing tissue ascorbic stores (Sies *et al*., 1992) as observed in rainbow trout fed oxidized dietary lipids (Hung and Slinger, 1980). Another interesting role of vitamin C as an antioxidant is the regeneration of tocopheroxyl radical, which has been showed to be feasible *in vitro* (Tappel, 1962; Packer *et al*., 1979; Niki *et al*., 1985). The increase in vitamin C content from 0 to 60 mg kg^{-1} did not influence the retention of α-tocopherol in Atlantic salmon as long as the fish were not vitamin C deficient (Hamre *et al*. 1997). Similar results were found in yellow perch (*Perca flavescens*) and channel catfish (Lee and Dabrowski, 2003; Yildirim-Aksoy *et al*., 2008). Moreover, in Atlantic salmon, tissue vitamin E levels were independent of vitamin C supplementation between 50 and 2750 mg kg^{-1} (White *et al.* 1993). On the other hand, there was a large drop in liver vitamin E concentration in Atlantic salmon that became vitamin C deficient (Hamre *et al*., 1997). Liver concentration of vitamin E was also increased by dietary vitamin C in vitamin E deficient yellow perch and channel catfish (Lee and Dabrowski, 2003; Yildirim-Aksoy *et al*., 2008). A parallel development of liver vitamin E, vertebrae hydroxyproline, growth and mortality was observed in vitamin E supplemented salmon in response to dietary vitamin C, suggesting that vitamin C status above deficiency is necessary to maintain the body stores of vitamin E (Hamre *et al.* 1997). These results may be taken as support of the hypothesis that vitamin C regenerates vitamin E *in vivo* in adults and juveniles fish (Figure 1.8). On the other hand, high dietary vitamin E levels appears to have a prooxidant effect in fish deficient in vitamin C, as tocopheroxyl radicals are accumulated in the membranes and may promote irreversible oxidation of the remaining vitamin C (Hamre, 2011).

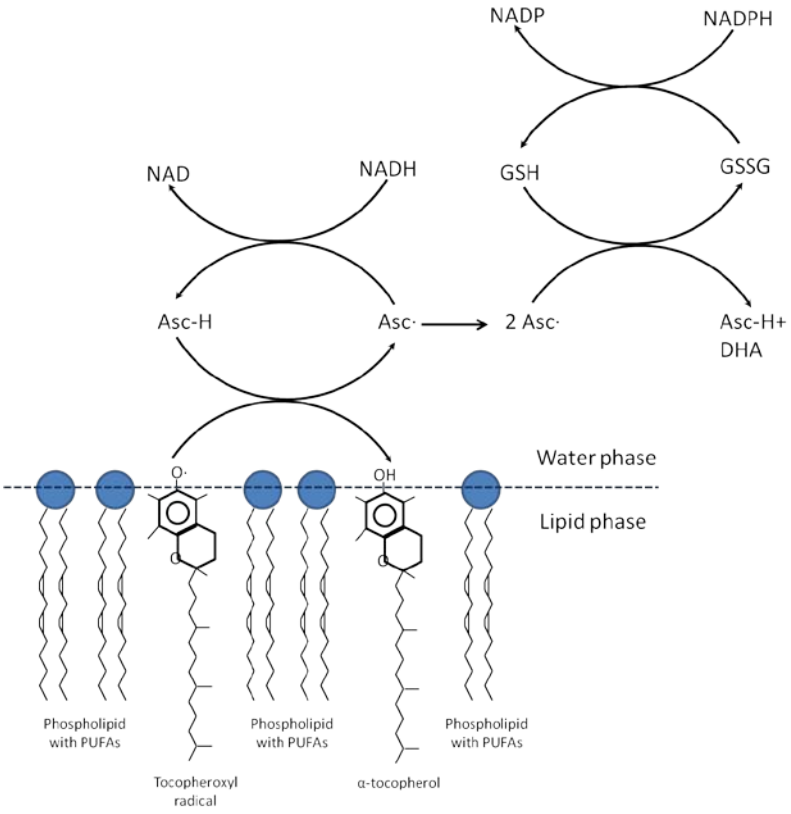

Figure 1.8 Proposed mechanism for regeneration of α-tocopherol from the tocopheroxyl radical according to Tappel (1962) in the cell lipid bilayer. Ascorbic acid (Asc-H) is oxidized in the process, producinf the resonance-stabilized tricarbonyl ascorbate free radical (Asc·). This free radical can be regenerated by glutathione (Mrtensson and Meister, 1991) either chemically or enzimatically or enzymatically by NADH (Meister, 1994; Winkler *et al*., 1994). To reach this, reduced glutathione (GSH) donates a hydrogen atom to two molecules of Asc·, giving place to the formation of Asc-H and its metabolite dehydroascorbic acid (DHA). Oxidized glutathione (GSSG) is reduced by GR at the expense of NADPH generated in the pentose phosphate shunt (Meister, 1994). This scheme is an adaptation from that showed at Hamre, 2011.

Fish larvae are particularly sensitive to vitamin C deficiency (Dabrowski *et al*., 1996), probably having higher vitamin requirements due to their rapid growth and high contents of PUFA in larval feeds (Table 1.4). Moreover, high ascorbic acid content has been found in fish eggs (Kossmann, 1988; Dabrowski and Bloom, 1994), which might be an indication of the importance of this micronutrient during early development. Addition of vitamin C to larval diets improved survival, growth performance, skeleton development, stress resistance and immune response in sea bass and turbot (Merchie *et al*., 1996) or sea bream (Atalah *et al*., 2010) larvae. Besides, it has been hypothesized that in early stages on embryogenetic development of marine organisms the low molecular weight antioxidants, such as vitamin C, play an important protective role

against oxygen damage, as an enhancement of the activity of antioxidant enzymes has been observed as larvae develop (Rudneva, 1999). Thus, control of vitamin C administered to broodstock, as well as adequate supplementation of vitamin C to young larvae are of vital importance to reach a balance oxidative status in marine fish larvae.

Table 1.4 Optimal vitamin C for different larval fish species

Species	Tested parameter	Requirement	Reference
Cirrhina mrigala	Growth Survival Behaviour Morphology	650-700 mg kg^{-1}	Mahajan and Agrawal, 1980
Clarias gariepinus	Growth	1600 mg kg^{-1}	Merchie *et al*., 1997
Cyprinus carpio	Growth Vitamin C content	45 mg kg^{-1}	Gouillou-Coustans *et al.*, 1998
Dicentrarchus labrax	Growth Survival Stress resistance	2500 mg kg^{-1}	Merchie *et al*., 1995
Dicentrarchus labrax	Growth Survival e Deformities	30-50 mg kg^{-1}	Darias *et al*., 2011
Scophtalmus maximus	Growth Survival	20-130 mg kg^{-1}	Merchie *et al*., 1997

1.6.2.2.3 Selenium

Selenium is a trace mineral and an essential micronutrient for vertebrates (Johansson *et al*., 2005), but also has the smallest window of any element between requirement and toxicity (Chassaigne *et al*., 2002; Polatajko *et al*., 2006). It is an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defence systems and immune function.

Selenium (Se) is widely distributed at low concentrations in freshwater (0.2-10 µg $liter^{-1}$) and seawater (approximately 0.09 µg $liter^{-1}$) (NRC, 1993). It also occurs naturally in foods and feedstuffs in organic complexes, primarily in the form of selenomethionine, selenocystine and selenocysteine. Fish meals and marine byproducts represent the best natural sources of Se among the common feedstuffs for fish. However, Bell and Cowey (1989), reported that selenium present in fish meal has low digestibility, whereas selenomethionine is highly digestible. The relative availability of Se in pure compounds is:

Selenite > *selenate* > *selenomethionine* > *selenide* > *elemental selenium*

In fish, Se is also involved in thyroid hormone and insulin function, maintenance of fertility as well as regulation of cell growth (Table 1.5; Lall, 2002; Kohlmeier, 2003). Besides, selenium can promote either bone formation or mineralization. However, excessive intakes of this mineral have deleterious effects on vertebrates skeletal tissue metabolism (NRC, 2005). Recent work by Penglase *et al.* (2010) has shown that feeding cod larvae with selenium-supplemented rotifers, causes a trend of increased deformities in individual vertebra. This could be caused by an alteration in skeletal mineralization in its ionic form, or via selenoenzymes as an antioxidant (Lall and Lewis-McCrea, 2007) or by regulating thyroid hormone ratios (Power *et al*., 2001).

Se is incorporated as selenomethionine at the active site of a wide range of proteins. In zebrafish (*Danio rerio*) a total of 18 selenoproteins have been identified, including three that do not have known orthologs in mammals (Kryukov and Gladyshev, 2000). One of the major Se function is as a component of the selenoproteins GPX, isoenzymes that protect lipid components and membranes at both the cellular and subcellular level from oxidative damage (Arteel and Sies, 2001). Other selenoproteins can have antioxidant functions as well, and can be observed at Table 1.5. Only selenomethionine can be incorporated into proteins, being the storage form of Se mainly in the skeletal muscle. In contrast, sodium selenite can be incorporated into active selenoproteins, such as GPX, but not into selenomethionine as storage protein in liver and muscle (Rider and Sweetman, 2008).

Se deficiency can lead to oxidative stress in organs (Gatlin *et al*., 1986; Bell *et al*., 1986, 1987), reduced growth (Wang and Lovell, 1997) and increased mortality (Gatlin *et al.*, 1986; Bell *et al*., 1987) in several fish species. The major effects of selenium toxicity are reduced growth, poor feed efficiency and high mortality. Besides, toxicity occurred in rainbow trout and catfish when dietary selenium exceeded 13 and 15 mg kg^{-1} respectively (Hilton *et al.,* 1980; Gatlin and Wilson, 1984). It must be taken into account that selenium requirements of fish varies with the form of selenium ingested, PUFA and vitamin E content of the diet, as well as concentration of waterborne selenium (Lall, 2002).

Table 1.5 Zebrafish selenoproteins and presumptive function. Adapted from Kryukov and Gladyshev (2000)

Selenoprotein	Zebrafish	Known function (mammals)
1. Glutathione peroxidase 1	GPX1a GPX1b	Hydroperoxide catabolism Sperm structure
2. Glutathione peroxidase 2 3. Glutathione peroxidase 4	GPX2 GPX4a GPX4b	
4. Thyroid hormone deiodinase 3	DI3	T4 activation T3 inactivation
5. Thioredoxin reductase 2	TR2	Protein thiol redox regulation Vitamin C recycling Synthesis of DNA
6. Thioredoxin reductase 3	TR3	
7. Selenoprotein P	SelPa	Selenium transport Antioxidant
8. Selenoprotein Pb	SelPb	
9. Selenoprotein W1	SelW1	Antioxidant
10. Selenoprotein W2	SelW2a SelW2b	
11. Selenoprotein T1	SelT1a SelT1b	Unknown
12. Selenoprotein T2	SelT2	Unknown
13. 15 kDa selenoprotein	Sel15	Cancer etiology??
14. Selenoprotein R	SelR	Unknown
15. Selenoprotein N	SelN	Unknown

Recent studies (Hamre *et al*., 2008a) have shown that Se content in rotifers is considerably low (0.08-0.09 mg kg^{-1} DW) than both fish requirements (0.5-0.3 mg kg^{-1} DW; NRC, 1993) and copepod levels (3-5 mg kg^{-1} DW) and may contain insufficient Se to meet larvae requirements. Therefore, Se could be one of the trace elements with a higher potential of being deficient in rotifers. Enrichment of rotifers with sodium selenite and sodium iodide proved to increase survival in Atlantic cod larvae, but no differences were observed in growth compared to the control group (Hamre *et al*., 2008b). Increase in the level of Se in rotifers enhanced the mRNA expression and activity of GPX in cod larvae (Penglase *et al*., 2010), suggesting that extra supplementation is needed to

protect larvae against lipid oxidation and the resulting oxidation products, which can be abundant in cultured live feed enriched with *n*-3 LC-PUFA. It is important to notice that vitamin E provides some compensation against Se deficiency (Gatlin *et al*., 1986; Awad *et al*., 1994), therefore, larvae could protect themselves from ROS by accumulating other antioxidant nutrients as it has been observed in cod larvae (Penglase *et al*., 2010).

1.6.3 Oxidative stress

Oxidative stress occurs when the ROS generation rate exceeds that of their removal (Sies, 1985; Figure 1.9). Its deleterious effects include oxidation of proteins and DNA, as well as peroxidation of unsaturated lipids in cell membranes. This produces unstable lipid hydroperoxides which are highly reactive, threatening the cell integrity. In addition these products can break down into free radicals that can perpetuate the destructive cycle of lipid-peroxidation chain reactions. The repair of proteins damaged by ROS appears restricted to the reduction of oxidized derivatives of the sulfur-containing amino acid residues. Repair of other kinds of protein oxidation has not been demonstrated. Instead, the damaged proteins are targeted for degradation to amino acid constituents by the action of various endogenous proteases, including cathepsin and calpain. In contrast, the oxidative damage to nucleic acids is subject to repair by highly efficient excision/insertion mechanisms. Nevertheless, modifications of cellular DNA upon exposure to ROS is the likely initial event involved in the induction of the mutagenic and lethal effects of various oxidative stress agents (Basu-Modak and Tyrrel, 1993).

In juvenile and adult fish some diseases have been related to free radical damage such as hemolisis (Kawatsu, 1969), anaemia (Cowey *et al*., 1984), jaundice (Sakai *et al*., 1989), liver degeneration (Cowey *et al*., 1984) or skeletal alterations (Hata and Kaneda, 1980; Watanabe *et al*., 1989; Lewis-McCrea and Lall, 2007). Among these skeletal alterations, one of the frequently most described in fish juveniles and adults is muscular dystrophy (Lovell *et al*., 1984; Gatlin *et al*., 1986; Frischknecht *et al*., 1994; Bowater and Burren, 2007).

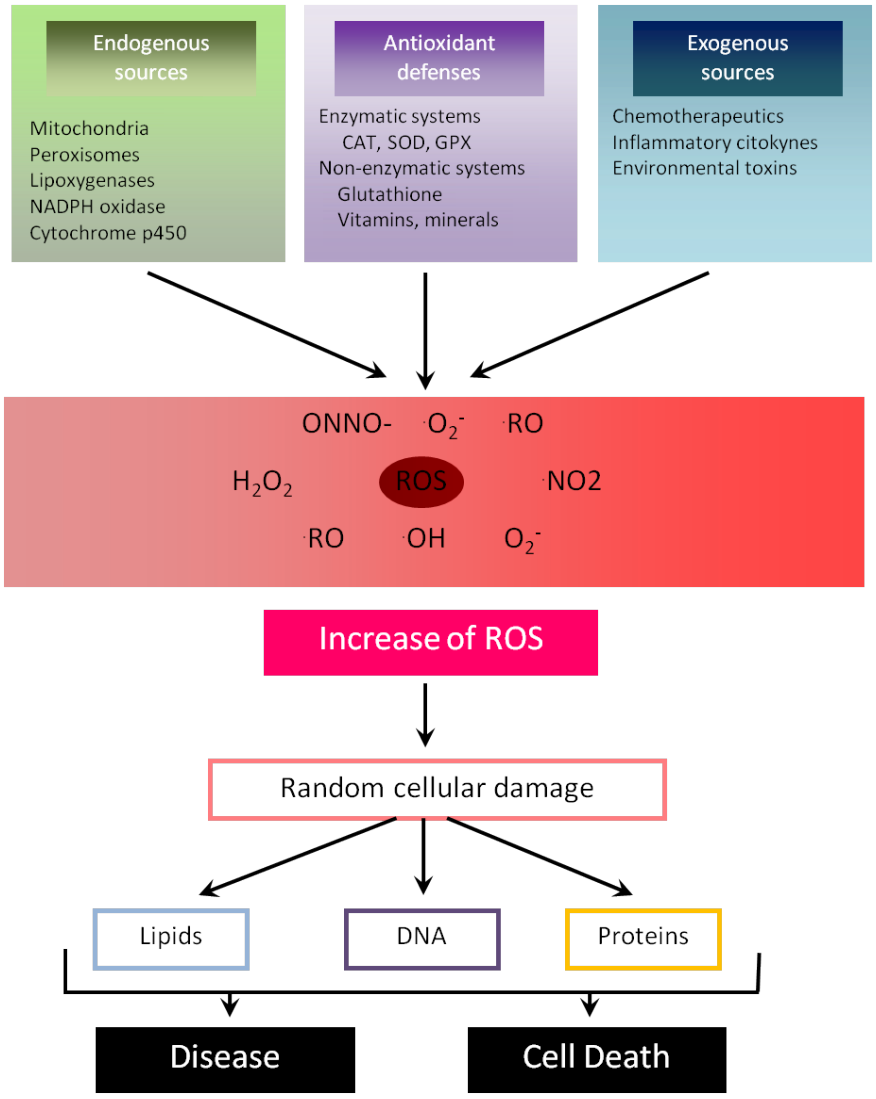

Figure 1.9 Sources and cellular responses to reactive oxygen species (ROS). An increase in ROS leads to random cellular damages on the different cellular components. If continued along time will cause disease or even cell death. Adapted from Finkel and Holbrook, 2000.

1.6.3.1 Auto-oxidation of lipids

Lipids oxidation, which can be defined as an autocatalytic process initiated by free radicals resulting in the deterioration of PUFA, is an important consequence of oxidative stress. ROS prefer to steal electrons from the lipid membrane of a cell, initiating lipid oxidation process. Auto-oxidation of lipids proceeds by a chain reaction whereby a single radical species has the ability to abstract a hydrogen atom from a methylene group of a PUFA, yielding a lipid radical (Figure 1.10). As a consequence, a self-sustaining reaction cycle is established, where the lipid peroxyl radical formed by one

turn of the cycle reacts with a new PUFA. In the absence of antioxidants, lipid oxidation may proceed as long as PUFAs are available for oxidation (Hamre, 2011). The lipid peroxidation chain can be terminated by two lipid radicals reacting to form a non-radical product or by quenching by a radical scavenger (Hølmer 1993; Frankel, 1998).

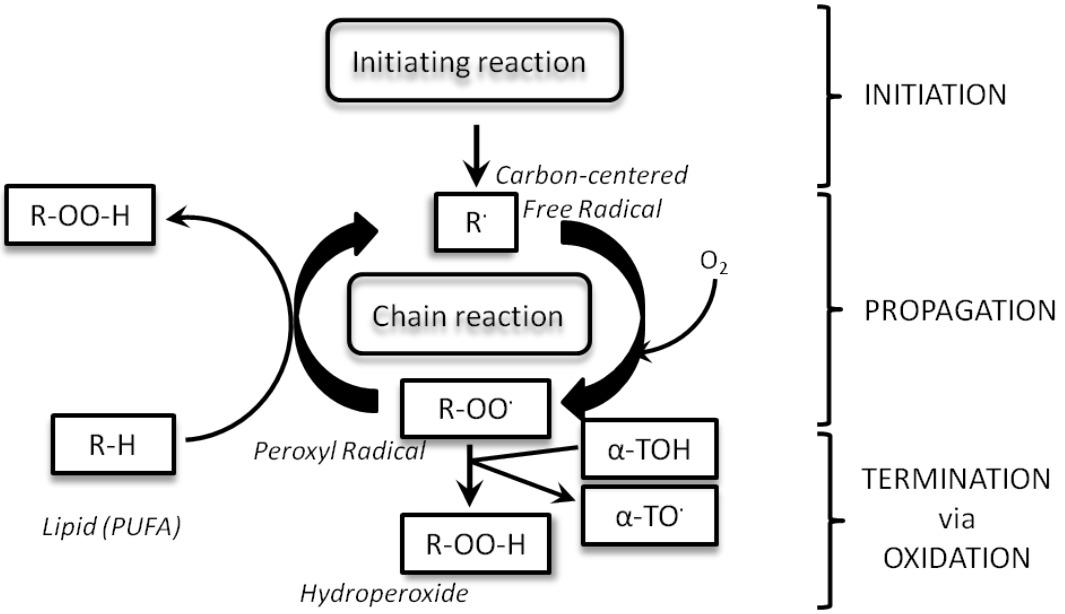

Figure 1.10 Scheme showing the three phases of the free radical chain mechanism of lipid peroxidation. The RH group contains the unsaturated unit common to all PUFA. In this case the reaction is quenched by α-tocopherol, but other radical scavenger can be implicated. Although PUFA are very vulnerable and the most probable target of free radicals attack, proteins could also suffer a peroxidative attack. Adapted from Burton and Traber, 1990.

In general, the overall effects of lipid peroxidation are a decrease in membrane fluidity, an increase in membrane permeability to normally impermeable substances and an inactivation of membrane-bound enzymes. The primary products of lipid oxidation are the conjugated dienes which are then converted into lipid hydroperoxides, which may undergo cleavage to form different secondary products of low molecular weight such as aldehydes and hydrocarbons (Hølmer, 1993; Frankel, 1998). Lipid hydroperoxides further oxidize, keeping the carbon chain intact into isoprostane, isofuran and mono- or dihydroxy fatty acids.

DHA oxidation specifically yields F4-type isoprostane, isofuran and mono- or dihydroxy DHA. Eight subfamilies of F4-isoprostanes could be formed from DHA owing to free radical attack at positions C_6, C_9, C_{12}, C_{15} and C_{18}. Furthermore, lipid hydroperoxides can lead to a complete loss of membrane integrity with the carbon chain disintegration resulting in the formation of different molecular species of aldehydes with different carbon length alkalenes and/or alkalenes as byproducts. The most stable product from DHA oxidation is 4-hydroxyhexenal (Van Kuijk *et al*., 1990). A combination of water and lipid-soluble antioxidants may be necessary to reduce these oxidation products. However, the effect of each individual antioxidant molecule can vary depending on the organisms and the specific tissue (Dietrich *et al*., 2002). Besides, lipid oxidation-induced damage to the lysosome membrane can result in hydrolytic enzymes escaping into the cell cytoplasm, further damaging the cell.

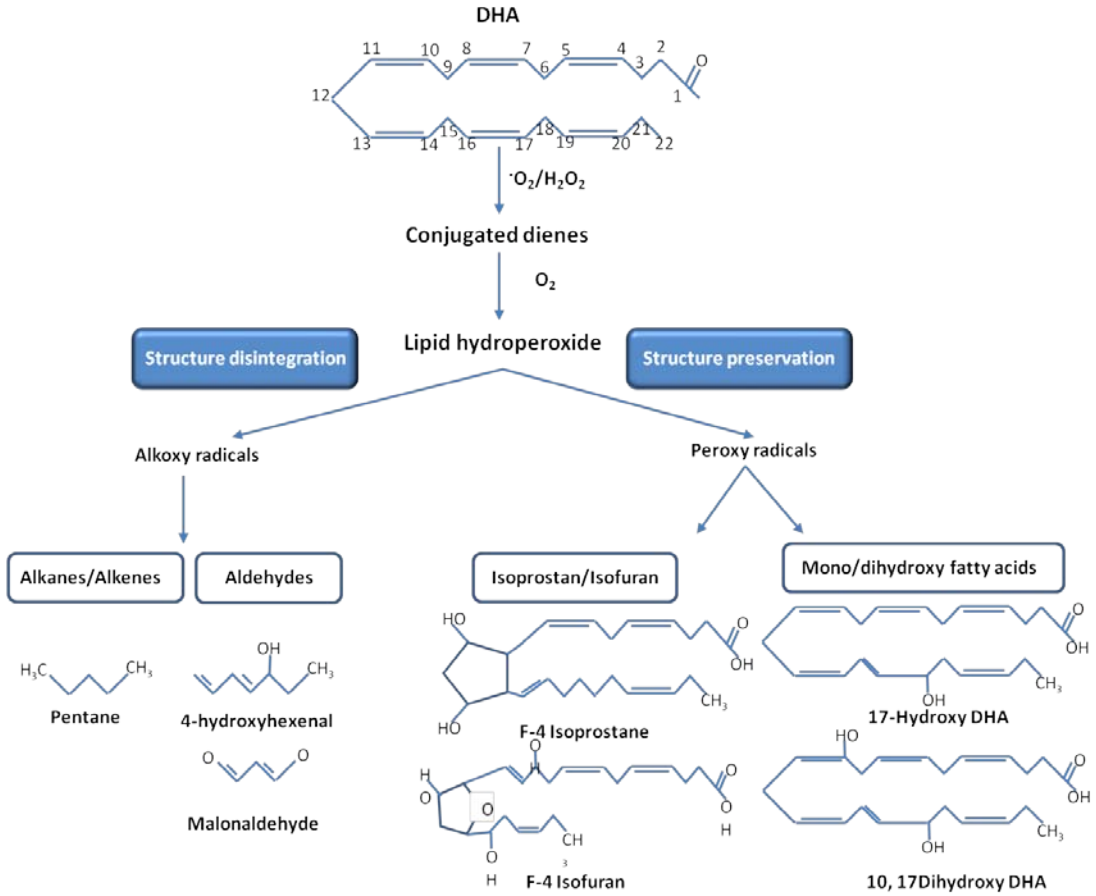

Figure 1.11 Non enzymatic oxidation of DHA is initiated after free radical attack, resulting in an unstable DHA-radical state that quickly undergoes isomerisation and rearrangement of double bonds. Lipid hydroperoxides further oxidize, keeping the carbon chain intact (structure preservation) or it can be disintegrated (structure disintegration), giving place to different byproducts. Several possible isomers exist within each class. Adapted from Siddiqui *et al*. (2008).

Fish tissues are at a high risk of suffering from lipid oxidation, as they contain relatively large quantities of PUFA. This risk will be even higher in marine fish larvae, as their PUFA requirement is more elevated and will be reflected in their tissues. However, susceptibility of fish to lipid oxidation rate depends, to a large extent, on the tissue fatty acid profile and levels and type of antioxidants present. Thus, to avoid *in vivo* lipid peroxidation, sufficient amounts of antioxidants must be included in diets.

1.7 Pathological effects of oxidation on fish tissues

Several studies have evidenced the adverse effects of oxidation on diverse marine and fresh water species. Apart from alteration of production parameters such as reduced growth or increased mortalities (Watanabe *et al*., 1970; Blazer, 1982; Wang *et al*., 2006) several pathological symptoms have been related to different sources of oxidative stress in adults and juveniles of different fish species (Table 1.6). As it can be observed in this Table, one of the most notorious signs is the appearance of muscular dystrophy.

1.7.1 The adverse effects of oxidative stress on musculoskeletal system

1.7.1.1 Muscular tissue

In teleost fish, axial musculatures are organized into a series of segmentally arranged myotomes that have a complex three-dimensional morphology. The individual myotomes are separated by collagenous sheets called myosepta and have a complex geometry associated with the requirements for mechanical stability during body bending (Figure 1.12; Van Leeuwen, 1999). The number and shape of the myotomes show a significant variation with ontogenetic stage, position along the trunk, body morphology, phylogeny and style of locomotion (Johnston *et al.,* 2011). Each myotome contains a superficial, wedge-shaped region lying directly beneath the lateral line, where the muscle fibres are arranged in a helical fashion, forming angles of up to 40º. This typical

orientation of the muscle fibres is associated with the need for constant amounts of sarcomere shortening at different body flexures (Rome and Sosnicki, 1990). In sea bass, as in many other fish species, there is an epaxial and a hypaxial myomere for each vertebra separated by the horizontal septa and lateral line (Figure 1.12).

Table 1.6 Some pathological effects of free radicals on several adult and juvenile fish species and tissues

Sign	Species	Source of oxidation	Author
Jaundice	*Seriola quinqueradiata*	Unknown	Sakai *et al*., 1989. 1998
Nutritional muscular dystrophy	*Cyprinus carpio*	VitE DEF	Watanabe *et al*., 1970
	Cyprinus carpio	OO+VitE DEF	Miyazaki, 1986
	Ictalurus punctatus	Se+VitE DEF	Gatlin *et al*., 1986
	Ictalurus punctatus	↑ FA	Lewis *et al.,* 1985
	Ictalurus punctatus	OO+VitE DEF	Murai and Andrews, 1974
	Lates calcarifer	VitE DEF	Bowater, 2007
	Salmo gairdneri	OFO	Cowey *et al*., 1984
	Salmo gairdneri	VitE+Se DEF	Bell *et al*., 1985
	Oncorhynchus mykiss	VitE, VitC, VitE+C DEF	Frischknecht *et al*., 1994
Haemolysis	*Salvelinus fontanalis*		Kawatsu, 1969
	Salmo gairdneri	OO+VitE/C DEF	Smith, 1979
	Salmo gairdneri	OFO+VitE/Ethoxyquin	Moccia *et al*., 1984
	Salmo salar	VitE+Se DEF	Poston *et al*., 1976
Skeletal abnormalities	*Hippoglossus hippoglossus*	OFO	Lewis McCrea and Lall, 2007
Ceroid pigment	*Seriola quinqueradiata*	Unknown	Sakai *et al*., 1989
	Salmo salar	OO	Roald *et al*., 1981

DEF: Deficiency; OO: Oxidized oil; OFO: Oxidized fish oil

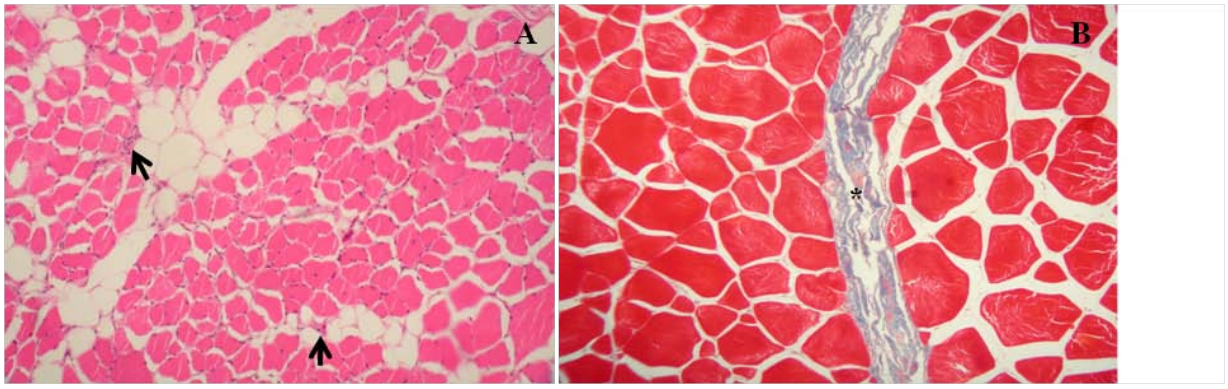

Figure 1.12 Transverse section of sea bream white muscle, haematoxylin and eosin staining (A) and Masson´s trichrome (B). (**A**) Presence of adipose tissue between muscle fibres can be observed as well as blood vessels (arrow) (x200). (**B**) Note the collagen myosepta stained in blue (x400).

The locomotor muscles of all groups of fish are very highly specialized to meet the wide range of force production that is required from the muscular system both during sustained cruising and also at high velocity bursts. The solution adopted universally to these conflicting requirements has been to divide the locomotor system into several parts containing different muscle fibres. Therefore, the locomotor muscles are organized in axial muscle formed mainly by fast-white fibres, covered by a thin layer of slow-red muscle fibres, and a layer of pink or intermediate muscle between them. Muscle colour is indicative of the degree of vascularization of each muscle type. The superficial red muscle, or slow muscle, appears dark because of its high myoglobin content, has abundant mitochondria in their centre and periphery, as well as, high fat and glycogen content (Figure 1.12; Johsnton, 1980; Shindo *et al*., 1986; Ayala *et al*., 1999). The red fibres are aerobic and have a high respiratory activity. These fibres are small in diameter (25 – 45 µm) and usually constitute less than 10% and never more than 30% of the myotomal musculature (Boddeke *et al*., 1959). The major ultrastructural features of this muscle type are high amount of subsarcolemmal and intermyofibrillar mitochondria of the lamellar type as well as lipid droplets. Transversally these fibres show a rectangular morphology. Generally this muscle type is used at high swimming speed.

The white muscle, also known as fast muscle, never represents less than 70% and shows the largest fibre diameters ranging between 50 and 100 µm or even larger (Figure 1.12). Ultrastructurally, it shows a marked radial orientation and retain a relatively uniform width. These fibres have a polygonal morphology, abundant myofilaments and scarce mitochondria. White muscle fibres are fast contracting and fatigue rapidly.

In accordance to their name, intermediate or pink fibres are intermediate in position between red and white muscle fibres. The relative amount of intermediate muscle differs both among fish species and developmental stages, in fact, in sea bass larvae this kind of fibres can be appreciated at the end of the larval stages (Scapolo *et al*., 1988; Veggetti *et al*., 1990; Ramírez-Zarzosa *et al*., 1995; López-Albors *et al*., 2005).

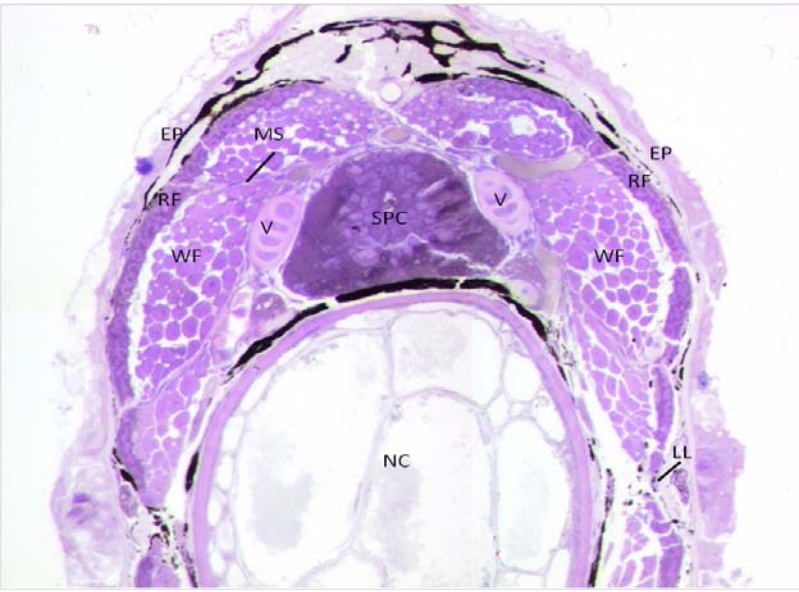

Figure 1.13 Fibre typing and structure of muscle fibre in cross sections of sea bass larvae, Toluidine blue. The trunk is divided into two lateral halves, supported by the vertebral column and skeletal processes. The superficial monolayer of red fibres (RF) can be observed in contact with the epidermis (EP). White fibres (WF), larger than red ones and displaced in several layers can be seen. NC notochord; LL lateral line; V vertebra; SPC spinal cord; MS myosepta.

Muscle growth in fish differs from that of mammals in that muscle recruitment continues throughout most of the life cycle (Greer-Walker, 1970; Stickland, 1983). Besides, both hyperplasia and hypertrophy occur during myogenesis in larval and adult muscle growth of sea bass, which reaches a large adult size (Weatherley *et al*., 1988). However, the number of myotomes is fixed before or shortly after hatching (Blaxter, 1988). Three phases of muscle formation can be distinguished in fish:

(1) Embrionic myogenesis: The formation of the embryonic fibre together with a population of undifferentiated myosblasts takes place.
(2) Stratified hyperplasia: This phase can be observed in yolk-sac larvae. Germinal zones of myoblast proliferation are observed at the dorsal and

ventral apices of the myotomes (Veggetti *et al*., 1990; Brooks and Johnston, 1993; Rowlerson *et al*., 1995).

(3) Mosaic hyperplasia: Myoblasts on the surface of the embryonic muscle fibres are activated. This process can continue throughout much of the adult life (Koumans *et al*., 1995; Johnston *et al*., 1995; Rowlerson *et al*., 1995).

It is known that several abiotic factors can affect fish myogenesis (Figure 1.13). Feeding is a key factor affecting the development and growth of fish muscles as suggested by the differences in size, morphology and proliferation rate of the myogenic progenitor cells isolated from the fed or starved fish (Fauconneau and Paboeuf, 2000). Besides, at larval stages, the supply of appropriate food that meets all the requirements of rapidly developing organs (including muscle) is crucial (Koumans and Akster, 1995). However, the incidence that lack or excess of determinate nutrients can have on fish marine larvae muscle is widely unknown. For instance, despite some reports have pointed out the effects that vitamin E and/or selenium deficiency can have on Atlantic salmon, channel catfish or barramundi (*Lates calcarifer*) adults or juveniles muscle (Poston *et al*., 1976; Lovell *et al*., 1984; Bowater and Burren, 2007), none of them relates to larval stages. Additionally, dietary lipid and/or protein level may have a direct effect on muscle oxidation as it has been shown in juvenile sea bass and rainbow trout (Álvarez *et al*., 1998).

Although the cell biology of myogenesis in teleosts is distinct from that described in mammals, the genes involved in growth regulation are apparently highly conserved (Watabe, 2001). However, slightly different regulatory mechanisms exist in the regulation of fish muscle development and growth. In teleost fish larvae, pluripotent stem cells become myoblasts, which are committed to a myogenic fate to form the Myogenic Progenitor Cell (MPC) population, involving the expression of myogenic regulatory factors (MRFs). Afterwards, MPCs are thought to undergo an asymmetric division to regenerate the MPC and provide a daughter cell committed to terminal differentiation. The MPC progeny may undergo a proliferation phase controlled by positive and negative signaling pathways and can migrate through the muscle and have several fates. Myoblasts in fast muscle can fuse to form myotubes in a myoblast-myoblast fusion event, which probably involves calpain. Calpains (EC 3.4.22.17) are calcium dependent cytosolic neutral proteases existing in two main ubiquitous forms, depending on the concentration of Ca^{2+} necessary for their activation: μ-calpain and m-calpain. Although it has been hypothesized that calpains may have a regulatory role in muscle growth, most reports in fish have been conducted to study the *post mortem* proteolytic activity of these

enzymes as well as their effect on flesh quality (Geesink *et al*., 2000; Chéreta *et al*., 2009; Caballero *et al*., 2009; Terova *et al*., 2011).

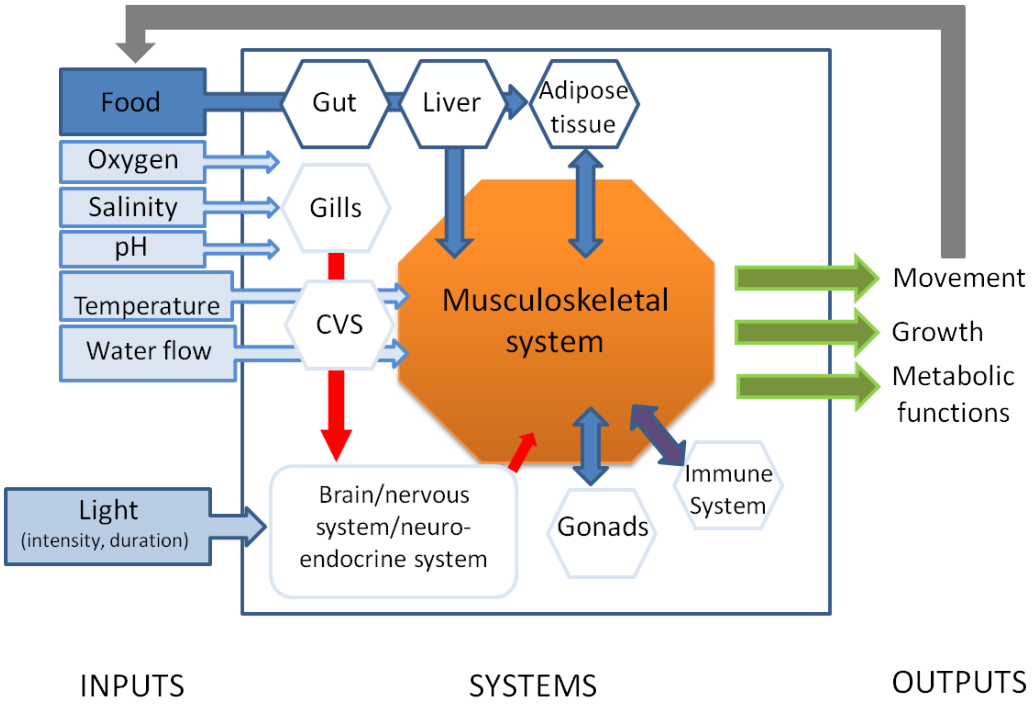

Figure 1.14 The environmental inputs and physiological systems that affect the functional outputs of skeletal muscle in teleost fish. If an appropriate nutritional supply is not given to fish larvae it may have an effect on the digestive system, liver and adipose tissue, reflecting their damages in musculoskeletal system. CVS: Cardiovascular system. Adapted from Johnston *et al*., 2006.

Once formed, myotubes initiate the programme of myofibrillargenesis and mature into muscle fibres. When viewed in longitudinal section, mature muscle fibres show transverse striations of alternating light (I) and dark (A) bands, with an amazing degree of evenness and regularity (Figure 1.14). A band is compound of thick (myosin) and thin (actin) myofilaments, whereas I band is exclusively formed of thin filaments. Each I band is dissected by a dark transverse line, the Z line. The structure remaining between two Z lines is denominated sarcomere and is the muscular contraction unit. In the centre of the A band there is a smaller band, exclusively compound of thick myofilaments and termed H band. In the middle of this H band exists another line called M line, where thick myofilaments are ingrained through different kind of proteins. Myofibrillargenesis is initiated on actin stress fibres formed on Z bodies (precursor of Z lines) prior to the integration and alignment of the thick filaments and associated proteins in a multi-step

process that appears to be conserved within vertebrates (Sanger *et al*., 2009). On the other hand, myosin is a hexamer consisting of two heavy protein chains (MyHC) and four light chains, including embryonic isoforms. Thus, MyHC are the main marker when studying the phenotypic determination of fibres and whole muscles during growth and adaptation. Both actin and myosin can be used as late markers of myogenesis, as they are expressed at the end of the myogenesis process.

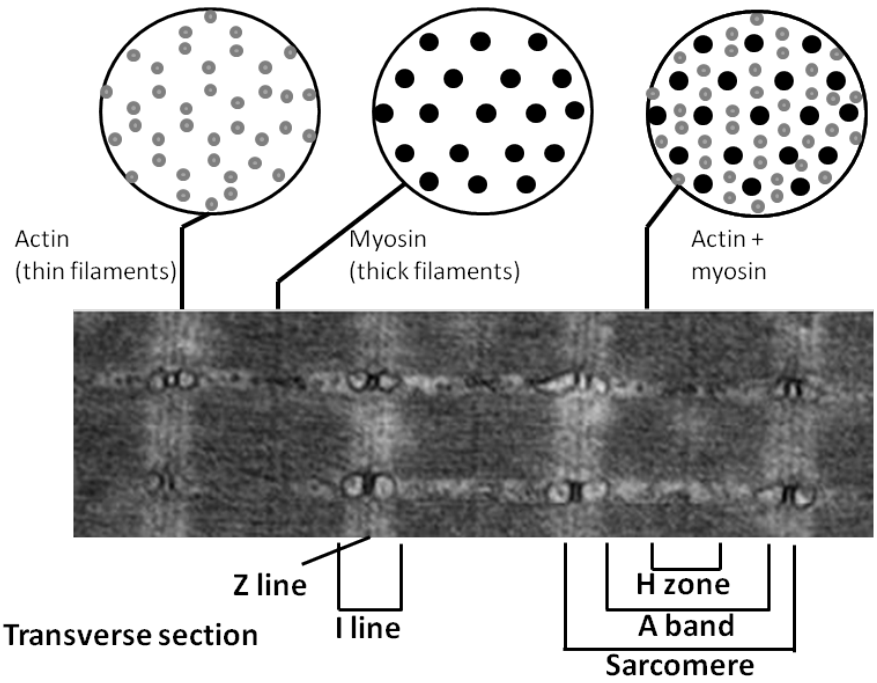

Figure 1.15 Schematic representation of the disposition of actin and myosin myofilaments in sea bass larvae white muscle (35 dph).

MPC are also called satellite cells, although some authors claim that they should not be called satellite cells, as MPC are not always found beneath the basal lamina of muscle fibres (Veggetti *et al*., 1990; Johnston *et al*., 2003). Besides, satellite cells, once activated, are responsible for postembryonic growth in teleosts. On the other hand, some works have analyzed the role of satellite cells in muscle regeneration in teleosts (Rowlerson *et al*., 1997), showing similar features compared to mammals muscle regeneration. In response to muscle injury, satellite cells are activated in order to proliferate and fuse to form new muscle fibres (Goldspink *et al*., 2001). These new fibres transiently express the developmental MyHC in a way that resembles those isoforms that are expressed during embryonic stages (Whalen *et al*., 1990).

The regulation of fibre mass is thought to be controlled by signaling pathways involving insulin-like growth factor I (IGF-I) and IGF-II. The IGF are single-chain growth

promoting polypeptides with structural homology to proinsulin. Depending on the biological context, IGF may stimulate cell growth, promote cell differentiation and inhibit apoptosis (Jones and Clemmons, 1995). IGF action can be influenced, both positively and negatively, by a family of IGF-binding proteins (IGFBP) and most, if not all, of their actions are mediated by the IGF-receptors (Jones and Clemmons, 1995). IGFs are one of the central pathways regulating protein synthesis in skeletal muscle. For instance, in zebrafish embryos, two IGF-II paralogues regulate midline development (White *et al*., 2009). Similarly, fish muscle has a substantially greater abundance of IGF-I receptors than insulin receptors (Parrizas *et al*., 1995). This indicates that IGF-I contributes more to the regulation of muscle function than insulin in fish, in contrast to mammals.

In mammals, it has been proved that various properties of skeletal muscle render it particularly susceptible to free radical injury, even when systemic oxidative stress takes place. Thus, muscle seems to be primarily, if not selectively, affected. This may relate to the high degree of susceptibility of muscle to oxidative stress by virtue of its requirement and ability to undertake rapid and coordinated changes in energy supply and oxygen flux during contraction. This property makes muscle very prone to oxidative injury as a result of increased electron flux and from the mitochondrial respiratory chain (Haycock *et al*., 1996). On the other hand, there is a very high concentration of myoglobin in muscle and it is known that such a heme-containing protein confers a greater sensitivity to free radicals (Ostdal *et al.*, 1997). Finally, the requirement of skeletal muscle membrane for PLs may render those membranes particularly susceptible to oxidative damage (Murphy and Kehrer, 1989).

Thus, muscle seems to be at a higher risk of suffering the attack of ROS than any other tissue. It is logical to think that antioxidant protection mechanisms should be enhanced in muscular tissues. However, it has been showed in Atlantic salmon that retention of α-tocopherol is particularly low in white muscle, especially if compared to other organs, like liver which showed an exponential retention of vitamin E (Hardie *et al*., 1990; Hamre and Lie, 1997). The linear model for α-tocopherol retention for muscle and the exponential model for liver are supported by other studies with tilapia (*Oreochromis aureus*; Satoh *et al*., 1987) and rainbow trout (Hung *et al*., 1980; Frigg *et al*., 1990; Puangkaew *et al*., 2005). Similar results are found with other antioxidant nutrients, like Se. Monteiro *et al*. (2009) found a lower retention of Se in muscle of *Brycon cephalus* fed a diet supplemented with Se in comparison to gills or liver. Besides, SOD, CAT and GPX activities showed to be lower in Manchurian trout (*Brachymystax lenok*, Pallas) larvae muscle compared to viscera, brain and gills (Zhang *et al*., 2009). Therefore, these conditions should be considered as additional possible causes of muscle injury in fish.

1.7.1.2 Bone-skeletal system

The skeletal system serves many physiological functions, including the support of the body structural integrity during development and locomotion. Besides, the skeleton also provides sites for muscle attachment, protects vital organs and serves as a mineral reservoir (Lall and Lewis-McCrea, 2007). Teleost fish show a high variety of skeletal tissues. Indeed, more than bone and cartilage, fish skeletal tissue is often best described as a continuous spectrum ranging from connective tissue to cartilage and to bone (Hall and Witten, 2007). Cartilage and several other tissues with histological characteristics between bone and cartilage have been identified in fish and play an important role in skeletal development (Benjamin, 1990; Beresford, 1993; Huysseune, 2000). Bone is a specialized vascularised connective tissue consisting of cells and a mineralized extracellular matrix. Before mineralization, the extracellular matrix is composed mainly by collagen type I that subsequently becomes mineralized through the osteoblast mediated deposition of hydroxyapatite (Hall and Witten, 2007; Nordvick, 2007). Cartilage is an avascular skeletal tissue composed of chondrocytes that are embedded in an extracellular matrix, primarily composed by collagen type II and proteoglycans (Witten *et al*., 2010). The chondroid bone is an intermediate tissue that is found, for instance, in the mandibullar and maxillary tissue of teleost fish and has intermediate characteristics of both bone and cartilage, but may be mineralized.

Different cells are involved in the formation and remodeling of axial skeleton bone. Osteoblasts are bone forming cells and their role is to secrete the non-mineralized bone matrix and control matrix mineralization. Osteoclasts are the cells involved in the resorption of the bone tissue and can be viewed as multinucleated macrophages. Osteocytes are cells trapped inside the bone matrix and are thought to be involved in the maintenance of bone substances and the exchange of ions from body fluids.

Several studies dealing with the impact of first feeding on fish development showed that different nutrients play a central role in the appearance of skeletal malformation when they are not supplied during the larval phase (Cahu, 2003). Besides, the development of skeletal disorders in larval and juvenile fish may be linked to a poorly understood relationship between nutrition, environment and genetic factors. Therefore to try to avoid the appearance of skeletal deformities in cultured larvae, adequate supplementation of nutrients such as vitamins, minerals or lipids is of vital importance.

Information of the role of oxidized lipids and free radicals in the development of skeletal abnormalities in teleost fish is limited. In humans, it is known that ROS

contribute mostly to bone remodeling by promoting bone resorption (Bai *et al*., 2005). This is probably the result of an inhibition of osteoblasts and a stimulation of osteoclasts ultimately causing a net bone loss (Parhami *et al*., 1997; Parhami, 2003). The reduction in bone formation accompanied by a stimulation in bone resorption could result in development of skeletal abnormalities, as observed in halibut fed oxidized lipids (Lewis-McCrea and Lall, 2007). Recently, studies with sea bream larvae showed that high dietary levels of DHA induced a higher percentage of skeletal deformities, finding in these larvae the highest value of TBARS, indicative peroxidative processes (Izquierdo *et al*., submitted). Vitamin E supplementation did not reduce the frequencies of abnormalities observed in juvenile halibut fed oxidized diets (Lewis-McCrea and Lall, 2007), neither in larval sea bream fed high DHA rotifers (Izquierdo *et al*., submitted), whereas vitamin E supplementation improved bone quality in adult mice who had been exposed to normal oxidative stress (Wang *et al*., 2000). Therefore, dietary oxidative products can cause deficiencies of antioxidant nutrients resulting in skeletal abnormalities. Further research on the effect of oxidized dietary lipid on skeletal development is required to understand the pathogenic effect on fish bone.

Presently, the production of juveniles is still a bottleneck in marine aquaculture. The use of a compound dry diet is crucial for sustaining production of high and constant quality juveniles. However, the exact requirements of marine fish larvae are not completely known. High dietary requirements of polyunsaturated fatty acids and particularly DHA are known to be increasing oxidative risk. However, little is known about the damages caused by free radicals in fish larvae and the potential effects of antioxidant nutrients on oxidative damages. Therefore, the objectives of this Thesis were:

1.- Determine the potential damage on sea bass larvae of high dietary levels of DHA. Traditionally high contents of PUFA, especially DHA, have been included in larval feeds to promote growth and survival. However, due to its high unsaturation, DHA is highly prone to peroxidation. This fact together with other characteristics can make marine fish larvae very susceptible of suffering oxidative stress on their tissues.

2.- Evaluate the antioxidant protective effect of vitamin E when different dietary levels of DHA are supplied. The inclusion of enough quantities of antioxidant nutrients must be included in diets to counteract the negative effects of ROS. Graded levels of α-tocopheryl acetate were tested in order to avoid oxidative stress adverse effects.

3.- Investigate the protective effect of the combination of vitamin E and selenium when high contents of DHA are included in the diet. A diet containing high DHA and vitamin E contents together with selenium supplementation was tested.

4.- Investigate the protective effect of the combination of vitamins C and E when high contents of DHA are included in the diet. To reach thos objective, high levels of vitamin C were added to a diet already containing

Chapter 2

General Materials and Methods

2.1 Experimental animals and conditions

2.1.1 Fish

Sea bass (*Dicentrarchus labrax*) larvae were obtained from natural spawnings from *Ecloserie Marine de Gravelines* (Gravelines, France, Chapter 3) and *Instituto de Acuicultura de Torre la Sal* (Castellón, Spain, Chapters 4, 5 and 6). During the first days of acclimation, water temperature was monitored through coolers (16ºC) and water flow was increased up to room temperature (19.5-20ºC). Larvae were cultured in keane tanks until they were 12 dph (Chapters 4, 5, 6 and 7) or 32 dph (Chapter 3), when they were randomly distributed into the experimental tanks and after an acclimation period of two days, fed one of the experimental microdiets.

2.1.2 Experimental conditions

All the experiments were carried out in *Instituto Canario de Ciencias Marinas* (ICCM, Telde, Canary Islands, Spain) facilities.

2.1.2.1 Green water pre-cultures

Sea bass recently hatched were incubated in a well-aerated 2000 L tank under an open circulating sea water system (7 water renovations d^{-1}) during the first five days. The sixth day, when larvae already had their mouth open, the sea water circuit was closed and larvae were cultured in green water. Everyday 20 L of *Nanochloropsis gaditana* (205 x 10^3 cells ml^{-1}) were added and the enriched rotifers density maintained at 10 ind ml^{-1}

(Figure 2.1). For this purpose fresh rotifers were supplied twice a day (8:00; 15:00). The non-ingested rotifers were removed from the tank by filtration, thus avoiding the presence of starved low nutritional value rotifers in the tank. Larvae were cultured under natural photoperiod (around 10 h light) for 12 (Chapters 4, 5, 6 and 7) or 32 (Chapter 3) days until larvae were transferred to the experimental tanks.

2.1.2.2 Experimental tank cultures

Each tank (170 L light grey colour cylinder fibreglass tanks) was supplied with filtered sea water (about 34 g L^{-1} salinity) previously stored at a 500 L tank for degasification. Water was filtered with a 50 µm mesh and entered in tanks at an increasing rate of 1.0 - 1.5 L min^{-1} in an open circulation system. Water was continuously aerated (125 ml min^{-1}), attaining 5-8 g L^{-1} dissolved O_2 and saturation ranged between 60 and 80%. Temperature and oxygen were daily measured by using an Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, USA). Light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia). The photoperiod was kept at 12 h light: 12 h dark. Tanks were daily manually cleaned between 18:00 and 20:00 with a hose by a siphon system.

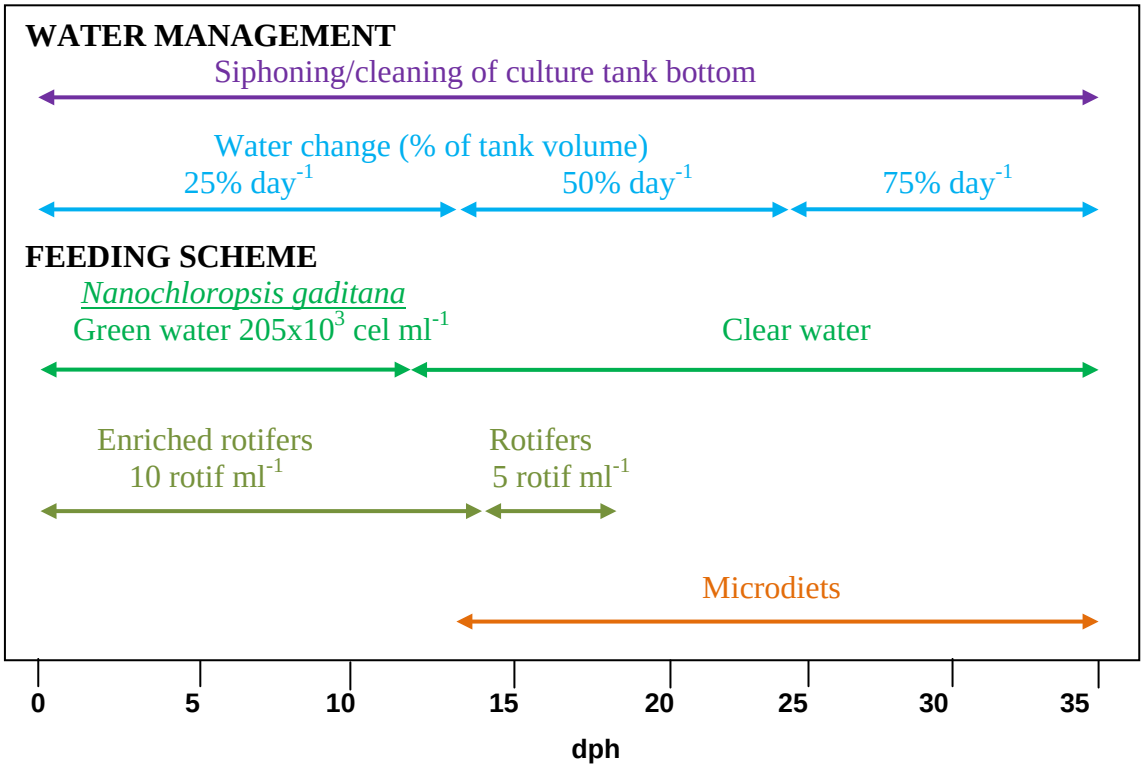

Figure 2.1 Scheme representation of the feeding sequence and cultivation routine during the experimental period.

2.2 Diets and feeding

2.2.1 Rotifers

Rotifers (*Brachionus plicatilis*) were cultivated in 1700 L cylindrical containers. The container was provided with a central aeration system through a porous stone that was maintained at 20 cm from the bottom. The cultures contained a combination of natural sea water (80%) and freshwater (20%). Rotifers, strain S-1, of 150-250 µm length, were inoculated at initial densities of 100 ind ml^{-1}. They were fed with 1.2 g fresh yeast extract 10^6 ind^{-1} d^{-1}, ration divided in two doses. Every four days the rotifers were carefully filtered through a 64 µm mesh, rinsed in water, the container washed and the culture resuspended in clean water at optimum culture densities of 100-250 ind ml^{-1}.

The rotifers were used to feed sea bass larvae during green water culture and during the first five days of microdiet trials. The rotifers supplemented along the green water cultures were previously enriched (24 h), at densities of 300 rotifers ml^{-1}, with emulsified DHA Protein Selco® (INVE, Belgium; 0.125 g Selco l^{-1} ration in two doses). During the first five days of microdiet testing, yeast-fed rotifers were directly supplemented in a co-feeding regime to the experimental larval tanks, providing no source of LC-PUFA (Figure 2.1).

2.2.2 Microdiets

Several isonitrogenous and isolipidic microdiets containing different levels of DHA, vitamin E, vitamin C or selenium were formulated. Diets were expected to contain around a 65% of protein and a 15% of lipids.

2.2.2.1 Microdiets formulation

EPA 50 and DHA 50 (Croda, East Yorkshire, United Kingdom) oils in tryglicerides form were used as sources of DHA and EPA. Vitamin E in α-tocopheryl acetate form was obtained from Sigma-Aldrich (Madrid, Spain) and ROVIMIX Stay-C-35 (ascorbyl monophosphate; Roche, Paris, France) was employed as vitamin C source. An organic

form of selenium extracted from yeast (Sel-Plex, Alltech Inc, Lexington, KY) was employed. The desired lipid content was completed with a non essential fatty acid source, oleic acid (Merck, Darmstadt, Germany). Soybean lecithin (Acrofarma, Barcelona, Spain) containing around 50% of polar lipids was used as a source of phospholipids. The attractants mixture according to Kanazawa *et al.* (1989), the hydro- and lipo-soluble vitamins mixture and minerals mixture according to Teshima *et al.* (1982) with some modifications is shown in Table 2.1. The protein source used was squid powder (Riber and Son, Bergen, Norway). To guarantee a best control of the microdiet fatty acid profile, squid powder was defatted three consecutive times with a chloroform:meal ratio of 3:1. Squid meal was suspended in three volumes of solvent and agitated for a few minutes. The particles of the mixture were filtered under a vacuum pump and the defatted meal was separated from the chloroform fraction. After each extraction, the defatted squid meal was spread in a laboratory tray and the remaining solvent was evaporated during 12 h at 38ºC. Fatty acid composition of these dietary components are shown (Table 2.2).

2.2.2.2 Microdiets preparation

The microdiets were prepared according to Liu *et al*. (2002). Firstly, squid powder was mixed with water soluble components (Table 2.1) in a mortar. Separately, oils and fat-soluble vitamins were combined to obtain a homogeneous mix which was afterwards joined together with the powder mix. Then gelatine was dissolved in warm water and added to the previously mixed ingredients. The paste was compressed, pelleted (Severin, Suderm, Germany) and dried in an oven at 38ºC for 24 h (Ako, Barcelona, Spain). Pellets were grounded (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain the desired particle size (from 125-500 µm). Diets were analyzed for proximal and fatty acid composition of dry basis and each diet was tested in triplicates (Chapters 3, 5 and 6) or quadruplicates (Chapters 4 and 7).

2.2.3 Feeding

Diets were manually supplied every 45 min from 8:00 to 19:00. Larvae were fed twice a day for at least five days with rotifers that had been fed only with baker´s yeast to avoid any *n*-3 LC-PUFA content. Initial density was 2 individuals ml^{-1} and then reduced to

1 individual ml^{-1}. To guarantee feed availability, daily dry supply was initially 2.0 g and increased 0.5 g each week.

Table 2.1 Mix of attractants, minerals and vitamins employed in the formulation of experimental microdiets

Vitamins	mg $100g^{-1}$ DW
Hydro-soluble vitamins	
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	180.000
Nicotinic acid	290.160
myo-Inositol	1450.900
Subtotal	2289.700
Lipo-soluble vitamins	
Retinol acetate	0.180
Ergocalciferol	3.650
Menadione	17.280
α-Tocopherol acetate	150.000
Subtotal	171.110
Choline chloride	2965.800
Total	5426.610

Attractants	mg $100g^{-1}$ DW
Inosine 5-monophosphate	500
Betaine	660
L-Serine	170
L-Tyrosine	170
L-Phenilalanine	250
DL-Alanine	500
L-Sodium aspartate	330
L-Valine	250
Glycine	170
Total	3000
Minerals	mg $100g^{-1}$ DW
NaCl	215.133
$MgSO_4 \cdot 7H_2O$	677.545
$NaH_2PO_4 \cdot H_2O$	381.453
K_2HPO_4	758.949
$Ca(H_2PO_4) \cdot 2H_2O$	671.610
FeC_6H_5O	146.884
$C_3H_5O_3 \cdot 1/2Ca$	1617.210
$Al_2(SO_4)_3 \cdot 6H_2O$	0.693
$ZNSO_4 \cdot 7H_2O$	14.837
$CuSO_4.5H_2O$	1.247
$MnSO_4 \cdot H_20$	2.998
KI	0.742
$CoSO_4 \cdot 7H_2O$	10.706
Total	4500.007

Table 2.2 Main fatty acids of the major dietary lipids sources used in the different experiments. (% total identified fatty acids)

	EPA 500	DHA 500	Oleic acid	Soy lecithin
14:0	0.20	0.08	0.50	0.07
15:0	n.d.	n.d.	0.02	0.04
15:1*n*-5	n.d.	n.d.	n.d.	0.02
16:0	0.22	0.61	6.69	17.29
16:1*n*-7	0.33	0.38	0.14	0.07
16:2*n*-6	0.19	0.12	n.d.	n.d.
16:2*n*-4	0.16	0.20	0.03	0.11
17:0	0.18	0.09	0.04	0.06
16:3*n*-3	0.11	0.07	n.d.	n.d.
16:4*n*-3	0.20	n.d.	n.d.	n.d.
18:0	0.62	2.64	1.56	3.50
18:1*n*-9	2.75	5.33	76.77	16.99
18:1*n*-7	1.14	0.97	n.d.	1.24
18:1*n*-5	0.04	0.03	n.d.	0.05
18:2*n*-9	0.35	n.d.	n.d.	n.d.
18:2*n*-6	1.49	0.71	13.20	54.12
18:2*n*-4	0.76	0.08	n.d.	n.d.
18:3*n*-6	0.47	0.30	n.d.	n.d.
18:3*n*-4	0.36	0.09	n.d.	n.d.
18:3*n*-3	0.95	0.27	0.16	5.41
18:3*n*-1	0.12	n.d.	n.d.	n.d.
18:4*n*-3	4.54	0.29	n.d.	n.d.
18:4*n*-1	0.51	n.d.	n.d.	n.d.
20:0	0.14	0.60	0.14	0.23
20:1*n*-9+*n*-7	0.65	2.75	0.41	n.d.
20:1*n*-5	n.d.	0.28	n.d.	n.d.
20:2*n*-9	0.41	0.03	n.d.	n.d.
20:2*n*-6	0.23	0.50	n.d.	0.03
20:3*n*-6	0.53	0.16	n.d.	0.03
20:4*n*-6	3.71	2.37	n.d.	n.d.
20:3*n*-3	0.10	0.37	n.d.	n.d.
20:4*n*-3	2.13	0.70	n.d.	n.d.
20:5*n*-3	62.91	9.93	0.03	0.03
22:1*n*-11	0.10	1.04	0.07	0.45
22:1*n*-9	0.93	0.62	0.05	n.d.
22:4*n*-6	1.60	0.50	n.d.	n.d.
22:5*n*-6	0.35	3.31	n.d.	n.d.
22:5*n*-3	1.87	3.60	n.d.	n.d.
22:6*n*-3	8.65	60.93	0.21	0.23

n.d., not determined.

2.3 Sampling

2.3.1 Biological parameters

At the beginning, final and intermediate points of each experiment, samples of live larvae unfed for 12 h were taken to determine total length and whole body dry weight.

2.3.2 Proximate analysis

To analyze biochemical composition, all the remaining larvae in each tank, after a starving period of 12 h, were collected, washed with distilled water and kept at -80ºC in air free labelled plastic sampling bags until analysis. Prior to the beginning of the dietary experiments, samples from the keane tanks were taken to know initial biochemical composition.

2.3.3 Histology

At each sampling point 30 sea bass larvae from each tank were collected and fixed in 10% buffered formalin. Other 30 larvae were fixed for 24 h at 4ºC in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for transmission electron microscopy (TEM) study.

2.3.4 Molecular biology

At each sampling point around 200 mg of unfed sea bass larvae were collected, washed in DEPC (Diethyl pyrocarbonate) water and conserved in 1000 µl of RNAlater (Sigma-Aldrich, Madrid, Spain) overnight at 4ºC, then RNAlater was removed and samples conserved at -80ºC until RNA extraction.

2.3.5 Activity test and final survival

Before the end of some of the experiments (Chapter 3 and 4) an activity test was conducted by handling 20 larvae tank^{-1} 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 h. Final survival was calculated by individually counting all the alive larvae at the beginning and at the end of the experimental trials.

2.3.6 Growth evaluation

Growth was determined by measuring dry body weight and total length. Whole body weight was determined by 3 replicates of 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 length of 30 anesthetised larvae from each tank was measured in a Profile Projector (V-12A Nikon, Nikon Co., Tokyo, Japan) at each sampling point.

2.4 Biochemical analysis

2.4.1 Proximate analysis

2.4.1.1 Moisture

Moisture content was determined by thermal oven drying to constant weight at 110ºC, with a first 24 h drying period, followed by 1 h periods until weight was nor reduced any further. Sample weight (approximately 100 mg) was recorded before drying and after each drying period, following the cooling in a dessicator. Moisture was expressed as a percentage of the weight according to Official Methods of Analysis (A.O.A.C., 1995), using the following equation:

$$Moisture\ (\%) = \frac{100 - (B - A) - (C - A)}{B - A}$$

Where:

A = Weight of empty flask

B = Weight of wet sample + flask

C = Weight of dry sample + flask

2.4.1.2 Ash

Ash content was determined by drying the samples (approximately 200 mg) in an oven at a temperature of 450ºC until a constant weight was attained (A.O.A.C., 1995).

2.4.1.3. Proteins

Proteins were estimated from total nitrogen present in the sample, using the Kjeldhal method (A.O.A.C, 1995) after the digestion of the sample (≈250 mg) with concentrated sulphuric acid at a temperature of 420ºC. Total nitrogen content was converted to total crude protein value by multiplying by the empirical factor 6.25.

2.4.1.4 Total lipids

Lipids were extracted following the method of Folch *et al.* (1957). The method starts taking a sample amount between 50-200 mg and homogenising it in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen Germany,) during 5 min in a solution of 5 ml of Chloroform:Methanol (2:1) with 0.01% of BHT. The resulting solution was filtered at reduced pressure through glass wool and 0.88% KCl added to increase the water phase polarity. After decantation and centrifugation at 2000 rpm during 5 min the watery and organic phases were separated. Once watery phase was eliminated, solvent was dried under nitrogen atmosphere and subsequently total lipids weighed.

2.4.2 Fatty acid methyl esters preparation and quantification

Fatty acid methyl esters (FAME) were obtained by acid transmethylation of total lipid with 1% sulphuric acid in methanol following the method of Christie (1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50ºC. Afterwards, fatty acid methyl esters were extracted with hexane:diethyl ether (1:1, v/v) and purified by adsorption chromatography on NH_2 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 mm, internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180ºC for the first 10 min, increasing to 215ºC at a rate of 2.5ºC min^{-1} and then held at 215ºC for 10 min, following the conditions described in Izquierdo *et al.* (1992). Fatty acid methyl esters were quantified by FIED and identified by comparison with external standards and well characterized fish oils (EPA 28, Nippai, Ltd. Tokyo, Japan).

2.5 Measurement of thiobarbituric reactive substances (TBARS):

The measurement of TBARS in triplicated samples was performed using a method adapted from that used by Burk *et al.* (1980). Approximately 20-30 mg of larval tissue per sample was homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol using a rotating probe homogeniser (Ultra-Turrax; IKA-Werke, T25 BASIC, Staufen, Germany). To this 2.95 ml of freshly prepared 50mM thiobarbituric acid solution was added before mixing and heating for 10 min at 100ºC. After cooling and removing protein precipitates by centrifugation (Sigma 4K15, Osterode and Harmz, Germany) at 2000 X *g*, the supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA) expressed as nmol MDA per g of tissue was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1} applying the following formula:

$$\text{nmol MDA g tissue}^{-1} = \frac{A}{0.156} \times \frac{50}{Sample\ weight}$$

2.6 Determination of vitamin E content

Vitamin E concentrations (α-tocopherol) were determined in diets and tissue samples using a reverse phase high-pressure liquid chromatography (HPLC) with UV detection after samples saponification. Vitamin E analysis was performed during a stay at the Institute of Aquaculture, University of Stirling (Scotland, UK).

2.6.1 Preparation of standards and calibration curve

Vitamin E quantification was achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich, Madrid, Spain) as an external standard. To prepare standards a stock solution of 1 mg ml^{-1} in 25 ml of methanol was prepared by adding 37 mg of oil to 25 ml of methanol in a volumetric flask and mix. From the 1 mg ml^{-1} stock solution 200 µl were removed and 10 ml added to give a working standard concentration of 20 µg ml^{-1} and absorbance read at 293 nm in a plastic cuvette using as blank methanol. The following equation was used to calculate the concentration of vitamin E:

$$\frac{A\ 293\ nm}{71} = \text{g vit E } 100\ ml^{-1}$$

Where, *A* is the absorbance and 71 is the molar extinction of vitamin E at 293 nm. Therefore,

$$\frac{\frac{g\ vit\ E}{100\ ml \times 10}}{20} = \text{µg vit E } 50\ \mu l\ \text{injection}^{-1}$$

The calibration curve is prepared using five concentrations ranging between 1-100 µg ml^{-1} taking volumes showed at Table 2.3.

Table 2.3 Dilutions employed for the preparation of α-tocopherol standard curve

Concentration (µg ml^{-1})	Stock solution (µl)	100 µg ml^{-1} vial (µl)	Methanol (ml)
100	400	-	3.6
50	200	-	3.8
25	100	-	3.9
10	-	400	3.6
1	-	40	3.96

Each concentration vial was measured on the spectrophotometer (CECIL CE 2021 series, CECIL instruments, Cambridge, UK) at 293 nm and the correct concentrations calculated. Standards solutions were randomly injected in triplicates onto HPLC and

store under nitrogen in the freezer (~-18ºC). The standard curve was run with each batch of samples.

2.6.2 Vitamin E extraction from feeds

Vitamin E extraction from the diets was conducted in triplicates according to McMurray *et al.* (1980). Approximately 1 g of feed was weighed and 25 ml of 6% (w/v) ethanolic pyrogallol was added to 100 ml stoppered cylinders that would be incubated at 70ºC for approximately 5 min. After this, 5 ml 60% KOH was added to the tubes that were vigorously shaken and return to the water bath for approximately 10 min, shaking the cylinders at approximately 5 min intervals. Next, tubes were removed from the water bath and cooled on ice before adding 40 ml chilled filtered water and 30 ml diluted iso-hexane + BHT. Each tube was vigorously shaken exactly for a minute to allow the layers to separate. To facilitate this process tubes were placed in a freezer (~ -18ºC) for approximately 10 min. Once the layers have separated, 20 ml of the top layer were removed to medium size tubes, solvent evaporated to dryness on a nitrogen evaporator and re-suspended in 5 ml of methanol. Seven ml of the solved extract were transferred to 7 ml glass vials and stored under nitrogen in the dark in a fridge prior to HPLC analysis.

2.6.3 Vitamin E extraction from larval tissues

Vitamin E extraction from larval tissues was conducted in triplicates according to Cowey *et al.* (1981). Approximately 50 mg of larval tissues were homogenised with a Ultra-turrax (IKA-Werke, T25 BASIC, Staufen, Germany) in 5 ml 2% (w/v) ethanolic pyrogallol and incubated for 5 min in a water bath at 70ºC. Once removed from the water, 1 ml 60% KOH was added to tubes that were flushed with nitrogen, stoppered, shaken and returned to the water bath for 20 min. Next, were removed, cooled on ice and 4 ml distilled water and 6 ml iso-hexane + BHT added. Tubes were whirlmixed for exactly one minute to extract the vitamin E into the iso-hexane layer (top). To facilitate layers separation tubes were placed in a freezer (~ -18ºC) for approximately 10 min. Once layers were separated, 4 ml of the top layer were removed, placed in a small tube, evaporated to dryness, re-dissolved in 1 ml methanol and transferred to 2 ml glass vials prior to analysis by HPLC.

2.6.4 Vitamin E quantification by HPLC

Conditions employed for vitamin E determination in feed and larval tissues are shown in Table 2.4.

Table 2.4 HPLC conditions for vitamin E determination

Chromatograph	Thermo liquid chromatograph equipped with an injection valve type 7125 (100 µl), a LDC 4100 and a multi-wavelength UV-VIS detector.
Column	150 x 4.60 mm reverse phase Luna 5µm C18 column (Phenomenox, California, USA).
Mobile phase	98% methanol (methanol:ultrapure water)
Flow	1.0 ml min^{-1}
Pressure	< 1000 psi
Detection	UV
Wavelength	293 nm
T° of column	Room temperature
Injection volume	50 µl
Run time	Approximately 25 min

Elution order was δ-tocopherol, β-tocopherol, γ-tocopherol and α-tocopherol. Retention time of α-tocopherol was estimated approximately at 12 min (Figure 2.2).

Calculations to know vitamin E content are:

$$\text{Vitamin E (µg/g)} = \frac{peak\ area}{average\ std\ area} \times \frac{V\ iso-hexane}{V\ layer\ removed} \times \frac{V\ MeOH}{V\ injected} \times \frac{1}{weight}$$

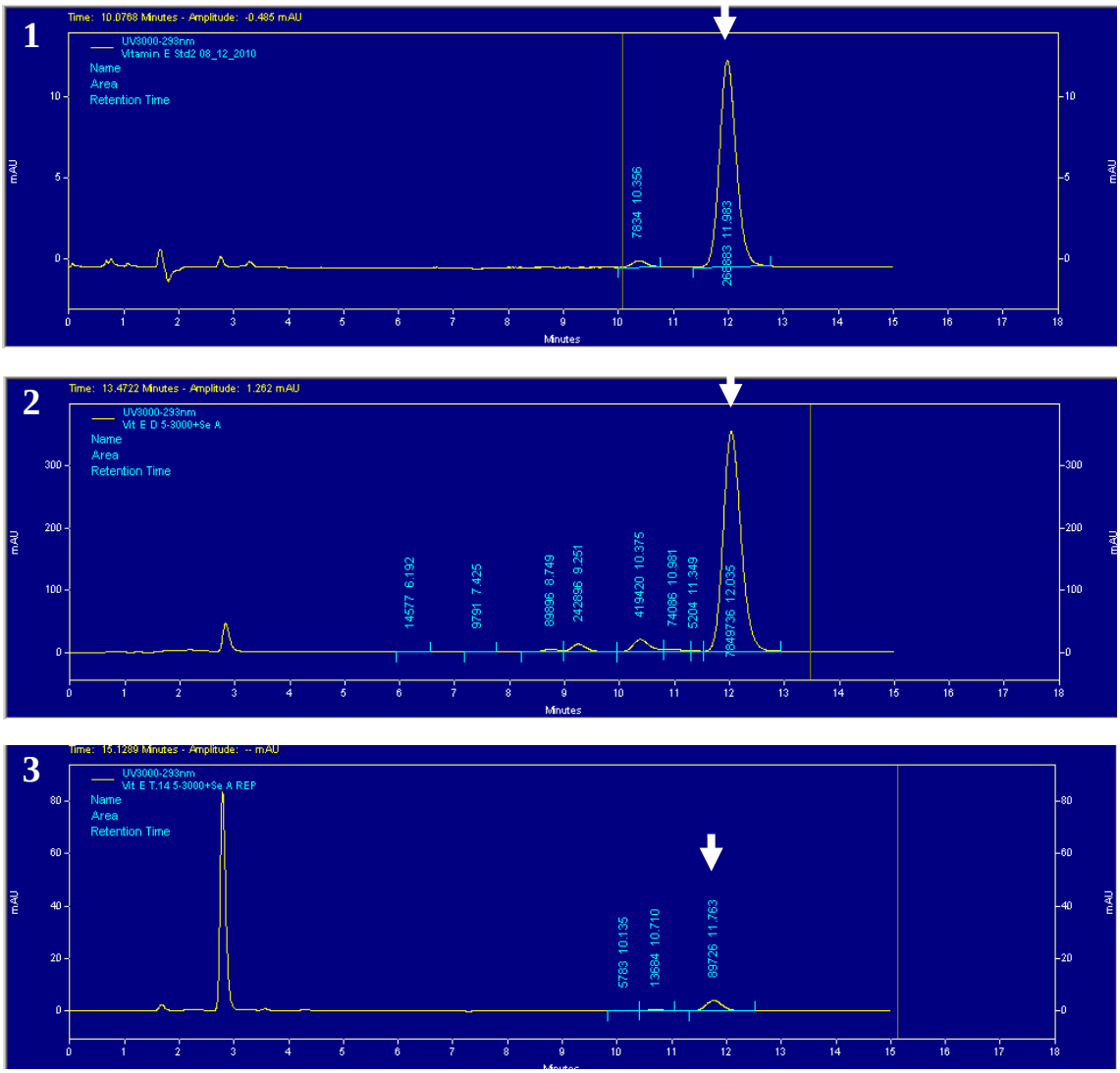

Figure 2.2 HPLC chromatograms showing identification of α-tocopherol (arrows) in standard (**1**), diet (**2**) and larvae (**3**).

2.7 Determination of vitamin C content

Ascorbil-2-monophosphate was extracted from feeds using a phosphate buffer and quantitated by reversed-phase HPLC with UV detection as developed by Roche Vitamins Ltd. Vitamin C analysis was performed during a stay at the Institute of Aquaculture, University of Stirling (Scotland, UK).

2.7.1 Vitamin C extraction from feeds

Samples were grinded and 5 g weighed into a 100 ml conical flask. 50 ml of 0.4 M phosphate buffer pH 3.0 was added and tubes stirred for 15 min at room temperature. Two aliquots of 1.5 ml were centrifuged (1610 x *g*) for 5 min at room temperature and supernatant was transferred into a 7 ml glass bottle. One ml was removed with a disposable syringe and filter through a disposable 0.45 µm filter unit into a 2 ml glass bottle. Vials were kept in fridge (4ºC) until HPLC analysis.

2.7.2 Vitamin C determination by HPLC

Conditions employed for vitamin C determination in feed and larval tissues are shown in Table 2.5. Retention time for ascorbic acid was estimated approximately 7.4 min (Figure 2.3).

Table 2.5 HPLC conditions employed for vitamin C determination

Chromatograph	Thermo liquid chromatograph equipped with an injection valve type 7125 (100 µl), a LDC 4100 and a multi-wavelength UV-VIS detector.
Column	150 x 4.60 mm reverse phase Gemini 5µm C18 column (Phenomenox, California, USA) fitted with a Gemini precolumn.
Mobile phase	30% eluant 1[a] and 70% eluant 2[b]
Flow	0.8 ml min^{-1}
Pressure	< 1000 psi
Detection	UV
Wavelength	293 nm
Tº of column	Room temperature
Injection volume	50 µl
Run time	Approximately 9 min
Absorbance range	0.1 AUFS

[a]Eluant 1: Dissolve 21.6 g KH_2PO_4 in 1.8 l of filtered water, add 4 ml of 1,5-dimethyhexylamine and adjust the pH to 3.0 with phosphoric acid. Adjust the volume to 2.0 l with filtered water. Filter through a white nylon 0.45 µm filter to degas the solvent.
[b] Eluant 2: Mix 900 ml of eluant 1 with 140 ml acetonitrile/ethanol 7:3 (v/v).

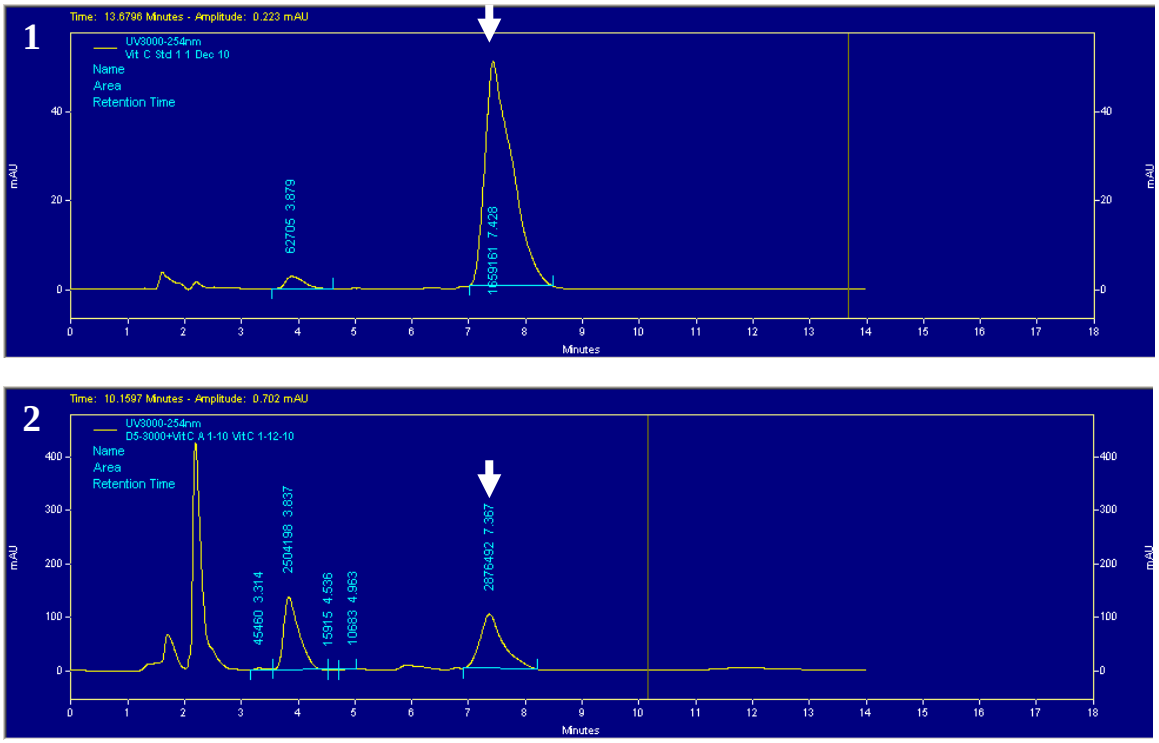

Figure 2.3 HPLC chromatograms showing identification of vitamin C (arrows) in standard (**1**) and diet (**2**).

2.7.3 Preparation of standards and calculation of their concentration

To prepare stock solution approximately 15 mg of 2-phospho-L-ascorbic acid tri sodium salt were weighed into a 20 ml volumetric flask, dissolved and volume adjusted with 0.4 M phosphate buffer pH 3.0. One ml of the stock solution was diluted to 25.0 ml using 0.4 M phosphate buffer pH 3.0 to make an approximately 0.1 mM solution.

The concentration of ascorbyl-2-monophosphate (C_{AMP}) was calculated in our working standard according to the following formula:

$$C_{AMP} = \frac{mst \times 100}{500} \times \frac{P}{100} \times 0.463$$

Where:

mst = the weight of tris-(cyclohexylammonium) ascorbic acid-2-phospate

1000 = Conversion from mg to µg; *mst* is the dilution factor

P = Purity of tris-(cyclohexylammonium) ascorbic acid-2-phospate

100 = Conversion of % purity

0.4363 = Conversion from tris-(cyclohexylammonium) ascorbic acid-2-phospate (MW = 553.7)

To calculate the response factor (RF) of ascorbyl-2-monophosphate (mVsml/µg) the next formula was applied:

$RF_{AMP} = A_{st}/C_{AMP}$

Where:

A_{st} = Mean peak area from injection of working standard solution (mVs)

C_{AMP} = Calculated concentration of ascorbyl-2-monophosphate

Experimental determinations are given as ascorbic acid equivalents (AAE)

$$C_{AMP}\ (mg/kg) = \frac{Asa}{RFamp \times msa} \times \frac{V1 \times V3}{V2}$$

$$C_{AAE}\ (mg/kg) = C_{AMP} \times \frac{17.61}{256.1} = C_{AMP} \times 0.6876$$

Where,

C_{AMP} = Content of ascorbyl-2-monophosphate in sample (mg kg^{-1})

C_{AAE} = Content of ascorbic acid equivalents in sample (mg kg^{-1})

A_{Sa} = Area obtained from the injection of the sample (mVs)

RF_{AMP} = Calculated response factor of ascorbyl-2-monophosphate (mVsml μg^{-1})

m_{sa} = weight of sample (g)

V_1 = Volume of the sample extract (ml)

V_2 = Volume of the aliquot taken for dilution (ml)

V_3 = Volume of the diluted sample solution (ml)

176.1 = MW of ascorbic acid (g mol^{-1})

256.1 = MW of ascorbyl-2-monophosphate (g mol^{-1})

0.6876 = Conversion factor of AMP to AAE

2.8 Selenium determination

Total selenium concentration was measured in total larvae and feeds. Samples were acidified in a microwave digestor (MarsXpress, CEM, Kamp-Lintfort, Germany) with 5 ml of 69% pure nitric acid. The resultant solution was poured after digestion into a 10 ml volumetric flask and made up to volume with distilled water. A total of 0.4 ml of this solution was then added to a 10 ml sample tube, 10 µl of the internal standard (Ga and Sc, 10 ppm) included and 0.3 ml of methanol added. The tubes were made up to volume with distilled water and total selenium measured in a collision/reaction cell ICP-MS (Thermo Scientific, Cheshire, UK) using argon and hydrogen as carrier gas. Collision/reaction cell ICP-MS is a technique that uses ion-molecule chemistry to eliminate polyatomic interferences from the mass spectrum of an ICP-MS. In this way, this technique has enabled ICP-MS to become virtually free of polyatomic interferences. Selenium determination was performed during a stay at Institute of Aquaculture, University of Stirling (Scotland, UK).

2.9 Histological analysis

2.9.1 Paraffin inclusion

Sea bass larvae were fixed in 10% buffered formalin for 1 or 2 days when they were processed. For its histological process larvae were dehydrated through graded alcohols (70-96º) thanks to Histokinette 2000 tissue processor (Leica, Nussloch, Germany), then xylene and finally embedded in paraffin wax (Jung Histoembedder, Leica, Nussloch, Germany). Paraffin blocks were sectioned at 3 µm on a microtome (Leica, RM2135, Leica Instruments, Nussloch, Germany) and stained with Haematoxilyn and Eosin (H&E) staining for its histopathological evaluation (Martoja and Martoja-Pearson, 1970). As well, several special staining techniques were employed, such as Periodic-acid-Schiff (PAS), Perl´s Prussian blue (PB), modified long Ziehl-Neelsen (ZN) and Sudan black (SB) staining (Martoja and Martoja-Pearson, 1970) to check the presence of different components (Table 2.6). The mounted sections were examined under light microscopy using a Olympus CX41 binocular microscope (Olympus, Hamburg, Germany) connected to an Olympus XC30 camera (Olympus, Hamburg, Germany), which was linked to a computer using image capturing software (CellB®, Olympus, Hamburg, Germany).

Table 2.6 Staining techniques employed in the present Thesis and their significance

Technique	Significance	Results
H&E	Histopathological evaluation	Collagen – Pale pink Muscle – Deep pink Acidophilic cytoplasm – Red Basophilic cytoplasm – Purple Nuclei – Blue Erythrocytes – Cherry red
PB	Demonstrates the presence of ferric iron, derivated from erythrocyte lysis	Ferric iron – Blue Nuclei – Red
PAS	Detects substances resultants from lipid peroxidation, such as lipofucsins or ceroid pigment	Ceroid – Magenta Nuclei – Blue
ZN	Detects substances resultant from oxidation of lipids and lipoproteins, such as lipofucsins or ceroid pigment	Ceroid – Magenta Nuclei – Blue Background – Pale magenta to pale blue
SB	Detects substances resultant from oxidation of lipids and lipoproteins, such as lipofucsins or ceroid pigment	Ceroid and red blood cells – Black Background – Pale grey

2.9.2 Resin inclusion

Ten larvae per tank were fixed for 24 h at 4º in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) and stored in cacodylate buffer until processed according to Millonig (Bancroft and Stevens, 1996). Samples were then rinsed in phosphate buffer and post-fixed for 1 h in 2% osmium tetraoxide in 0.2 M potassium ferrocyanide and dehydrated in acetone. Each larva was then embedded in an Araldite (Durcupan, Fluka, Buchs, Switzerland) resin block and kept in the oven at 60ºC overnight. Serial transverse and longitudinal larvae thick sections were cut at 1 µm on the ultramicrotome (Leica LKB Ultratome Nova, Nussloch, Germany), stained with 1% toluidine blue in 1% sodium

tetraborate and examined under light microscopy (Hoffman *et al*., 1983). This inclusion technique is used as a method to select regions to obtain ultrathin sections for TEM and to obtain a higher resolution for optical microscopy.

Once the region was selected, thin sections were cut at 50 nm with a diamond blade, placed on a copper grid and stained with lead citrate before its observation at a ZEISS EM 910 (Carl ZEISS, Oberkochen, Germany) transmission electron microscope at the Electron Microscope Service of University of Las Palmas de Gran Canaria. Microphotographs were taken employing a proscan Slow-scan CCD-Camera (Froscan Elektronische Systeme, Germany) which was linked to a computer using an image capturing software (Soft Imaging System, Germany).

2.9.3 Whole mount staining

A whole mount staining protocol was applied in order to determine tissue mineralization and the incidence of deformities in early larval stages at Chapters 4 and 5. At 35 dph (end of the experimental trial) 100 larvae per tank were stored in buffered formalin to examine skeletal deformities and 50 larvae to determine the ossification level. Larvae were stained with Alizarin red S to demonstrate bone following the protocol of Vandewalle *et al.* (1998) (Table 2.7). Larvae from the different experimental groups were stained simultaneously in order to prevent any technical variability.

After staining larvae were measured by a Profile Projector (V-12A Nikon, Nikon Co., Tokyo, Japan) under a magnification objective of 50X and classified in three total length size classes (<10 mm; 10-12 mm and >12 mm). Larvae belonging to the second size class were considered for deformities study and observed under a stereomicroscope (Leica DM2500, Nussloch, Germany). Deformities were classified in three groups attending to their localization:

- **Head deformities**: Include deformities such as pugheadness, alteration of brachistegals rays and opercula.
- **Lordosis**: Defines an inward curvature of the vertebral column.
- **Kyphosis**: Defined as the curvature of the upper spine.
- **Others**: Including deformities such as scoliosis, vertebral compression or coiled vertebrae.

Table 2.7 Single staining protocol according to Vandewalle *et al.* (1998)

	Step	Duration	Solutions
Hydratation	Ethanol 95%	1 h	For 100 ml: 95 ml absolute ethanol 5 ml distilled water
	Ethanol 95%	1 h	
	Ethanol 95%	1 h	
	Ethanol 75%	1 h	For 100 ml: 75 ml absolute ethanol 25 ml distilled water
	Ethanol 40%	1 h	For 100 ml: 40 ml absolute ethanol 60 ml distilled water
	Ethanol 15%	1 h	For 100 ml: 15 ml absolute ethanol 75 ml distilled water
	Distilled water	1 h or overnight	
Tissue digestion	Trypsin solution	1 h	90 mg porcine pancreas trypsin 70 ml distilled water 30 ml saturated solution of $Na_2B_4O_7$
Ca staining	Alyzarin red	1´30 h	1 gL^{-1} alizarin red in 0.5% KOH solution
Clearing	Gylcerin:KOH 1:3	12-24 h	For 100 ml: 25 ml glycerine 75 ml KOH (0.5%)
	Gylcerin:KOH 1:1	12-24 h	For 100 ml: 50 ml glycerine 50 ml KOH (0.5%)
	Gylcerin:KOH 3:1	12-24 h	For 100 ml: 75 ml glycerine 25 ml KOH (0.5%)
Storage	Glycerin		Pure glycerine with some grains of thymol to avoid the proliferation of microorganisms

The surface corresponding to bone in whole larvae was visualized and quantified using a computerized image analysis package (Image-Pro Plus®, Media Cybernetics, Maryland, USA) after staining with Alizarin red S. A list of image processing commands was used, encompassing the selection of pixel colour range and quantification. Selecting ranges of pixel values in colour images allowed the pixels associated with red to be distinguished. A mask was applied in order to turn all the selected pixels in bright objects. The number of selected pixels was then quantified using a particle analysis operation and by counting the area of all bright objects (in pixels). Larval size was estimated by calculating the surface areas (in pixels) covered by whole stained larvae.

2.10 Molecular biology

All processes indicated in this section were performed during a stay at the University of Insubria, Department of Biotechnology and Lifer Sciences (Varese, Italy). Studied genes are showed in Table 2.8. Catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzyme gene sequences were reconfirmed prior to its use for quantitative real time PCR (RT-PCR). Primers of these genes were ordered to Eurofins (Ebersberg, Germany) (Table 2.9).

Table 2.8 Genes studied in the present Thesis

Gene	Accession number
Superoxide dismutase	FJ860004
Catalase	FJ860003
Glutathione peroxidase	FM013606
Insulin-like Growth Factor I	AY800248
Insulin-like Growth Factor II	AY839105
α-Actin	FJ716131
Myosin heavy chain	DQ317302
Calpain 1	FJ821591

2.10.1 Total RNA extraction

Total RNA was extracted from sea bass larvae (≈200 mg) using PureYield RNA Midiprep System (Promega, Milan, Italy). Larvae were homogenised on ice in 2ml of Lysis buffer with 40 µl of β-Mercaptoethanol using a rotating probe homogeniser (Ultra-Turrax; IKA-Werke, T25 BASIC, Staufen, Germany). The rotating probe was washed between samples with NaOH 1M, Ethanol 100º and DEPC water. Afterwards lysates were incubated on ice for 10 min to complete the lysis and 2 ml were transferred to a disposable tube. To clear lysates 4 ml of RNA Dilution Buffer was added and mixed thoroughly by inverting the tube 3-4 times and then vortexing. Then 1 ml of Clearing Agent was added and once again mixed and vortexed. Afterwards tubes were placed in a water bath at 70ºC and incubated for 5 min to denature the samples and cooled at room temperature for at least 5 min. Samples were placed inside one Pureyield™ Clearing Column in a 50 ml collection tube and centrifuged at 12000 x *g* for 10 min at 20-25ºC temperature. Lysate discard was saved and 4 ml isopropanol were added. RNA purification was performed using a Vacuum Manifold (Promega, Milan, Italy), so centrifugation was not needed at this point. Lysates samples were poured into a PureYield™ Binding Column and vacuum was applied allowing the mixtures to pass through the column. Successively 20 and 10 ml of RNA wash solution were added to columns and allowed to pass through. After a 3 min vacuum to dry the columns was applied, 1 ml Nuclease-Free Water was added to the columns, incubated at room temperature for 2 min and vacuum applied. The purified RNA was stored at -80ºC in RNase-free-microcentrifuge-tubes.

2.10.2 RNA quality check

RNA measurements and quality checks were performed using a Bio-Rad SmartSpec Plus spectrophotometer (Bio-Rad, Milan, Italy). Purity was assessed the absorbance ratio at 260:280, indicating a ratio higher than 1.8 a high level of purity and that the sample is not contaminated by protein (McKenna *et al*., 2000). Furthermore, RNA degradation was checked by running 2µg of total RNA on a 1% agarose gel stained with ethidium bromide after separation by gel electrophoresis. Samples were run at 90 V for 45 min and then gel visualized on an UV transluminator (Bio-Rad UV Transluminator 2000, Milan, Italy).

2.10.3 Synthesis of cDNA

Complementary DNA (cDNA) is typically generated from messenger RNA (mRNA) by action of a retroviral reverse transcriptase which reverse transcribes a single strand molecule of RNA into single stand cDNA. After DNAse treatment (Invitrogen, Milan, Italy), 3 µg of total RNA was reverse transcribed into cDNA in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated at 65ºC for 5 min, chilled on ice and then 4 µl of 5x reverse transcription buffer, 2 µl 0.1M dithiothreitol (DTT), 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT) added. After incubation at 37ºC for 50 min, reaction was stopped by heating at 75ºC for 15 min.

2.10.4 Cloning and sequencing

To amplify selected primers sequences, touch up PCR was performed. The selection of this specific kind of PCR was due to the wide variety of melting temperatures of the primers (Table 2.9). In this sense, with a touch up PCR, the initial temperature of 54ºC increases to 0.5ºC in each cycle, arriving to a final temperature of 72ºC. A total of 30 cycles (10 touchdown) of the PCR amplification were performed for all primer sets, using an automated Thermal Cycler (Mycycler, Bio-Rad, Italy).

Table 2.9 Oligonucleotide primer sequences and melting temperatures used for PCR to produce standard curves

Oligo name	Sequence (5´→3´)	TmºC
SOD_T7	*GTAATACGACTCACTATAGGG*GTTGGAGAC CTGGGAGATGT	73.4
SOD antisense	CTCCTCATTGCCTCCTTTTC	57.3
CAT_T7	*GTAATACGACTCACTATAGGG*ATGGTGTGG GACTTCTGGAG	73.4
CAT antisense	AGTGGAACTTGCAGTAGAAACG	58.4
GPX_T7	*GTAATACGACTCACTATAGGG*AGTTAATCC GGAATTCGTGAGA	71.3
GPX antisense	TGAGTGTAGTCCCTGGTTGTTG	60.3

An aliquot of 4 µl of the resulting cDNA was amplified using 25 µl GoTaq Green Master Mix (Promega, Milan, Italy) in 50 µl of final volume containing 5 µl high fidelity buffer, 10 mM dNTPs and 50 pmol of each designed primer.

A total of 31 PCR amplification cycles (eight touchup) were performed for all primer sets, using an automated Thermal Cycler (MyCycler, BioRad, Milan, Italy). An aliquot of each sample was then electrophoresed on a 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer (BioRad, Milan, Italy) and bands were detected by ethidium bromide. Samples ran with a 100 bp+1.5 Kb DNA ladder to control molecular weight of DNA and with a negative control consisting of reaction mixture without cDNA, confirming in that way the absence of genomic contamination (Figure 2.4).

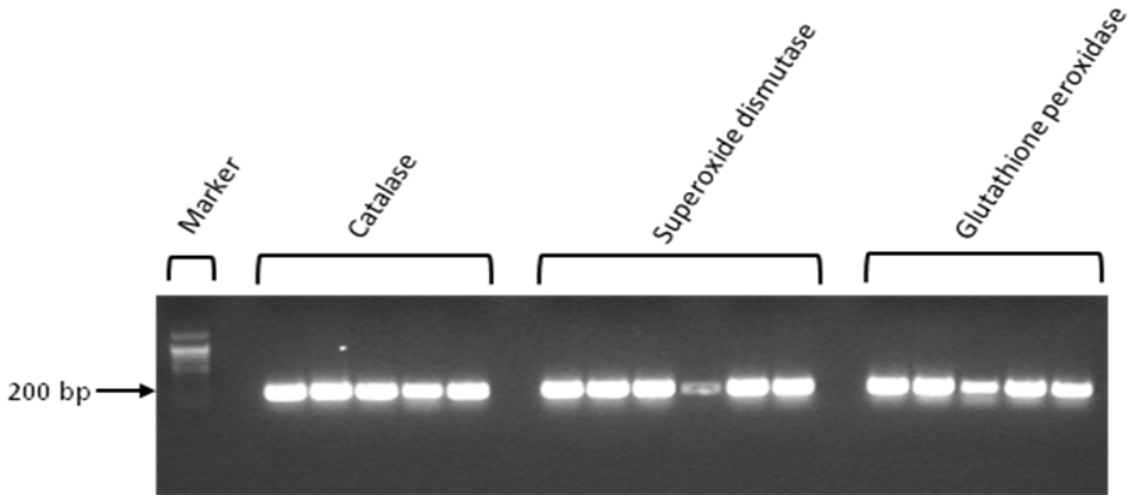

Figure 2.4 Agarose gel showing PCR products of each designed primer.

Prior to cloning, the PCR product was purified by cutting a DNA fragment of the predicted molecular weight from the gel and processing it with Wizard Clean-up System (Promega, Milan, Italy) according to the manufacturers instructions. After gel slice was dissolved it was placed on one SV Minicolumn and incubated one minute at room temperature. Columns were then centrifuged at 14000 x *g* for 1 min, liquid discarded and washed with 700 µl of Membrane Wash Solution diluted with 95% ethanol. After centrifugation the mini column was placed on a new microcentrifuge tube and eluted with 50 µl of Nuclease-Free water. The purified PCR product concentration was then measured using a SmartSpec Plus spectrophotometer (Bio-Rad, Milan, Italy). The

amount of PCR product to include in the cloning reaction was calculated using the following formula:

$$ng\ Insert = \frac{ng\ Vector \times Kb\ Insert}{Kb\ Vector} \times Insert : Vector\ molar\ ratio$$

Where:

ng vector= 50 ng

Kb vector= 3.0 Kb

Insert:Vector molar ratio= 3:1

Kb size of insert = In this case the genes had a length of 200 bp, so 0.2 Kb

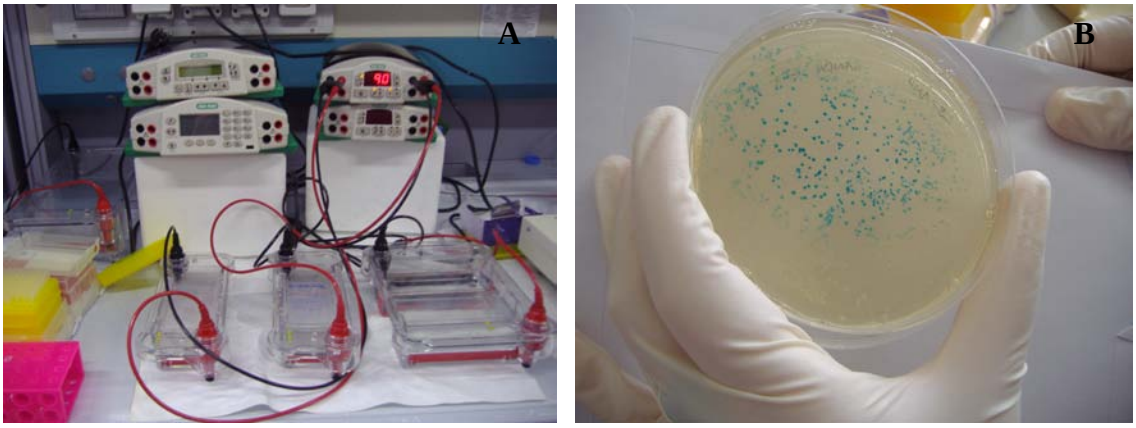

Figure 2.5 (**A**) Electrophoresis system employed in the present Thesis. (**B**) Plate containing blue and white colonies after transfection of *E. coli* with vector containing PCR products.

The cloning vector used in this study was pGEM®-T Easy cloning vector system (Promega, Milan, Italy; Figure 2.3). The ligation of the PCR product was performed by the intrinsic topoisomerase activity associated with the commercial cloning vector preparation. The cloning reaction was performed overnight at 4ºC and next day 50 µl of *Escherichia coli* bacteria (Promega, Milan, Italy) was added to this mixture and incubated in ice for 30 min. A heat shock was then performed in a water bath (40ºC) without shaking for 45 sec and tubes immediately returned to ice for 2 min. 940 µl of SOC medium (Sigma) was then added and the mixture was incubated at 37ºC for 2 h on a shaker mixer. The mixture containing the transformed cell was then spread on a 3 selective LB Agar plates per gene and incubated overnight at 37ºC. The selective LB

Agar plates contained ampicilin (100µg ml^{-1}), X-gal and IPTG. As a result, only the cells containing the plasmid which contained ampicilin resistant gene would grow. Moreover, the selection of cells containing a ligated PCR fragment was based on the colorimetric metabolic reaction of X-gal by Laczα gene, as the ligation of the PCR product would result in disruption of the Laczα reading frame leading to the production of white colonies. Five white colonies per gene were solved in 20µl of RNase-Free Water, being used 4µl of this solution to make a new PCR.

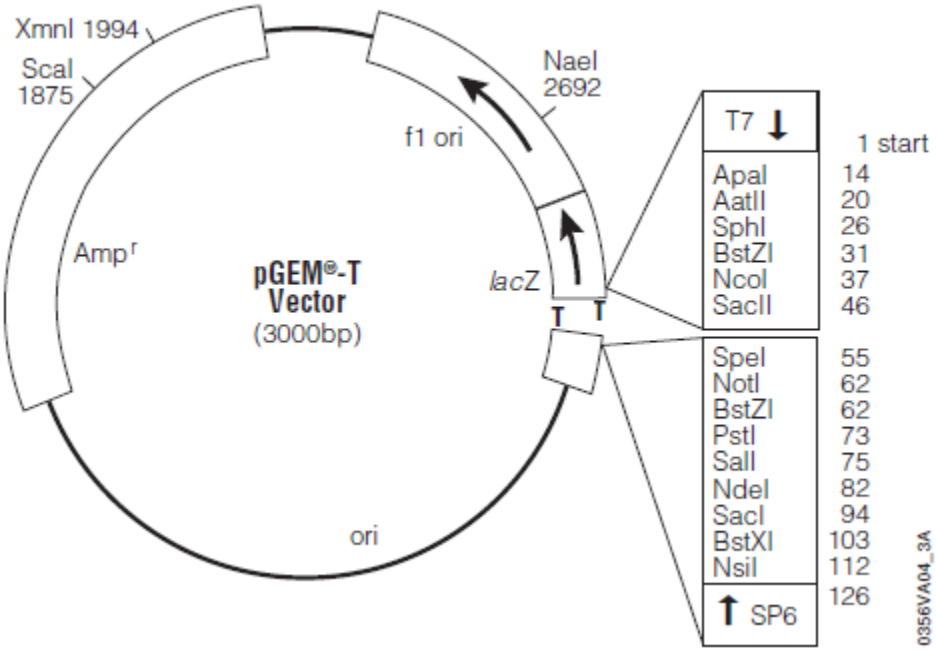

Figure 2.6 pGem – T Vector Map and sequence and sequence reference points.

According to the quality of the bands, one colony will be chosen and grown overnight at 37ºC in 10 ml of LB Agar containing ampicilin. The plasmid vector was purified using the Pureyield Midiprep System Kit (Promega, Milan, Italy) as described in the manual. The column was eluted with 30 µl of RNase-Free Water, concentration calculated with a SmartSpec Plus spectrophotometer (Bio-Rad, Milan, Italy) and conserved at -20ºC. Extracts were dried up and subsequently sent to BMR Genetics (Padova, Italy) for its gene sequencing in both directions (T7 and SP6).

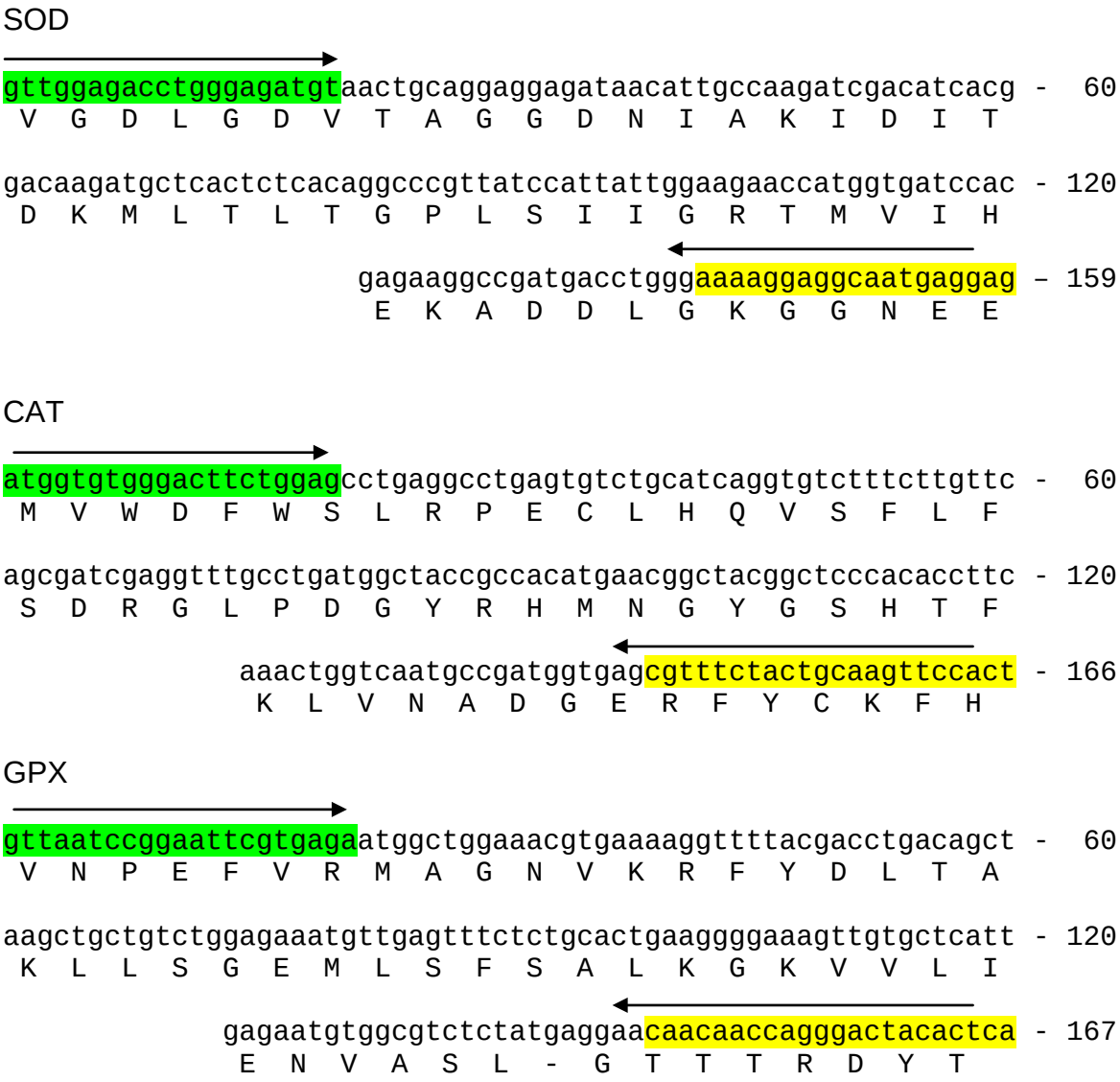

Figure 2.7 Obtained nucleotide sequences from sea bass antioxidant enzymes genes and predicted aminoacid sequences. Arrows indicate the position and sequences of primers used to amplify the cDNAs.

2.10.5 Quantitative real-time RT-PCR

2.10.5.1 Generation of *in vitro*-transcribed cRNAs for standard curves

The number of each target gene transcript copies could be absolutely quantified by comparing them with a standard graph constructed using the known copy number of

mRNAs of each gene. For this, a forward and a reverse primer were designed (Table 2.7) based on the mRNA sequences of *Dicentrarchus labrax*. These primer pairs were used to create templates for the *in vitro* transcription of cRNAs for each gene. The forward primers were engineered to contain a T7 or a T3 phage polymerase promoter gene sequence to their 5' end and used together with the reverse specific primer in a conventional RT-PCR of total sea bass larvae RNA. RT-PCR products were then checked on a 2.5% agarose gel stained with ethidium bromide, cloned using pGEM®-T Easy cloning vector system (Promega, Milan, Italy) and subsequently sequenced in the SP6 direction.

In vitro transcription was performed using T7 or T3 RNA polymerase and other reagents supplied in the Promega RiboProbe *In Vitro* Transcription System kit according to the manufacturer's protocol.

The molecular weight (MW) of the *in vitro*-transcribed RNAs were calculated according to the following formula:

$$MW = [(n^o\ of\ A\ bases) \times 329.2) + (n^o\ of\ U\ bases) \times 306.2) + (n^o\ of\ C\ bases) \times 305.2) + (n^o\ of\ G\ bases) \times 345.2)] + 159.$$

Spectrophotometry at 260 nm gave a concentration of each cRNA. Therefore, the concentration of the final working solutions were calculated and expressed as n° of molecules μl^{-1} (Table 2.10).

2.10.5.2 Generation of standard curves

The cRNAs produced by *in vitro* transcription were used as quantitative standards in the analysis of experimental samples. Defined amounts of cRNAs at 10-fold dilutions were subjected in triplicates to real-time PCR using one-step TaqMan EZ RT-PCR Core Reagents (Applied Biosystems, Italy), including 1X Taqman buffer, 3 mM MnOAc, 0.3 mM deoxynucleotide triphosphates (dNTP) except deoxythymidine triphosphate (dTTP), 0.6 mM deoxyuridine triphosphate (dUTP), 0.3 µM forward primer, 0.3 µM reverse primer, 0.2 µM FAM-6 (6-carboxyfluorescein-labeled probe), 5 units *rTH* DNA polymerase, and 0.5 units AmpErase UNG enzyme in a 25 µl reaction. AmpErase® uracil-N-glycosylase (UNG) is a 26-kDa recombinant enzyme encoded by the

Escherichia coli uracil-N-glycosylase gene. UNG acts on single-and double-stranded dU-containing DNA. It acts by hydrolyzing uracil-glycosidic bonds at dU-containing DNA sites. The enzyme causes the release of uracil, thereby creating an alkali-sensitive apyrimidic site in the DNA. The enzyme has no activity on RNA or dT-containing DNA. For TaqMan® assays, AmpErase® UNG treatment can prevent the reamplification of carry over PCR products from previous PCR reactions. When dUTP replaces dTTP in PCR amplification, AmpErase® UNG treatment can remove up to 200,000 copies of amplicon per 50 µl reaction. RT- PCR conditions were: 2 min at 50°C, 30 min at 60°C, and 5 min at 95°C, followed by 40 cycles consisting of 20 s at 92°C, 1 min at 62°C. The Ct (cycle threshold) values obtained by amplification were used to create standard curves for target genes (Figure 2.8).

Table 2.10 Molecular weights and number of molecules μl^{-1} of genes

Gene	Molecular weight	Molecules μl^{-1}
Superoxide dismutase	51833.8	3.25×10^{12}
Catalase	53428.2	5.53×10^{12}
Glutathione peroxidase	54530.6	3.43×10^{12}
Insulin growing factor I	181336.2	1.07×10^{12}
Insulin growing factor II	208412.8	1.44×10^{11}
Myosin	112235.0	1.39×10^{11}
α- actin	156736.6	5.69×10^{11}
Calpain 1	191785.2	5.21×10^{11}
Cathepsin-L	144192.0	1.00×10^{12}

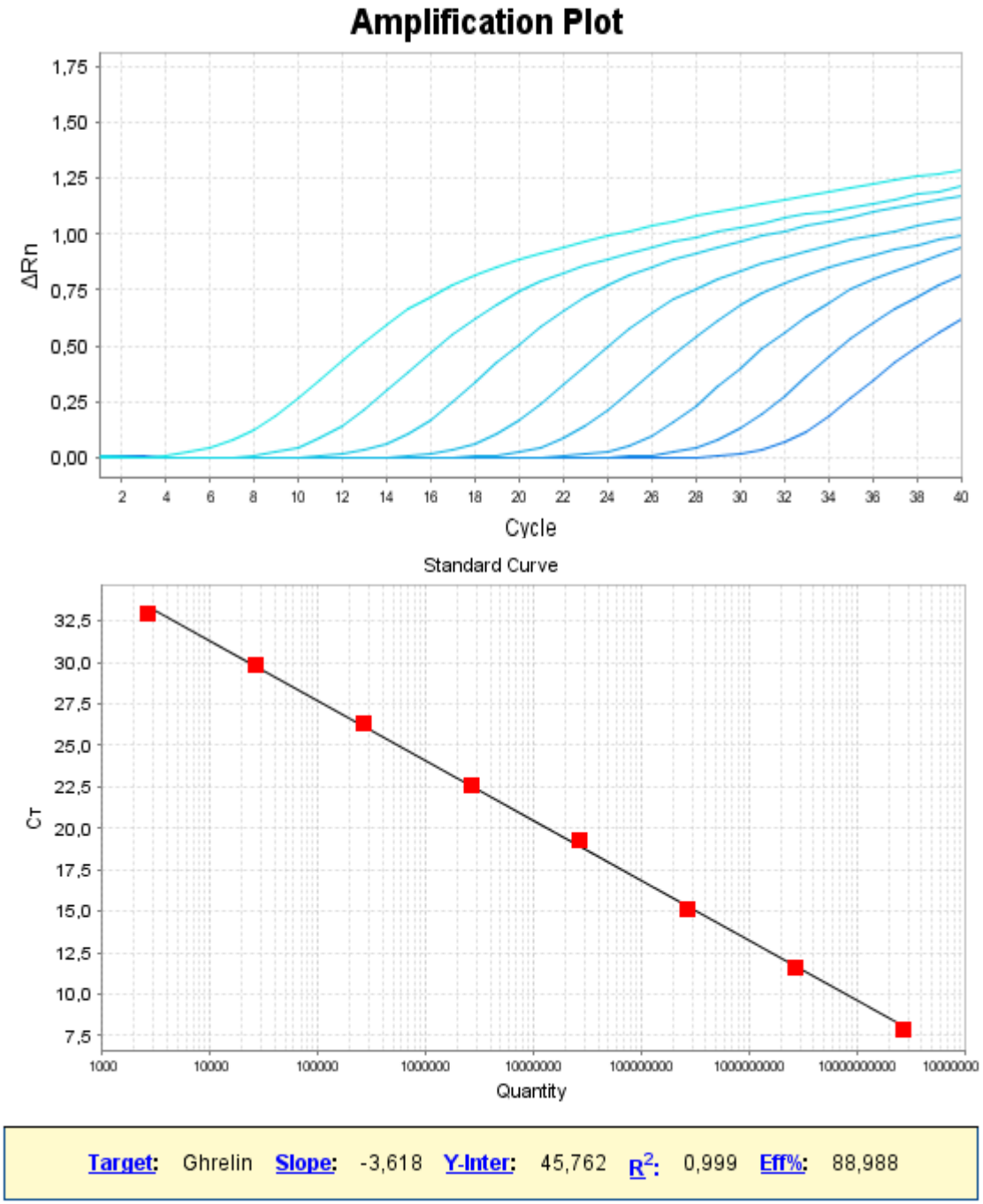

Figure 2.8 Standard curve and amplification plot of myosin heavy chain gene.

2.10.5.3 Quantitation of transcripts by one-step RT-PCR TaqMan® system

A hundred nanograms of total RNA extracted from the experimental samples were subjected, in parallel to triplicates of 10-fold-diluted, defined amounts of standard

cRNAs, to real-time PCR under the same experimental conditions as for the establishment of the standard curves. Real-time Assays-by-Design™ PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems (ABI; Monza, Italy). TaqMan® probes of the obtained target genes are showed at Table 2.11.

Table 2.11 TaqMan® probes of genes employed in the present Thesis

Genes	Kind of primer	Sequence (5´→3´)
CAT	Forward primer	ATGGTGTGGGACTTCTGGAG
	Reverse primer	GCTGAACAAGAAAGACACCTGATG
	TaqMan® probe	CAGACACTCAGGCCTCA
SOD	Forward primer	TGGAGACCTGGGAGATGTAACTG
	Reverse primer	TCTTGTCCGTGATGTCGATCTTG
	TaqMan® probe	CAGGAGGAGATAACATTG
GPX	Forward primer	AGTTAATCCGGAATTCGTGAG
	Reverse primer	AGCTTAGCTGTCAGGTCGTAAAAC
	TaqMan® probe	AATGGCTGGAAACGTG
IGF-I	Forward primer	GCAGTTTGTGTGTGGAGAGAGA
	Reverse primer	GACCGCCGTGCATTGG
	TaqMan® probe	CTGTAGGTTTACTGAAATAAAA
IGF-II	Forward primer	TGCAGAGACGCTGTGTGG
	Reverse primer	GCCTA CTGAAATAGAAGCCTCTGT
	TaqMan® probe	CAAACTGCAGCGCATCC
Calpn	Forward primer	ACTTTACAGGCGGCGTGA
	Reverse primer	GGCTCTGCTGATGATGTTGTAGA
	TaqMan® probe	TCAGATCGTACATTTCCG
α-actin	Forward primer	CCTCTTCCAGCCTTCCTTCA
	Reverse primer	TGTTGTAGGCGGTCTCATGGATA
	TaqMan® probe	CCAGCAGACTCCATACCGA
MyHC	Forward primer	TGGAGAAGATGTGCCGTACTCT
	Reverse primer	CGTGTCATTGATTTGACGGACATTT
	TaqMan® probe	AACTGAGTGAACTGAAGACC

TaqMan® PCR was performed on a StepOne Real Time PCR System (Applied Biosystems; Monza, Italy). To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

2.10.5.4 Sample quantification

Data from TaqMan® PCR runs were collected with StepOne™ Software v 2.0. Ct values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA (nº molecules/ng total RNA).

2.11 Statistical analysis

The statistical analysis showed within this Thesis were performed using the SPSS software package (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005). A significance level of 5% (P<0.05) was used for all tests performed.

The arithmetic or sample mean was used to provide as an estimate of the population mean together with the standard deviation (SD) to represent sample distribution and was calculated for each parameter measured.

2.11.1 Parametric testing

Parametric tests were performed based on the assumptions that the observations were made at random and that the test variances independent. Furthermore, the sample variances must be homogeneous and the data normally distributed. Where data failed to meet these requirements, non-parametric tests were employed (Section 2.11.2).

A Levene´s test was used to determine the normality and homogeneity of variances and once met the assumptions, were analyzed using a one-way analysis of variance (ANOVA) and where data differed significantly (P<0.05), Duncan multiple comparison *post-hoc* test was applied using a SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005). For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

where Y_{ij} is the mean value of the tank, μ is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

In addition, the two variables general linear model (GLM) features was used to analyze the molecular biology data sets. In this sense, the factor "sampling time" was incorporated into the model, thus allowing to observe differences between variables diets, effect of time and interactions. The model employed was:

$$Y_{ijk} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + \varepsilon_{ijk}$$

Where Y_{ij} is the mean value of the tank, μ is the mean population, α_i is the fixed effect of the diet, δ_j is the fixed effect of the time, $(\alpha\delta)_{ij}$ is the interaction between diet and time and e_{ij} is the residual error.

2.11.2 Nonparametric testing

Data failing to meet the assumptions for parametric tests or categoric data were analyzed using nonparametric statistical methods. A Kruskall-Wallis test was performed using a SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005). Means bearing significant differences (P<0.05) were further tested using squared Chi *post-hoc* test.

Chapter 3

α-tocopherol in weaning diets for European sea bass (*Dicentrarchus labrax*) improves survival and reduces tissue damage caused by excess dietary DHA contents

This work was published in Betancor *et al.* (2011) *Aquaculture Nutrition*, **17**(2): e112-e122.

Keywords:

DHA
larvae
myopathy
oxidative stress
sea bass
vitamin E

ABSTRACT

The objective of the present study was to investigate the combined effect of several dietary contents of vitamin E and polyunsaturated fatty acids, mainly DHA, on growth, survival, biochemical composition and tissue morphology of sea bass along early development. A feeding experiment was conducted in sea bass larvae using five different diets with the same proximate composition and different ratios of DHA concentrated fish oil (1, 3 and 5 % dry weight) and vitamin E (α-tocopheryl acetate) (150 and 300 mg 100 g^{-1} dry weight). DHA was readily deposited in fish tissues and associated to higher sea bass mortalities probably due to increased peroxidation risks. Besides, the elevation of dietary DHA contents up to 5% severely increased the incidence of muscular lesions and the presence of ceroid pigment within hepatocytes. However, elevation of dietary vitamin E levels markedly reduced the incidence of these symptoms in sea bass, increasing the tissue content in several polyunsaturated fatty acids and improving growth and stress resistance. Moreover, when sea bass was fed diets containing high vitamin E levels, fish showed a significant improvement in growth when dietary DHA was raised from 1 to 3 %. Therefore, in sea bass larvae a ratio of 3% DHA and 300 mg 100 g^{-1} vitamin E seems to be adequate to achieve a good larval performance and to avoid muscular lesions.

3.1 Introduction

Most marine fish larvae require high amounts of *n*-3 LC-PUFA (long chain polyunsaturated fatty acid) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in their diets as essential fatty acids for normal growth and development, differing from freshwater species that can produce these fatty acids from linolenic acid (Sargent *et al*., 1999). Dietary levels of these essential fatty acids in marine fish larvae vary over a range between 0.3 and 39 g kg^{-1} (formulated diet or live prey) dry weight (DW; Izquierdo, 1996). Besides, an increase of dietary *n*-3 LC-PUFA in larval sea bream (*Sparus aurata*) up to 8% improves larval growth and survival (Liu *et al*., 2002); meanwhile inclusion levels of 1.5% promote maximum growth in sea bream juveniles (Ibeas *et al*., 1994), proving the essentiality of these fatty acids in larval stages. 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. On the other hand, fish larval tissues are known to be very rich in those polyunsaturated fatty acids (PUFA) (Salhi *et al*., 1997) and require the action of antioxidants to protect them intra- and extra-cellularly from free radical compounds (Sargent *et al*., 1997).

Vitamin E (α-tocopherol; α-TOH) 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). Thus, for physiological protection against oxidation, the increasing in dietary PUFA must be combined to an increase in α-TOH (Sargent *et al*., 1997). Mourente *et al.,* (1999a) reported that *Dentex dentex* larvae fed *Artemia* enriched with apparently supra-optimal levels of *n*-3 LC-PUFA showed significantly lower α-TOH contents and poorer performance indicating increased oxidative stress. Tocher *et al*., (2003) have shown that supplementation of α-TOH to an oxidized diet improves sea bream juveniles growth and reduces lipid peroxidation products content in sea bream and turbot (*Psetta maxima*) tissues. Indeed, tissue α-TOH contents are closely related with tissue PUFA 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). Dietary vitamin requirements of fish decrease as the age and weight of the animal increases and as the growth rate decreases (Amezaga and Knox, 1990). Growth rates are much higher for larvae than for adult fish (Gatesoupe 1994), and therefore, young fish are more susceptible to a lack of vitamins in their food than are juveniles or

subadults (Dabrowski, 1986). Thus, early nutritional studies have showed that α-TOH is essential for marine fish larvae (Watanabe *et al*., 1970; Murai and Andrews, 1974; González *et al*., 1995). In gilthead sea bream larvae, González *et al*. (1995) found that the increase in microdiet α-TOH content improves larval growth and survival.

Lipid peroxidation as a consequence to an imbalance between PUFA and antioxidants will damage the biomembranes, producing several pathological conditions in fish (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). However, there is a complete lack of studies regarding the combined effect of dietary PUFA levels and α-TOH and its relation on potential pathological damages on fish larvae.

Thus, the objective of the present study was to investigate the combined effect of several dietary contents of α-TOH and PUFA, mainly DHA, on growth, survival, larval fatty acid composition and tissue morphology of sea bass along early development.

3.2 Materials and methods

3.2.1 Fish

Sea bass (*Dicentrarchus labrax)* larvae were obtained from natural spawnings from France (Ecloserie Marine de Gravelines, Nord-Pas-de-Calais), the experiment was carried out in the *Instituto Canario de Ciencias Marinas* facilities (Telde, Canary Islands, Spain). A trial was conducted to test 5 microdiets in triplicates. Larvae were previously fed a commercial microdiet until they reached 34 days old. 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 $tank^{-1}$ 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 (34 g L^{-1} salinity) at an increasing rate of 1.0 - 1.5 L min^{-1} 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^{-1}), attaining 5-8 g L^{-1} dissolved O_2 and saturation ranged between 60 and 80%.

3.2.2 Diets

Five isonitrogenous and isolipidic experimental microdiets (pellet size<250 µm) similar in their EPA content and different in DHA and vitamin E content were formulated (Table 3.1) using concentrated fish oils EPA500 and DHA500 (CRODA, East Yorkshire, England, UK) as sources of EPA and DHA. The α-TOH (DL- α- Tocopheryl Acetate) contents were tested in 150 and 300 mg 100 g^{-1} DW (Sigma-Aldrich, Madrid, Spain). Given the scarcity of works focusing on the initial requirements of α-TOH in marine fish larvae, these levels were based on previous studies performed by Tocher *et al.* (2002) and Ortuño *et al.* (2000) in fry sea bream. 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), EPA500 and DHA500 were added in different quantity to obtain the desired ratios, and the oleic acid was added to equalize the lipid content in each diet (Table 3.1). The microdiets were prepared according to Liu *et al*. (2002) by mixing squid powder and water soluble components, then the lipid and fat soluble vitamins and, finally, warm water solved gelatine. The paste was pelleted and dried in oven at 38ºC 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. Diets were manually supplied; fourteen times per day every 45 min from 9:00-19:00. Daily feed supplied was 2.0 and 2.5 g $tank^{-1}$ during the first and second week of feeding, respectively.

3.2.3 Growth and survival

Before the end of the experiment an activity test was conducted by handling 20 larvae $tank^{-1}$ 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 live larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105 ºC 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish $tank^{-1}$ at the beginning, in the middle and at the end of the trial.

Table 3.1 Proximate composition of the experimental diets.

Dietary DHA/vitamin E	*1/150*	*1/300*	*3/150*	*3/300*	*5/150*
EPA g kg^{-1} (DW)	27.29	27.41	27.05	27.08	24.40
DHA g kg^{-1} (DW)	7.60	8.53	30.80	31.48	58.70
α-TOH mg kg^{-1} (DW)	1490	2960	1560	2900	1520
Oleic acid g kg^{-1}	82.60	88.08	64.03	63.97	37.45
Lipid (DW)	17.05	17.50	17.97	18.16	17.88
Protein (DW)	70.07	69.86	69.82	70.23	69.95
Humidity (%)	9.65	11.40	10.69	9.38	9.71

3.2.4 Biochemical analysis

Besides, all the remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition analysis 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 *et al.,* 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*. (1992) and identified by comparison to previously characterized standards and GLC-MS.

3.2.5 Histopathological sampling

Thirty larvae from each tank were collected at the beginning, middle and end of the feeding trial and fixed in 10% buffered formalin for 1 or 2 days, dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax. Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm, and sections were stained with Haematoxilin and Eosin (H&E) for histopathologic evaluation. Sections were further stained by the Periodic Acid-Schiff´s (PAS), Ziehl-Neelsen and Sudan Black methods to control the presence of ceroid pigments and with Prussian blue for iron pigments (Martoja and Martoja-Pearson, 1970).

3.2.5 Statistical analysis

Survival and growth data were tested for normality and homogeneity of variances, not requiring any transformation. Chi-squared test was employed for incidence of muscular lesions and ceroid pigment. Survival and growth data were treated using one-way ANOVA. Means were compared by Duncan's test ($P<0.05$) using SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005). For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

3.3 Results

All experimental microdiets were well accepted by larvae. For a given dietary vitamin E content, increase in dietary DHA significantly reduced larval survival at the end of the experiment (Figure 3.1), whereas for a given dietary DHA content, elevation of dietary vitamin E significantly improved total length and resistance to an air exposure stress (Figures 3.2 and 3.3). Increase in DHA did not improve total length at the lowest dietary α-TOH contents, but it significantly improved ($P=0.003$) total length at dietary α-TOH values of 300 mg 100 g^{-1} (Figure 3.2). Lowest growth was found in larvae fed the highest DHA levels (5/150). The one way ANOVA analysis showed a significant and positive effect of the elevation of DHA from 1 to 3%, being larvae fed this later level a 4% larger. But further elevation of DHA levels up to 5% significantly reduced total length, being these larvae a 7% smaller. This analysis also showed a positive significant effect of α-TOH on final total length, being larvae fed with 300 mg a 6% larger.

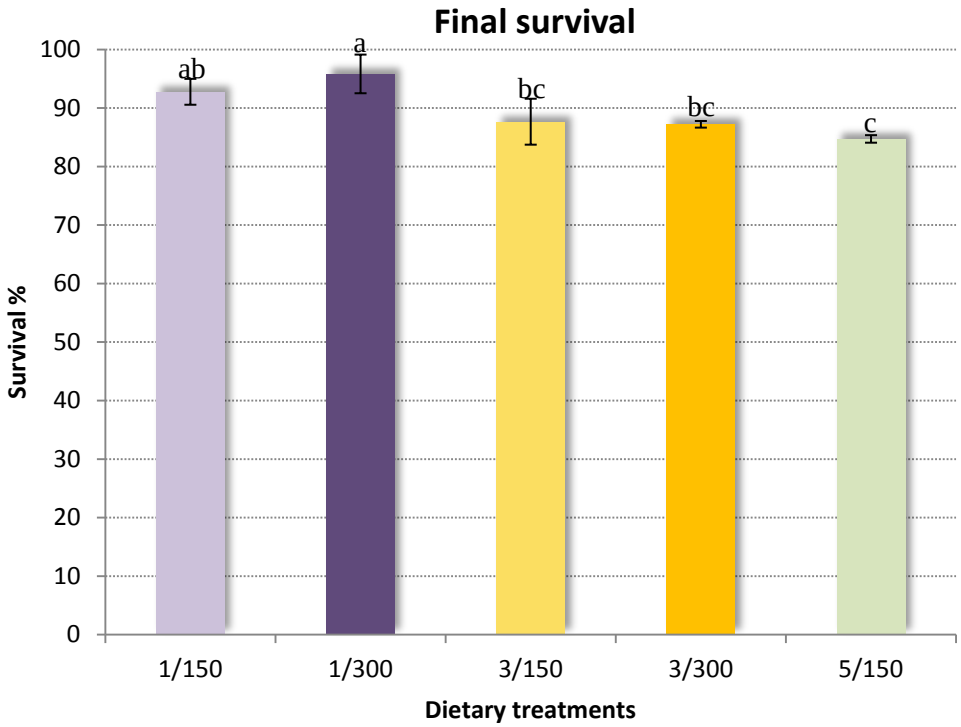

Figure 3.1 Survival rate of sea bass fed the experimental diets containing several DHA and α-TOH contents for 14 days.

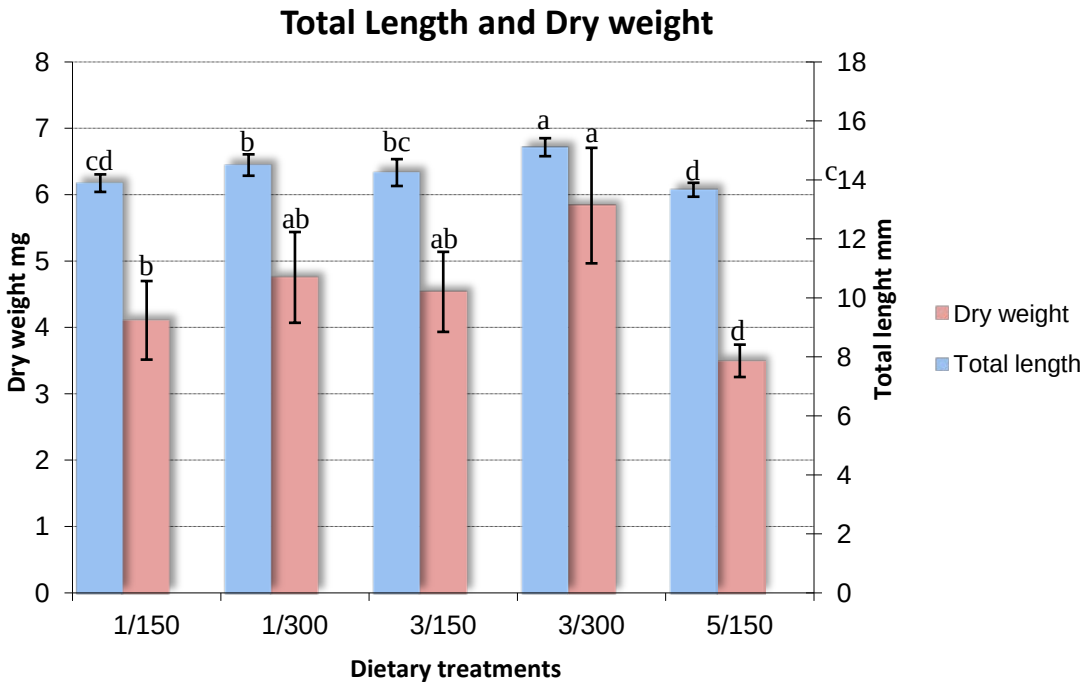

Figure 3.2 Total length and dry weight of sea bass after 14 days of feeding the experimental diets containing several DHA and α-TOH contents

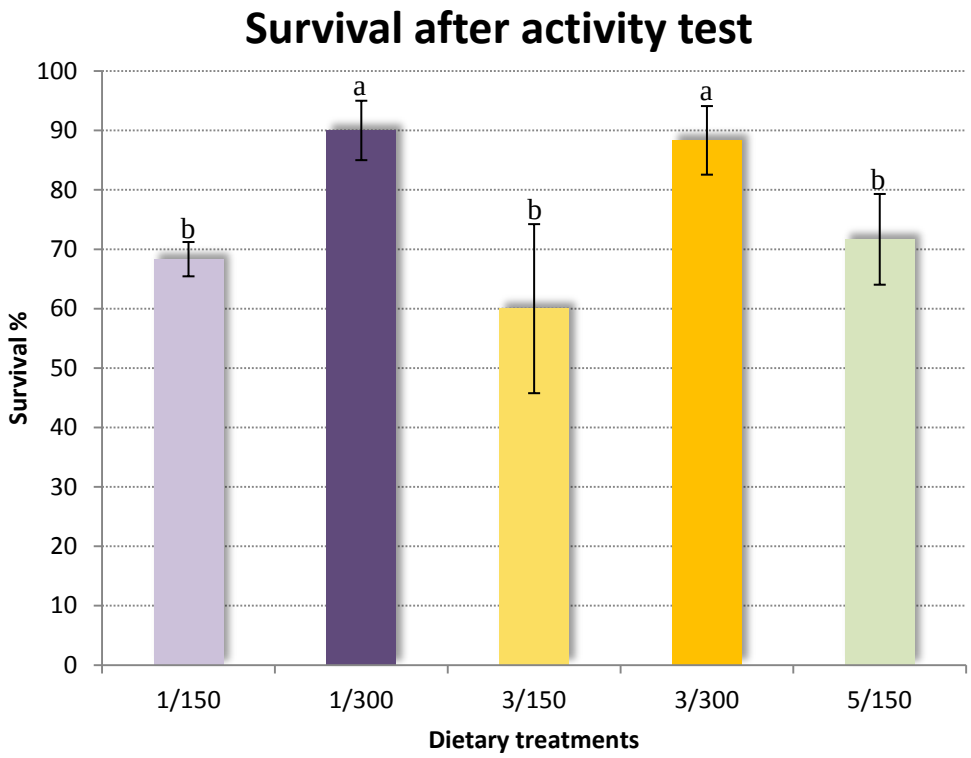

Figure 3.3 Survival of sea bass larvae 24 hours after the activity test.

Diets fatty acids compositions were similar in saturated content, whereas they differed in their monounsaturated and polyunsaturated fatty acids contents (Table 3.2). Diets containing about 1% DHA (1/150 and 1/300) showed 28.47 % higher monoenoic fatty acids and 27.79 % higher *n*-9 fatty acids than diets containing 3 or 5 % DHA (3/150, 3/300 and 5/150) due to the higher oleic acid content in the former diet. Elevation of dietary DHA also increased *n*-3, *n*-3 LC-PUFA and *n*-6 fatty acids. Total lipid content of sea bass larvae did not vary among dietary treatments, moreover, fatty acid composition of the larvae generally reflected the fatty acid composition of the diet (Table 3.3). However, in larvae fed 1% DHA diets, elevation of α-TOH from 150 to 300 mg 100 g^{-1}, increased EPA in a 12.79 %, *n*-3 in a 5.20 % and *n*-3 LC-PUFA in a 5.82 %, whereas in larvae fed 3% DHA, elevation of α-TOH from 150 to 300 mg 100 g^{-1} increased arachidonic acid in a 1.44 %, DHA in a 1.62 % and *n*-6 in a 23.78 % increase.

Microscopic lesions were observed in skeletal muscle tissues. These lesions showed a hyaline degeneration characterized by eosinophilic and pale cytoplasm which lost the normal cytoplasmatic striations and the adjacent nucleous (Figure 3.5, A-B). Afterwards, cytoplasm became flocculated or granular as the myofibre starts to fragment (Figure 3.5, C-D). Mononuclear infiltrate, mainly lymphocytes and macrophages surrounded the affected fibres, crossing the basal lamina and clearing cytoplasmatic

debris (Figure 3.5, E-F). A higher incidence of muscular lesions was found in larvae fed lower α-TOH content (1500 mg 100 g^{-1}), which was increased by the increase in dietary DHA up to 5% (Figure 3.6). A lower incidence of muscular lesions was found in fish fed higher dietary α-TOH diets (300 mg 100 g^{-1}) (Figure 3.6) showing a 11.6% of the observed larvae lesions at 48 dph. The incidence of these lesions was increasing along the experiment as a consequence of feeding the experimental diets. For instance, 13.6% of larvae fed diet 5/150 presented muscular lesions at 41 dph, increasing up to 34.6% at 48 dph. A negative correlation was found, at the end of the feeding trial, between percentage of lesions incidence and fish growth ($y = -0.0721x + 15.293$; $R^2 = 0.8684$). No lesions were found at larvae at the beginning of the feeding trial.

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

Dietary DHA/vitamin E	*1/150*	*1/300*	*3/150*	*3/300*	*5/150*
12:0	0.05	0.05	0.04	0.03	0.02
14:0	1.53	1.58	1.27	1.26	0.77
14:1*n*-7	0.13	0.03	0.10	0.10	0.06
14:1*n*-5	0.23	0.37	0.17	0.16	0.09
15:0	0.25	0.23	0.26	0.25	0.17
15:1*n*-5	0.14	0.26	0.12	0.12	0.06
16:0ISO	0.06	0.14	0.01	0.06	0.07
16:0	5.71	5.93	6.47	6.40	5.78
16:1*n*-9	3.39	2.86	2.45	2.45	1.42
16:1*n*-7	0.17	3.44	0.13	0.13	0.07
16:1*n*-5	0.09	0.13	0.10	0.09	0.07
16:2*n*-6	0.51	0.52	0.41	0.40	0.22
16:2*n*-4	0.06	0.07	0.05	0.08	0.08
17:0	0.22	0.22	0.23	0.27	0.26
16:3*n*-4	1.17	1.16	0.85	0.84	0.50
18:0	1.17	n.d.	1.88	1.86	2.40
18:1*n*-9	48.45	50.33	35.63	35.23	20.94
18:1*n*-7	3.03	3.54	2.31	2.50	2.05
18:1*n*-5	0.32	0.31	0.24	0.17	0.12
18:2*n*-6	8.03	8.05	7.71	7.80	7.51
18:2*n*-4	0.32	0.30	n.d.	0.26	0.19
18:3*n*-6	n.d.	0.14	n.d.	n.d.	0.27
18:3*n*-3	1.06	1.10	1.07	1.08	1.10
18:4*n*-3	1.07	1.05	0.96	0.95	0.78
18:4*n*-1	n.d.	0.11	0.09	n.d.	0.07
20:0	n.d.	n.d.	0.17	0.18	0.32
20:1*n*-9	0.95	0.97	0.97	1.19	1.57
20:1*n*-7	n.d.	n.d.	n.d.	n.d.	0.14
20:2*n*-9	n.d.	0.07	0.18	n.d.	0.27
20:3*n*-9	n.d.	0.10	n.d.	0.13	n.d.
20:4*n*-6	0.46	1.03	1.30	1.30	1.66
20:4*n*-3	0.52	0.51	0.57	0.56	0.59
20:5*n*-3	16.00	15.66	15.05	14.91	13.65
22:1*n*-11	n.d.	0.15	0.14	n.d.	0.34
22:4*n*-6	n.d.	0.00	0.85	0.87	1.76
22:5*n*-3	0.45	0.47	1.05	1.05	1.81
22:6*n*-3	4.46	4.87	17.14	17.33	32.83
Saturated	8.99	8.16	10.38	10.29	9.79
Monoenoics	56.89	56.62	42.35	42.14	26.93
n-3	23.56	23.66	35.82	35.89	50.75
n-6	9.00	9.74	10.27	10.37	11.41
n-9	52.78	51.76	39.23	38.99	24.21
n-3 LC-PUFA	21.43	21.52	33.79	33.85	48.87
ARA/EPA	0.17	0.38	0.48	0.48	0.68
EPA/DHA	21.06	18.37	4.87	4.74	2.33
Oleic a./DHA	63.75	59.03	11.57	11.19	3.57
Oleic a./*n*-3 LC-PUFA	13.88	14.01	6.16	6.02	2.52
n-3/*n*-6	15.34	13.88	19.41	19.05	24.88

Data are means ± SD; n.d., not detected

Table 3.3 Lipid levels (g kg $^{-1}$) and fatty acid composition (% total identified fatty acid) of total lipids from sea bass at 34 dph and after 14 days of feeding the experimental diets (48 dph)

Dietary DHA/vit E	*Initial*	*1/150*	*1/300*	*3/150*	*3/300*	*5/150*
Total lipids (g kg^{-1})	291±0.0	189±1.9	164±1.9	172±2.1	167±1.1	175±1.0
14:0	0.5±0.1	0.9±0.1	0.8±0.1	0.9±0.1	0.9±0.1	0.7±0.1
14:1*n*-7	1.5±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0
15:0	0.3±0.1	0.4±0.0	0.2±0.1	0.3±0.0	0.3±0.0	0.3±0.0
15:1*n*-5	0.1±0.0	0.2±0.1	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0
16:0	19.0±1.2	14.0±2.1	12.1±1.7	14.8±1.4	15.3±2.7	13.9±1.4
16:1*n*-9	n.d.	0.9±0.1	0.8±0.2	0.8±0.1	0.8±0.1	0.620.0
16:1*n*-7	2.9±0.21	2.2±0.2	2.0±0.5	1.9±0.2	1.9±0.3	1.3±0.1
16:1*n*-5	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0
16:2*n*-6	n.d.	0.5±0.0	0.5±0.1	0.4±0.1	0.5±0.0	0.3±0.0
16:2*n*-4	0.2±0.0	0.2±0.1	0.1±0.0	0.2±0.1	0.2±0.1	0.3±0.1
17:0	0.5±0.0	0.4±0.1	0.4±0.1	0.5±0.1	0.5±0.1	0.5±0.0
16:3*n*-4	0.1±0.0	0.9±0.1	0.8±0.2	0.8±0.1	0.8±0.1	0.5±0.1
16:4*n*-3	0.6±0.0	0.6±0.1	0.5±0.1	0.6±0.1	0.6±0.1	0.5±0.0
16:4*n*-1	0.3±0.0	0.7±0.0	0.6±0.1	0.6±0.0	0.6±0.1	0.4±0.0
18:0	7.3±1.0	6.2±1.0	5.1±0.6	6.5±0.4	6.6±1.2	6.6±0.6
18:1*n*-9	11.7±4.2	31.2±3.3	27.5±7.0	26.3±3.0	26.4±4.1	18.4±2.1
18:1*n*-7	2.6±0.9	4.1±0.5	3.4±0.8	3.6±0.2	3.7±0.5	2.0±1.7
18:2*n*-6	15.7±0.3	5.8±0.2	5.6±0.9	6.0±0.17	5.8±0.5	5.8±0.4
18:2*n*-4	0.1±0.0	0.2±0.0	0.2±0.1	0.3±0.0	0.2±0.0	0.2±0.0
18:3*n*-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0
18:3*n*-3	1.4±0.0	0.5±0.0	0.5±0.1	0.6±0.0	0.6±0.0	0.6±0.0
18:3*n*-1	0.7±0.0	0.1±0.0	0.2±0.0	0.2±0.1	0.1±0.0	0.1±0.0
18:4*n*-3	n.d.	0.4±0.1	0.5±0.2	0.4±0.0	0.4±0.0	0.4±0.0
20:0	n.d.	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0
20:1*n*-9	2.1±0.23	2.0±0.3	1.6±0.4	2.0±0.2	1.9±0.3	2.0±0.2
20:2*n*-9	n.d.	0.8±0.1	0.7±0.1	0.9±0.0	0.9±0.1	1.0±0.1
20:3*n*-9	n.d.	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
20:4*n*-6	1.7±0.0	2.0±0.3	2.0±0.3	2.2±0.3	2.3±0.3	2.5±0.2
20:4*n*-3	0.2±0.0	0.3±0.0	0.3±0.1	0.2±0.0	0.2±0.0	0.2±0.0
20:5*n*-3	7.4±0.0	10.3±3.6	11.6±2.7	8.3±1.8	8.4±2.2	7.9±1.2
22:1*n*-11	1.1±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
22:4*n*-6	0.3±0.0	0.3±0.0	0.2±0.0	0.8±0.2	0.9±0.2	1.5±0.1
22:5*n*-3	1.2±0.2	0.9±0.3	1.0±0.2	0.9±0.2	0.9±0.2	1.0±0.1
22:6*n*-3	17.7±3.9	11.0±3.7	11.0±3.0	19.0±7.0	19.3±6.9	28.4±6.3
Saturated	27.7±4.2	22.3±3.3	19.0±2.5	23.6±2.0	24.2±4.2	22.7±2.3
Monoenoics	20.4±5.4	41.4±4.4	36.0±8.9	35.5±4.0	35.5±5.5	24.9±4.7
n-3	28.5±6.1	24.1±7.8	25.4±6.1	29.9±9.0	27.6±10.6	39.2±7.6
n-6	19.7±0.0	8.8±0.1	8.5±1.2	7.7±3.1	9.6±0.7	10.4±0.2
n-9	13.8±4.9	34.3±3.7	30.0±7.5	29.3±3.2	29.3±4.5	21.0±2.8
n-3 LC-PUFA	26.5±6.5	22.5±7.3	23.9±5.6	28.4±8.8	26.1±10.5	37.6±7.5
ARA/EPA	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.3±0.0	0.3±0.0
EPA/DHA	0.4±0.0	0.9±0.0	1.1±0.1	0.4±0.1	0.4±0.0	0.3±0.0
OLA/DHA	0.7±0.9	3.1±1.2	2.7±1.2	1.5±0.7	1.6±0.8	0.7±0.2
OLA/*n*-3 LC-PUFA	0.4±0.1	1.6±0.6	1.3±0.5	1.0±0.4	1.2±0.5	0.5±0.2
n-3/*n*-6	1.6±0.8	2.7±0.9	3.0±0.7	4.9±4.0	2.9±1.3	3.8±0.8

Data are means ± SD. Values within the same row bearing different superscript letter are significantly different ($P< 0.05$); n.d., not detected. LC-PUFA, long chain polyunsaturated fatty acid; ARA, arachidonic acid; EPA, eicosapentenoic acid; DHA, docosahexaenoic acid; OLA, oleic acid

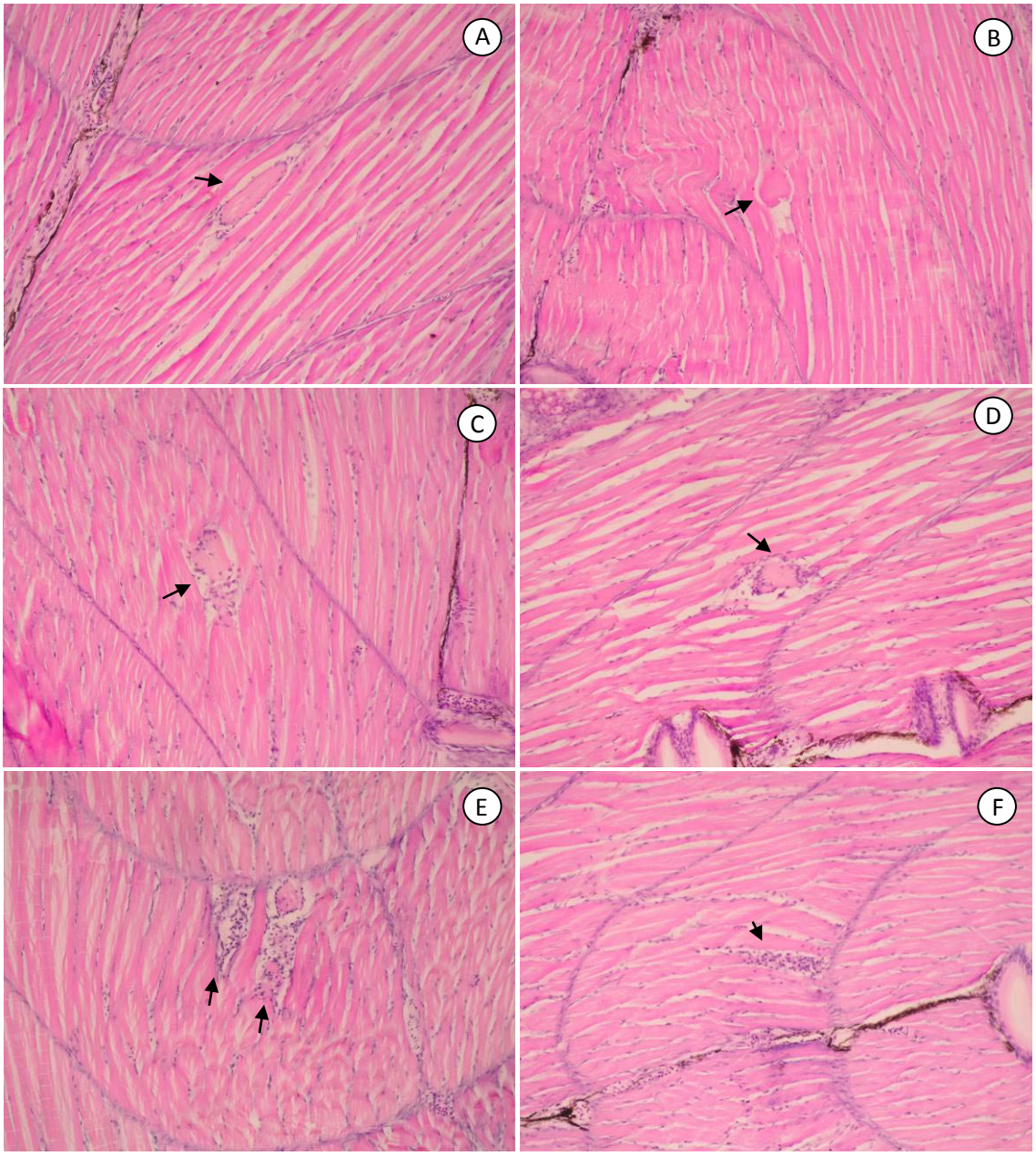

Figure 3.4 Muscular lesions found in larvae fed diet 5/150 at 48 days old, Haematoxilin & Eosin staining (x400). **A/B**- Initial lesion showing fibre swelling (arrows); **C/D**- Fibres start breaking down (arrows); **E/F**- Intense mononuclear infiltrate that traverse the basal lamina of muscular fibre (arrows).

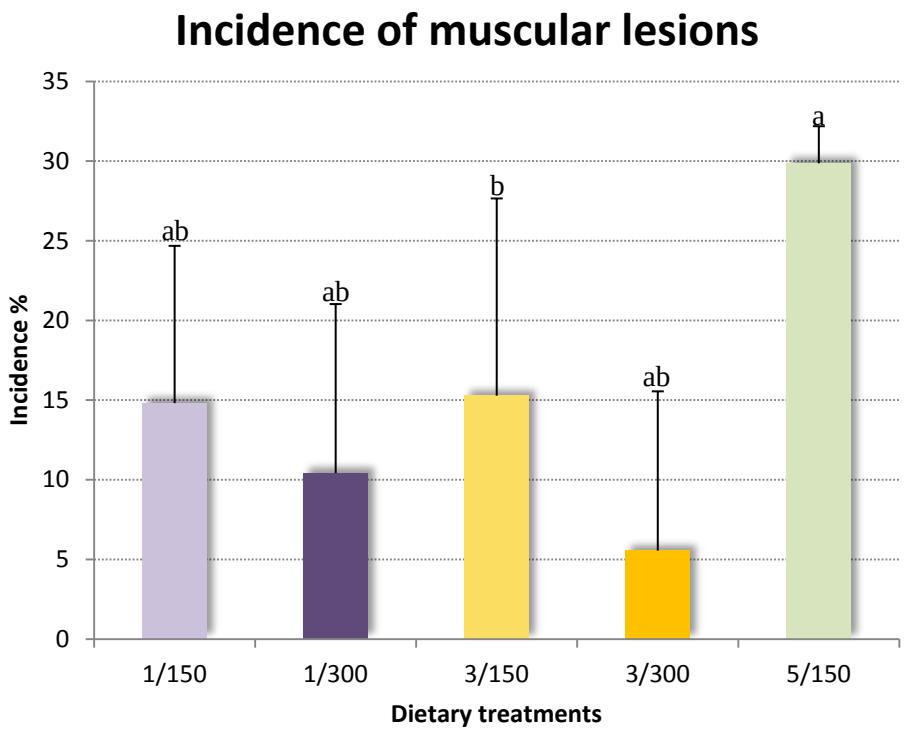

Figure 3.5 Incidence of muscular lesions in sea bass feeding the experimental diets containing several DHA and vitamin E. Larvae were considered injured when some kind of lesion showed in Figure 3.4 was observed.

Another morphological alteration was observed in the hepatocytes which showed a pink (H&E) vacuolar intracytoplasmatic pigment (Figure 3.7, A-B). This material stained Sudan Black and PAS positively, moderately Ziehl–Neelsen and Prussian Blue negative, suggesting its ceroid nature. A higher incidence of ceroid hepatocytes was found in larvae fed the low α-TOH content (150 mg 100 g^{-1}), which was increased by the rise in dietary DHA up to 5% (Figure 3.8). A lower incidence was found in fish fed higher dietary α-TOH diets (300 mg 100 g^{-1}) (Figure 3.8), hence the highest incidence being found in fish fed diet 5/150 (40.4%) and the lowest in fish fed diet 1/300 (11.1%) (P=0.002) (Figure 3.8). The incidence of ceroid pigment was increasing along the experimental period, observing no pigment at the beginning of the trial, being the 68.7% of the observed larvae affected at 48 dph. No other morphological alterations or pathologies were found in liver or any other tissue.

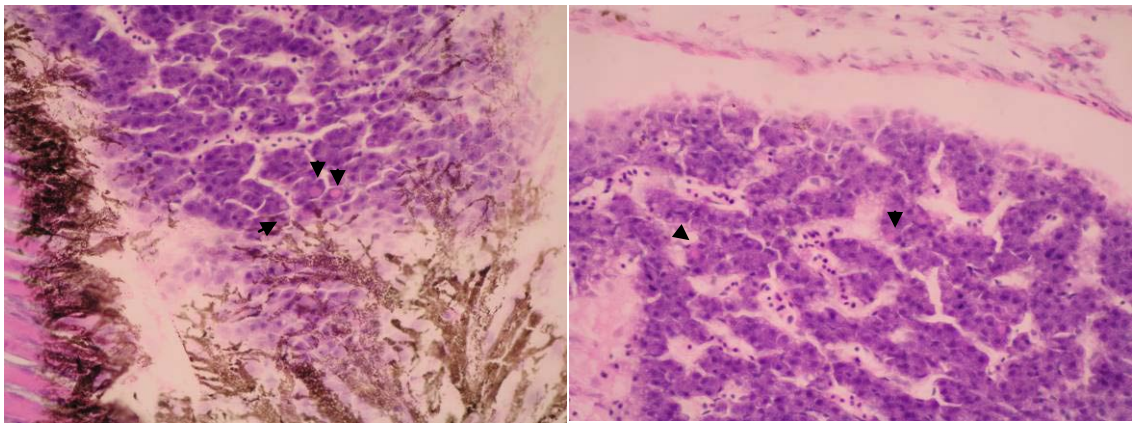

Figure 3.6 Vacuoles of ceroid pigment (arrows) within hepatocytes fed diet 5/150 at 48 days old (x400). H&E (**A**) and PAS (**B**) staining.

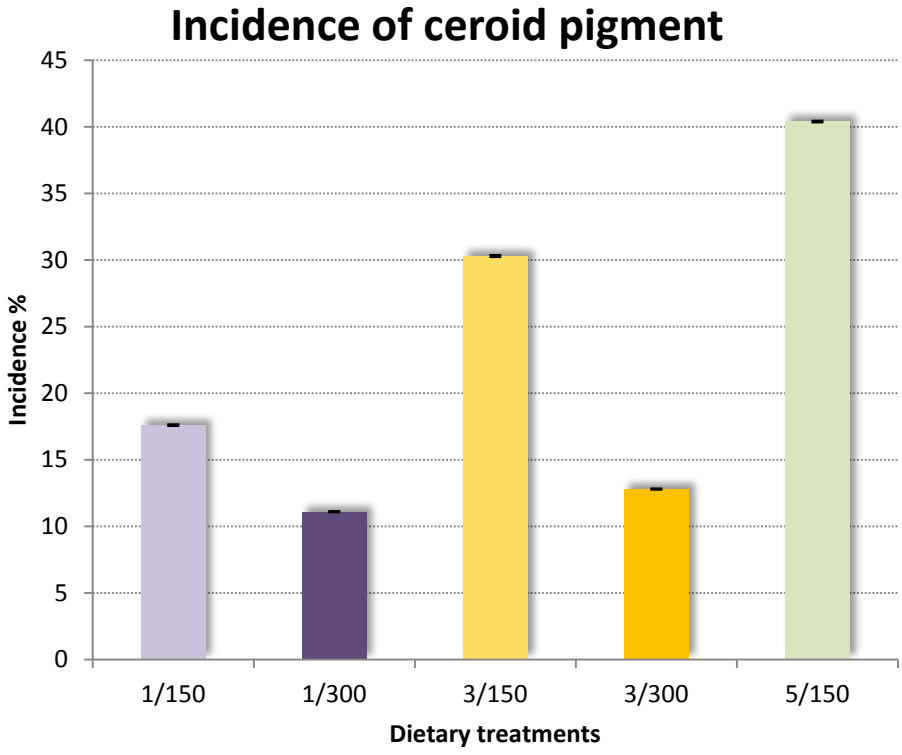

Figure 3.7 Incidence of ceroid pigment depending on the diets.

3.4 Discussion

DHA was readily deposited in fish tissues reaching almost 30% of total lipids in fish fed the highest dietary contents. But, since elevation of dietary DHA markedly increases peroxidation risks, the higher sea bass mortalities found in fish fed increased DHA levels suggested the proliferation of free radicals derived from this fatty acid and the formation of toxic oxidized compounds. Indeed increased DHA multiplies two, four and eight times the oxidation potential of the diet in comparison to the same increase in arachidonic, linolenic or linoleic acid, respectively (Zhang and Chen, 1997). Oxidation of PUFA produces compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons, several of them being toxic, binding to proteins amino groups, nucleic acid and phospholipid bases and damaging membrane lipids, proteins and DNA (Frankel, 1998). Thus, lipid peroxidation is highly deleterious in fish, resulting in damage to cellular and sub-cellular membranes (Puangkaew *et al*., 2005), structural proteins and different fish tissues and organs (Watanabe *et al*., 1970; Moccia *et al*., 1984; Sakai *et al.,* 1989; Bai and Lee, 1998). Indeed, in the present study the elevation of dietary DHA contents up to 5% severely increased the incidence of muscular lesions, including hyaline degeneration and fragmentation of myofibres, which denoted the severe damage in both membranes and structural proteins, necrosis and mononuclear infiltrates. Another pathological finding in sea bass associated to the high dietary DHA levels was the presence of ceroid pigment within hepatocytes, which has been also found to be related to an imbalance between anti- and pro-oxidants (Porta *et al.,* 2002). It has been shown that the presence of ceroid pigments can be used as an estimation of lipid peroxidation as it is usually associated to other consequences of oxidative stress (Moccia *et al*., 1984; Sakai *et al*., 1998).

Both muscular distrophy and ceroid pigment deposits have been described among the symptoms of deficiency in α-TOH (Lovell *et al.,* 1984; Gatlin *et al.,* 1986; Frischknecth *et al.,* 1994; Bowater and Burren, 2007), which being a fat-soluble vitamin it is deposited in the lipid fraction of tissues, including biomembranes, and plays an important role in protecting them from lipid peroxidation. In the present study, elevation of dietary α-TOH levels markedly reduced the incidence of these symptoms in sea bass, increasing the fish tissue content in several PUFAs, which denotes the antioxidant and protective role of this vitamin, and improving survival. Moreover, an increase in PUFA could be seen in fishes fed with diets deficient in α-TOH (Baker and Davies, 1996; Bell *et al*., 2000). The cause of this phenomenon is not clear, but it is likely that α-TOH has an

effect on fatty acids desaturation and elongation (Mourente *et al*., 2007). The kind and degree of incidence of muscular lesions associated to dietary α-TOH imbalances differ among fish species, nutritional status, age, size, diet quality and feeding period (Moccia *et al.,* 1984). For instance, α-TOH liver storage can greatly prevent the development of this pathology in rainbow trout (Cowey *et al.,* 1981) and young Atlantic salmon showed severe muscular dystrophy when fed a α-TOH deficient diet (Poston *et al.,* 1976), whereas this type of lesion could not be found in bigger fish (Bell *et al.,* 1985). On the other hand, it is known that DHA is a significant component of the cell membrane phospholipids of the skeletal muscle (Infante, 1987; Salem *et al*., 2001) and is profoundly susceptible to oxidative stress (Song *et al*., 2000). It has been suggested that DHA-containing phospholipids are important for very active $Na^{+}K^{+}$ATPase (Else and Wu, 1999) and Ca^{2+}ATPase (Infante, 1987; Infante *et al*., 2001) in muscle. This increased metabolism probably results in a greater consumption of oxygen, being these phospholipids important substrates for damage by the free radicals (Hulbert *et al*., 2002). These data could explain the fact that the adverse effects of a DHA/α-TOH imbalance are seen before in the muscular tissue than in any other localization.

The present study suggests that larvae and very young marine fish are very sensitive to oxidative imbalances what is probably related to several factors such as the high water resorption which occurs during this later period of metamorphosis, the high oxidative metabolism, fast growth or the high requirements for PUFAs (Fontagnè *et al*., 2006; Fontagnè *et al,.* 2008). In addition, it is possible that larval marine fish may not have sufficiently well- developed antioxidant capability, as many of their biological and physiological systems are poorly developed (Mourente *et al*., 1999b). Thus, a too high elevation of dietary PUFA, and particularly DHA, may easily cause an oxidative imbalance in this very young fish even when α-TOH is supplied in high amounts in the diet as in the present experiment, increasing the anti-oxidant requirements. In fact, copepods, natural preys for these fish have a very high content of several antioxidant nutrients such as α-TOH, Se, vitamin C, phospholipids or carotenoids (Hamre *et al.,* 2008a). Moreover, muscular lesions in such a young larvae could interfere in the normal development of larvae, increasing the potential to develop skeleton deformities. Rainbow trout fry syndrome, characterized by several pathologies including muscle degeneration, has been also associated with an increased incidence of vertebral deformities (Madsen and Dalsgaard, 1999).

Ceroid pigment within hepatocytes has been associated with numerous pathological conditions in which the main pathogenic factor is the deficiency of α-TOH or

imbalances between anti- and pro-oxidants. In this study ceroid pigment did not affect extensively the liver denoting the mildness of the pathology, since fish given diets containing rancid oil show severe ceroidosis in addition to myopathy (Miyazaki, 1995). Moreover, in acute cases ceroid deposits are found mainly in Kupffer cells and secondarily inside of hepatocytes (Lovell *et al.,* 1984; Lewis *et al.,* 1985) since hepatocytes containing ceroid pigments are phagocytized by Kupffer cells and macrophages, as it has been shown in liver of other animal species (Elleder *et al.,* 1995; Terman and Brunk, 1998; Isobe *et al.,* 2008). In sea bass, this process was not complete and ceroid pigments were directly found in the hepatocytes what could be related to a milder affection denoted by the low incidence, the appearance only in restricted areas of the liver and the lack of affection in other tissues. The presence of this pigment exclusively in the liver could be due to the active lipid metabolism and accumulation in this organ, which might motivate the lipid peroxidation and consequently the ceroid pigment deposition.

Evolution of α-TOH content in sea bass body along larval development shows a steady increase from the first days of feeding until the end of metamorphosis (Guerriero *et al.,* 2004), suggesting the high requirement of α-TOH during early development. In the present study, the best growth was performed by fish larvae fed diets containing 300 mg 100 g^{-1} DW α-TOH. Similarly, elevation of dietary α-TOH levels from 50 mg 100 g^{-1} to 300 mg 100 g^{-1} in microdiets, significantly improves growth of larval gilthead sea bream (Atalah *et al.,* 2011). Growth improvement by dietary α-TOH elevation has been also found in juveniles of Atlantic salmon (*Salmo salar*) (Hamre *et al.,* 1994) or rainbow trout (*Onchorhynchus mykiss*) (Cowey *et al.* 1984; Frischknecht *et al.,* 1994). In juvenile gilthead sea bream, increase in α-TOH dietary contents improves growth particularly when oxidized oils are present in the diet (Tocher *et al.* 2003), associating once more the beneficial effect of α-TOH to their antioxidant properties. However, Puangkaew *et al.,* (2005) noticed that in rainbow trout high doses of dietary α-TOH did not promote growth, denoting the interaction between dietary *n*- 3 LC-PUFA and α-TOH. In this sense, in the present study, sea bass larvae fed diets containing 300 mg 100 g^{-1} α-TOH showed a significant improvement in growth when dietary DHA was raised from 1 to 3 %. DHA is an essential fatty acid for fish growth (Watanabe *et al.,* 1989; Watanabe, 1993; Izquierdo, 1996), as it is incorporated in cell membrane regulating membrane integrity and function (Izquierdo, 2005). Nevertheless, the positive effect of α-TOH 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).

Finally, sea bass fed high α-TOH also showed a significantly improved stress resistance. In gilthead sea bream, increase in α-TOH enhanced resistance to both chronic and acute stress (Montero *et al.,* 2001), the fish fed higher α-TOH showing lower post-stress plasma cortisol levels (Montero *et al.,* 1998). The addition of dietary α-TOH has been also shown to increase stress resistance in mammals as showed by the decrease in plasma corticosteroids (Watson and Petro, 1982; Mudron *et al.,* 1996).

In summary, elevation of dietary DHA increased DHA deposition in sea bass tissues increasing peroxidation risks and causing increased mortalities and incidence of muscular and hepatic lesions. However, increase in dietary α-TOH markedly reduced the incidence of these symptoms in sea bass, protecting PUFA from oxidation and improving growth and survival after activity test. Elevation of dietary α-TOH levels up to 300 mg 100 g^{-1} significantly improved fish growth in terms of total length. Finally, sea bass fed high vitamin E also showed a significantly improved stress resistance.

Chapter 4

Oxidative status and histological changes in sea bass larvae muscle in response to high dietary content of DHA

This study is in press at *Journal of Fish Diseases* as Betancor *et al.* (2012)

ABSTRACT

In previous studies we observed dystrophic alterations in muscle of 48 day-old sea bass fed imbalanced DHA and vitamin E diets. In order to better understand the whole pathological process associated to oxidative stress, a deeper histological study was performed feeding 14 day-old sea bass larvae with microdiets containing different ratios of DHA/vitamin E (1/150, 5/150 and 5/300). Larvae fed diet 1/150 showed no lesions in contrast to larvae fed diet 5/150 and 5/300 where the highest incidence of muscle lesions and TBARS content was observed. Semithin sections showed focal lesions consisted of degenerated fibres with hypercontracted myofilaments and extensive sarcoplasm vacuolization affecting both red and white muscle. Ultrathin sections of these degenerating muscle fibres showed diffuse dilatation of sarcoplasmic reticulum, disorganized myofilaments and autophagic vacuoles eventually containing myelin figures and dense bodies. In addition, some monocyte - macrophage cells were observed among injured fibres as numerous satellite cells. The results of the present work reinforced the conclusions observed in our previous study and demonstrate the pathological potential of free radicals in sea bass larvae musculature, which could not be attenuated by dietary vitamin E. The implication of other nutrients related to cell protection against oxidative stress is being studied at present.

Keywords:

Sea bass
larvae
oxidative stress
DHA
muscle dystrophy
ultrastructure

4.1 Introduction

It is widely known that marine fish larvae have higher requirements of docosahexaenoic acid (DHA; 22:6*n*-3) than juveniles (LeMillinaire, 1984; Hernández-Cruz *et al.*, 1999; Mourente *et al*., 1999a; Izquierdo, 2005) to reach a good culture performance in terms of growth and survival (Mourente *et al*., 1993; Brinkmeyer and Holt, 1998; Furuita *et al*., 1998; Copeman *et al*., 2002; Liu *et al*., 2002) and stress resistance (Watanabe and Kiron, 1994; Kanazawa, 1997). Moreover, these high requirements are reflected in the large larval DHA content, accounting for 30% of the total larval tissue fatty acid. Due to the high unsaturation of this fatty acid, it is very susceptible to lipid peroxidation. Thus, larval tissues may be more at risk of suffering peroxidative attack than adult tissues (Hamre *et al*., 2010).

Lipid peroxidation may be initiated by any reactive oxygen species (ROS) that has sufficient reactivity to substract a hydrogen atom from a polyunsaturated fatty acid (PUFA) side chain in membrane lipids (Aruoma, 1998). ROS are continuously produced as byproducts of various metabolic pathways in all organisms. To counter its potentially deleterious effect an array of endogenous and exogenous antioxidant mechanisms has evolved (Yu, 1994). The endogenous antioxidants comprise of a series of enzyme scavengers of oxyradicals and other free radicals. Among exogenous antioxidants, vitamin E-type compounds may be widely regarded as primary lipid-soluble antioxidants (Wang and Quinn, 1999), as they are present among the lipid constituents of cell membranes and lipoproteins. These compounds are not produced by animals and must be obtained from the diet. It is therefore important to supplement marine fish larval diets with vitamin E (Hamre *et al.,* 2010), as the requirement of this vitamin must increase in diets containing high concentrations of PUFA (Stéphan *et al*., 1995; Atalah *et al*., 2010).

When an imbalance between the generation and removal of ROS by cellular defences occurs a status of oxidative stress takes place. This status may lead to the oxidation of various cellular constituents like lipids, proteins or DNA. Such condition may lead to acute cellular dysfunction, chronic tissue degeneration or even cell death if such changes accumulate (Rando, 2002). In fish some diseases have been related to free radical damage such as hemolysis (Kawatsu, 1969), anaemia (Cowey *et al.*, 1984), jaundice (Sakai *et al*., 1989), liver degeneration (Cowey *et al.,* 1984) or skeletal alterations (Watanabe *et al*., 1989; Lewis-McCrea and Lall, 2007). Among these skeletal alterations, one of the most described in fish juveniles and adults is muscular

dystrophy (Lovell *et al*., 1984; Gatlin *et al*., 1986; Frischknecht *et al*., 1994; Bowater and Burren, 2007). This muscular disorder has been associated to a vitamin E deficiency (Bowater and Burren, 2007), suggesting the importance of this nutrient in the prevention of *in vivo* peroxidation.

In a previous study, an increase in the incidence of muscle lesions was observed in sea bass larvae, *Dicentrarchus labrax*, fed increasing levels of DHA, particularly when vitamin E levels were low (Chapter 3). Several reasons may explain the higher susceptibility of muscular tissues to free radical injury. For instance, muscle is prone to oxidative injury as a result of increased electron flux due to its requirement and ability to undertake rapid and coordinated changes in energy supply and oxygen flux during contraction (Haycock *et al*., 1996). There is also a very high concentration of myoglobin in muscle, and it is known that such a heme – containing protein may confer a greater sensitivity to free radical induced damage through the conversion of hydrogen peroxide to more reactive species (Ostdal *et al*., 1997). Finally, the requirement of skeletal muscle membranes for phospholipids containing large proportions of polyunsaturated fatty acids may render those membranes particularly susceptible to oxidative stress (Murphy and Kehrer, 1989).

The objective of the present study was to evaluate the oxidative status and the structural changes in sea bass larvae musculature resulting from the use of different dietary DHA and vitamin E ratios.

4.2 Materials and methods

4.2.1 Fish

The experiment was carried out at the *Instituto Canario de Ciencias Marinas* facilities (Telde, Canary Islands, Spain). Sea bass, *Dicentrarchus labrax,* larvae were obtained from natural spawnings from the *Instituto de Acuicultura de Torre de la Sal* (Castellón, Spain). Prior to the start of the feeding experiment, larvae were fed enriched yeast-fed rotifers until they reached 14 days post hatching. Larvae (total length 7.55 $\pm$ 0.55 mm, dry body weight 0.20 $\pm$0.00 mg) were randomly distributed into the experimental tanks (n=12) at a density of 1000 larvae tank^{-1} and fed one of three experimental diets in quadruplicate, for 21 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 (34 ppm salinity) at an increasing rate of 1.0 - 1.5 L min^{-1} during the feeding trials. Water entered the tank from bottom to top. Water quality was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min^{-1}), attaining 5-8 g L^{-1} dissolved O_2 and saturation ranged between 60 and 80%.

4.2.2 Diets

Three isonitrogenous and isolipidic experimental microdiets (pellet size<250 μm) similar in their eicosapentaenoic acid (EPA; 20:5*n*-3) content but different in DHA and vitamin E content were formulated (Table 1) using concentrated fish oils EPA500 and DHA500 (CRODA, East Yorkshire, England, UK) as sources of EPA and DHA. Vitamin E (DL- α- Tocopherol Acetate; Sigma-Aldrich, Madrid, Spain) was tested in 150 and 300 mg $100g^{-1}$ dry weight (DW) contents and DHA in 1 and 5 g 100 g^{-1} DW, giving 3 different diets depending on their DHA/vitamin E ratio (1/150, 5/150 and 5/300). The protein source (squid meal) was defatted 3 times with a chloroform:squid meal ratio of 3:1 (v:v) to allow complete control of the fatty acid profile of the microdiet. The microdiet based on defatted squid meal (2.6% lipid content), EPA500 and DHA500 were added in different quantities to obtain the desired ratios. Oleic acid was added to equalize the lipid content in each diet (Table 1). The microdiets were prepared according to Liu *et al*. (2002) by first mixing the squid powder and water soluble components, followed by lipid and fat soluble vitamins and finally, warm water solved gelatine. The paste was pelleted (Severin, Suderm, Germany) and oven dried at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain particle sizes below 250 μm. Diets were analyzed for proximate and fatty acid composition of dry basis. Diets were manually supplied every 45 minutes from 9:00 to 19:00h. The amount of feed supplied was 2, 2.5 and 3 g $tank^{-1}$ per day during the first, second and third week of feeding respectively.

4.2.3 Growth and survival

Before the end of the experiment a vitality test was conducted by handling 20 larvae/tank out of the water in a scoop net for 1 minute. Larvae were subsequently allocated into another tank supplied with clean seawater and aeration to determine survival after 24 hours. Final survival was calculated by individually counting all live larvae at the beginning and at the end of the experiment. Growth was determined by

measuring dry body weight (105 ºC 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish tank^{-1} at the beginning, middle and end of the trial.

Table 4.1 Main lipid ingredient composition and analyzed lipid, protein and vitamin E contents of the experimental diets.

Dietary DHA/vitamin E	*1/150*	*5/150*	*5/300*
EPA g kg^{-1} (DW)*	26.25	17.85	18.32
DHA g kg^{-1} (DW)*	7.28	37.4	38.9
Vitamin E mg kg^{-1} (DW)	1480	1530	2970
Oleic acid (DW)$^{\#}$	84.2	53.02	50.91
Lipid (DW)	15.70	16.30	15.52
Protein (DW)	65.71	65.59	66.00
Humidity (%)	11.16	11.48	9.99

4.2.4 Biochemical analysis

At the end of the trial, remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition and TBARS analysis, following a 16 hour starvation period. Moisture, protein (A.O.A.C. 1995) and lipid (Folch, Lees and Stanley 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of total 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*., (1992) and identified by comparison to previously characterized standards and GLC-MS.

The measurement of thiobarbituric acid reactive substances (TBARS) in triplicate samples was performed using a method adapted from that used by Burk *et al*., (1980). Approximately to 20-30 mg of larvae per sample was homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol. To this 2.95 ml of freshly prepared 50mM thiobarbituric acid solution was added before mixing and heating for 10 min at 100ºC. After cooling and removing protein precipitates by centrifugation (Sigma 4K15, Osterode am Harz, Germany) at 2000 X *g*, the supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA), expressed as µmol MDA per g of tissue, was calculated using the extinction coefficient 0.156 µM^{-1} cm^{-1}.

4.2.5 Histopathological sampling

Thirty larvae from each tank were collected every seven days from the beginning of the feeding trial, fixed in 10% buffered formalin for 1 or 2 days, dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax. Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm, before stained with Haematoxilin and Eosin (H&E) for histopathological evaluation (Martoja and Martoja-Pearson, 1970). To determine the incidence of muscular lesions all larvae were observed, such that when a single muscular lesion was present the larva was considered as injured. Results are expressed as injured larvae in relation to total observed larvae.

Ten larvae per tank were fixed for 24 hours at 4ºC in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). Samples were then rinsed in phosphate buffer and post-fixed for 1 hour in 2% osmium tetraoxide in 0.2 M potassium ferrocyanide. Each larva was then embedded in an Eppon/Araldite resin block. Serial transverse and longitudinal larvae thick sections were cut at 1 µm, contrasted with toluidine blue and examined under light microscopy (Hoffman *et al*., 1983). Ultra-thin sections were cut at 50 nm and stained with lead citrate before observing with a ZEISS EM 910 (Berlin, Germany) transmission electron microscope (TEM) at the Electron Microscope Service of University of Las Palmas de Gran Canaria.

4.2.6 Statistical analysis

Survival and growth data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation. Chi-squared test was employed for incidence of muscular lesions and TBARS content. Survival and growth data were treated using one-way ANOVA. Means were compared by Duncan's test (P<0.05) using SPSS for Windows statistical software, Version 14.0 (SPSS Inc., Chicago, IL, USA, 2005). For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

4.3 Results

All experimental diets were well accepted by larvae. Dietary increase of either DHA or vitamin E alone did not significantly affect larval growth, whereas raising both nutrients in diet 5/300 significantly improved the final total length in comparison to diet 1/150 (Figure 4.1). A positive correlation was found between *n*-3 LC-PUFA larval content and total length (y = 0.0671 + 6.6688; R^2 = 0.8127). No significant differences were observed in neither the final dry weight nor survival at the end of the experiment or following the activity test.

Dietary fatty acids compositions were similar in saturated content, whereas diets containing about 1% DHA (1/150) showed higher monoenoic fatty acids than diets containing 5% DHA (5/150 and 5/300), due to the higher oleic acid content in the former diet (Table 4.2). Elevation of dietary DHA (5/150 and 5/300) increased *n*-3 and *n*-3 LC-PUFA fatty acids contents. Fatty acid composition of the larvae generally reflected the fatty acid composition of the diet (Table 4.3). However, in larvae fed 5% DHA diets, the elevation of vitamin E from 150 to 300 mg $100g^{-1}$ increased by 10.43% *n*-3 LC-PUFA and 17.46% EPA in larvae fed diet 5/300 compared to larvae fed 5/150.

The level of lipid peroxides, as indicated by TBARS content (µmol g^{-1} larval tissues), was significantly higher in those larvae fed diets with the highest DHA content regardless of dietary vitamin E (Figure 4.2).

Table 4.2 Main fatty acids (% total identified fatty acids) of experimental diets fed to European sea bass for 21 days

	1/150	5/150	5/300
14:0	0.45	0.35	0.36
15:0	0.02	0.07	0.07
16:0ISO	0.00	0.04	0.05
16:0	7.43	6.94	6.97
16:1*n*-7	0.28	0.51	0.51
16:1*n*-5	0.02	0.04	0.03
16:2*n*-6	0.05	0.10	0.10
16:2*n*-4	0.12	0.28	0.29
17:0	0.08	0.12	0.12
16:3*n*-3	0.04	0.09	0.09
16:4*n*-3	0.07	0.03	0.04
18:0	1.76	3.44	3.44
18:1*n*-9	49.58	31.19	29.95
18:1*n*-7	0.54	0.47	0.72
18:1*n*-5	0.31	0.26	0.04
18:2*n*-9	0.23	0.17	0.06
18:2*n*-6	13.47	10.71	10.52
18:2*n*-4	0.14	0.11	0.09
18:3*n*-6	0.05	0.23	0.22
18:3*n*-4	0.04	0.09	0.09
18:3*n*-3	0.80	0.87	0.85
18:3*n*-1	0.04	n.d	n.d.
18:4*n*-3	1.05	0.57	0.61
18:4*n*-1	0.12	0.05	0.06
20:0	0.16	0.57	0.59
20:1*n*-9+7	0.68	2.59	2.62
20:1*n*-5	0.05	0.37	0.31
20:2*n*-9	0.08	0.10	0.06
20:2*n*-6	0.07	0.37	0.38
20:3*n*-6	0.12	0.15	0.16
20:4*n*-6	1.00	1.76	1.90
20:3*n*-3	0.07	0.27	0.28
20:4*n*-3	0.48	0.53	0.55
20:5*n*-3	15.44	10.20	10.47
22:1*n*-11	0.34	0.79	0.83
22:1*n*-9	0.20	0.62	0.60
22:4*n*-6	0.01	0.06	0.07
22:5*n*-6	0.05	1.43	1.49
22:5*n*-3	0.36	1.44	1.51
22:6*n*-3	4.16	22.00	22.90
Saturated	9.90	11.50	11.55
Monoenoics	52.00	35.77	34.29
n-3 FA	22.47	35.99	37.29
n-6 FA	14.82	14.81	14.85
n-3 LC-PUFA	20.51	34.44	35.70
n-3/*n*-6	1.52	2.43	2.51

n.d., not determined. FA, fatty acids; LC-PUFA, long chain polyunsaturated fatty acids

Table 4.3 Lipid levels (g kg^{-1}) and main fatty acid compositions of total lipids from sea bass larvae fed experimental diets for 21 days (% total identified fatty acid)

	Initial	1/150	5/150	5/300
Total lipids (g kg^{-1})	287±0.0	188±1.7	170±1.9	168±2.1
14:0	1.1±0.0	0.5±0.1	0.5±0.1	0.5±0.0
15:0	0.2±0.1	0.5±0.3	0.4±0.2	0.4±0.2
16:0	16.4±3.3	14.4±1.2	15.6±3.7	13.8±0.4
16:1*n*-7	9.7±1.7	0.7±0.2	0.8±0.1	1.6±0.4
16:1*n*-5	0.5±0.0	0.2±0.1	0.2±0.0	0.3±0.0
16:2*n*-6	0.9±0.1	0.3±0.0	0.4±0.0	0.4±0.0
16:2*n*-4	0.9±0.0	0.5±0.1	0.7±0.2	0.7±0.0
17:0	1.0±0.0	0.2±0.0	0.3±0.0	0.3±0.0
16:4*n*-3	0.4±0.1	0.5±0.1	0.4±0.0	0.5±0.1
16:4*n*-1	0.2±0.0	0.6±0.1	0.2±0.0	0.3±0.1
18:0	7.4±0.8	8.7±0.5	9.8±2.5	8.2±0.2
18:1*n*-9	16.1±2.1	26.1±2.3^{a}	17.4±1.1^{ab}	16.2±0.1^{b}
18:1*n*-7	6.3±0.4	2.1±0.3	2.0±0.4	2.3±0.2
18:1*n*-5	0.6±0.1	0.2±0.0	0.2±0.1	0.2±0.0
18:2*n*-6	3.7±1.1	7.8±1.2	5.0±0.0	4.9±0.2
18:3*n*-3	0.8±0.1	0.3±0.1	0.4±0.0	0.4±0.1
20:0	0.2±0.1	0.3±0.0	0.6±0.2	0.4±0.0
20:1*n*-9	2.0±0.3	2.3±0.2	2.4±0.2	2.2±0.1
20:1*n*-5	0.5±0.2	0.1±0.0	0.2±0.0	0.2±0.0
20:2*n*-6	0.7±0.5	0.9±0.1	1.0±0.1	1.0±0.1
20:4*n*-6	3.3±0.7	2.8±0.1	3.3±0.1*	3.4±0.1*
20:5*n*-3	7.6±0.3	9.5±0.6	6.3±1.4**	7.4±0.4*
22:1*n*-9	0.3±0.0	0.3±0.0	0.4±0.1	0.6±0.0
22:5*n*-6	0.6±0.2	0.4±0.1	1.2±0.2	1.3±0.0
22:5*n*-3	1.6±0.2	1.3±0.1	0.8±0.2	1.0±0.0
22:6*n*-3	14.2±4.2	16.8±3.0	27.2±7.5	29.4±2.7**
Saturated	26.2±3.3	24.6±2.2	27.2±4.8**	23.7±0.4*
Monoenoics	36.6±2.7	32.0±3.2	20.7±2.3	24.0±0.9
n-3 FA	25.4±3.4	28.4±3.7	35.8±8.7*	39.1±2.0*
n-6 FA	9.7±1.1	4.5±0.2	5.8±0.1	6.6±0.6
n-3 LC-PUFA	23.7±2.1	27.7±3.6	34.5±6.5*	38.1±2.1**
n-3/*n*-6	2.6±0.7	6.4±0.8	6.2±0.9	5.9±0.8

Each value represents mean ± SD (n=12). * $P < 0.05$, ** $P < 0.005$ versus larvae fed diet 1/150.

Histopathological examination revealed segmental necrosis affecting the axial musculature in studied fish larvae (Figure 6.3). The earliest recognizable stage in necrosis was the swelling of affected muscle fibres, which stood up in sharp contrast to the adjacent fibres that were often displaced by pressure (Figure 6.3A). In addition, affected fibres showed typical features of hyaline degeneration as marked eosinophilia, loss of striations and marked oedema (Figure 4.3, A and C). Necrotic fibres were phagocytized by macrophages (Figure 4.3, B and D) which first appeared at the periphery of affected fibres. The incidence of these muscular lesions at the end of the experimental period was higher in those dietary treatments containing 5% of DHA, whereas no lesions were found in larvae fed 1% of DHA (Figure 4.4). The increase in vitamin E content from 150 to 300 mg did not reduce the incidence of muscle injuries,

with muscular lesions increased through the experimental period. Moreover, a positive correlation was found at the end of the feeding trial between the TBARS content and the incidence of muscular lesions (y = 0.0408 – 4.781; R^2 = 0.9122).

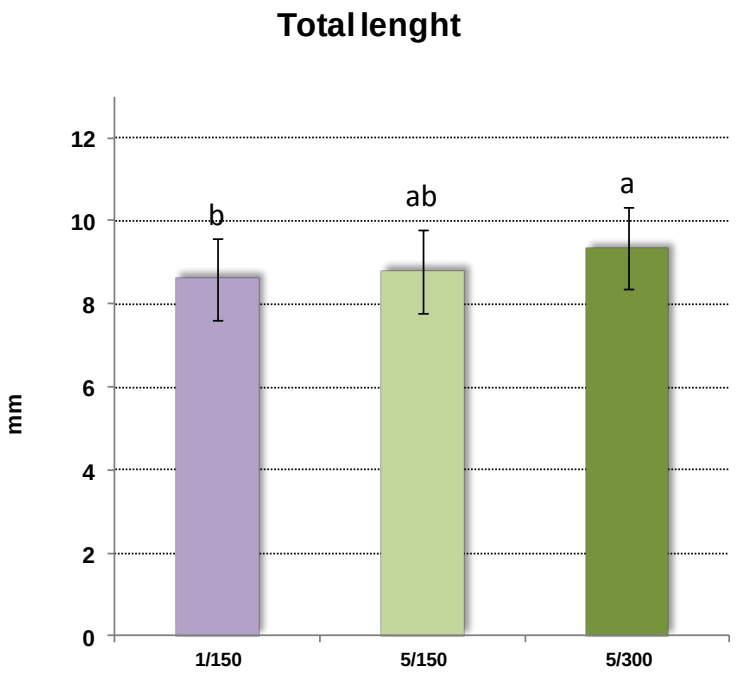

Figure 4.1 Total length of sea bass larvae (35 dph) following 21 days of feeding the experimental diets containing different DHA and vitamin E contents. Each value represents mean ± SD (n=120).

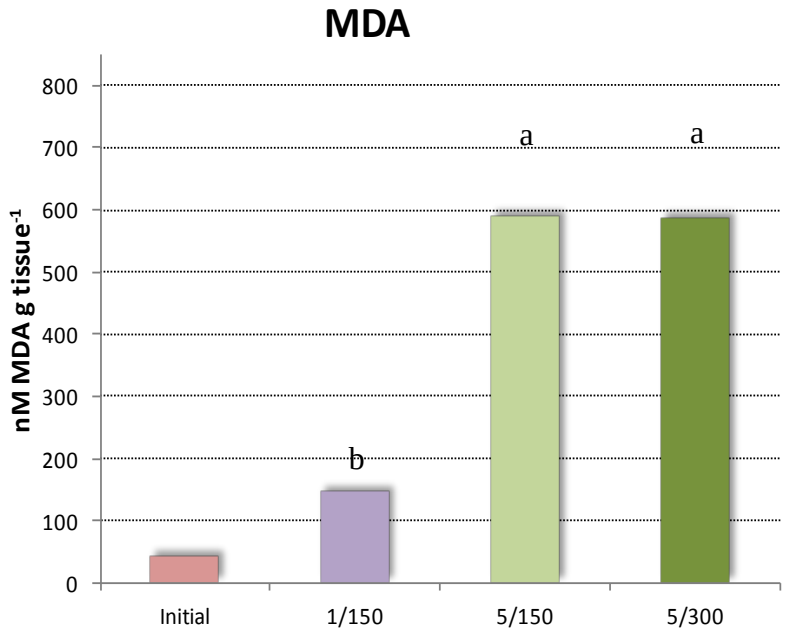

Figure 4.2 TBARS content in sea bass larvae larvae (35 dph) following 21 days of feeding the experimental diets containing different DHA and vitamin E contents. Each value represents mean ± SD (n=12).

More detailed features of these lesions could be observed in thick sections (Figure 4.5). Although not all muscle fibres were affected, both the red and white muscle were involved. A marked oedema was observed between muscular fibres, observing the loss of the normal myotome architecture (Figure 4.5, A and B). Transversal sections showed a great variation in the size of individual fibres, some of which presented a dark cytoplasm most probably related to myofilament concentration. Necrotic fibres showed protein coagulation in their sarcoplasm as well as irregular staining (Figure 4.5, C). Additionally, clear vacuoles could be observed inside some muscular fibres that apparently did not show any other alteration (Figure 4.5, A and B).

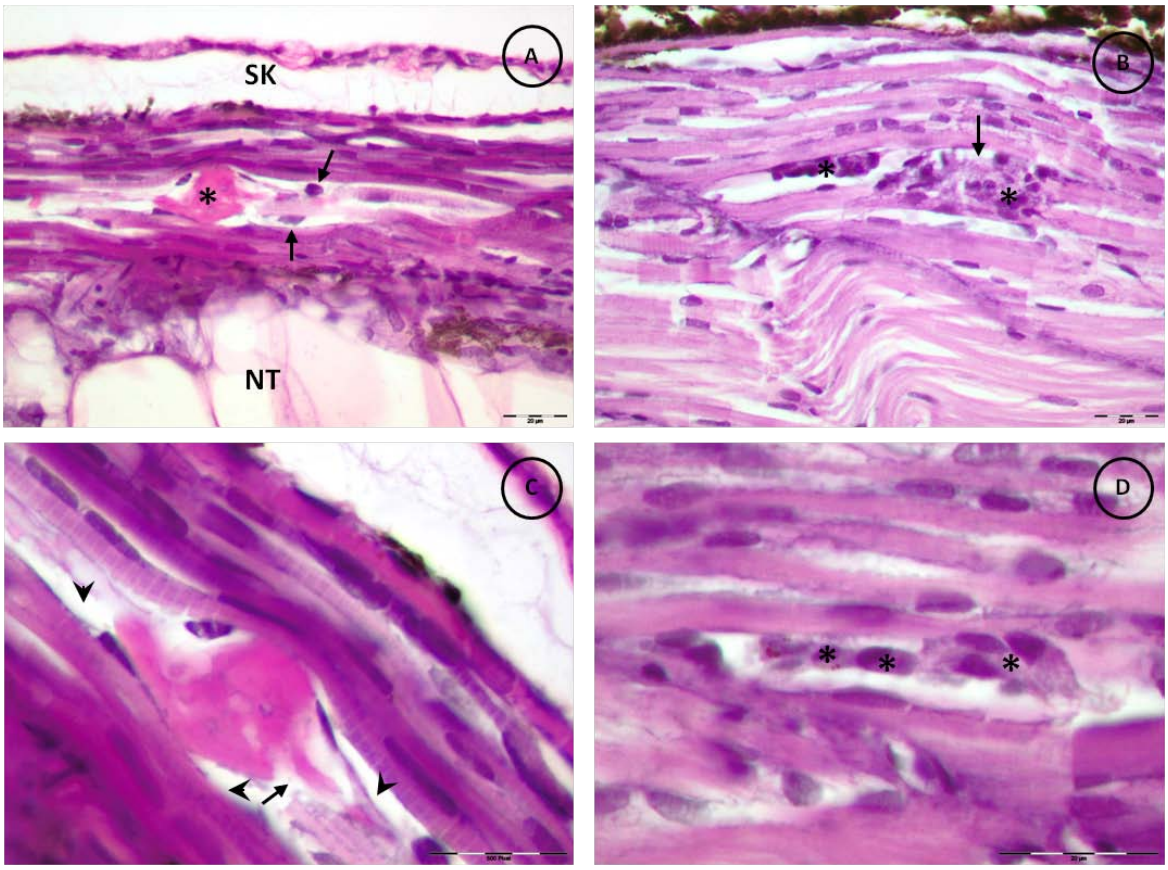

Figure 4.3. Muscular lesions found in sea bass larvae fed diets 5/150 and 5/300 at 35 dph, longitudinal sections, haematoxylin and eosin staining. (**A**) Swollen portion of a muscular fibre (*), partially fragmented showing eosinophilic cytoplasm and the presence of some macrophages (arrow). (**B**) Muscular debris (arrow) surrounded by a severe inflammatory infiltrate (*) undergoing phagocytic removal. (**C**) More detailed feature of necrotic fibre showed in Figure 3A where presence of flocculated cytoplasm and partial fragmentation (arrow) can be appreciated. A marked oedema can be appreciated (arrowhead). (**D**) Macrophages aggregate (*) phagocytosing necrotic muscle fibre. SK, skin; NT, notochord.

Figure 4.4 Incidence of muscular lesions (%) after 3 weeks of feeding the experimental diets. Each value represents mean ± SD (n=120).

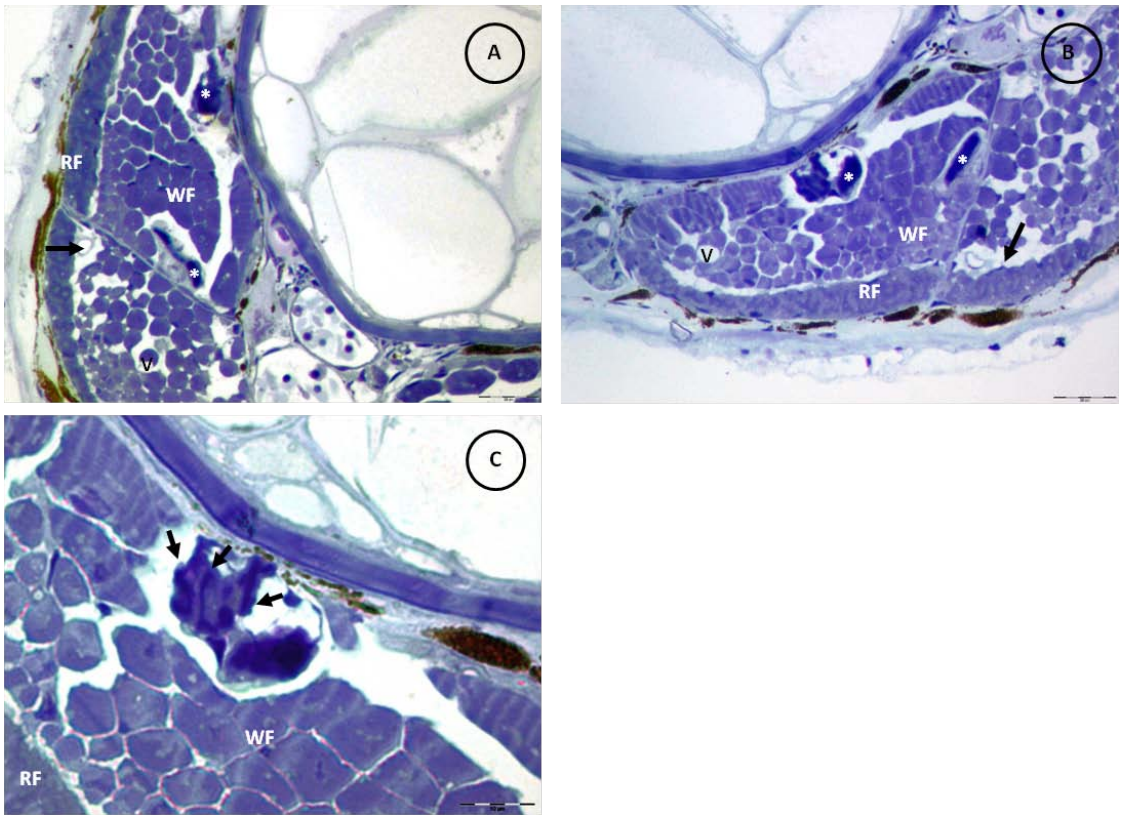

Figure 4.5 Sea bass larvae fed diets 5/150 and 5/300 transversal thick sections, toluidine blue staining. (**A** and **B**) We can observe loss of regular architecture and oedema (arrow) between red (RF) and white fibres (WF), vacuoles (V) inside affected muscle fibres and swollen cells (*).(**C** and **D**) More detailed features of necrotic fibres are showed, observing the irregular staining of the sarcoplasm due to the presence of hypercontraction bands (arrow).

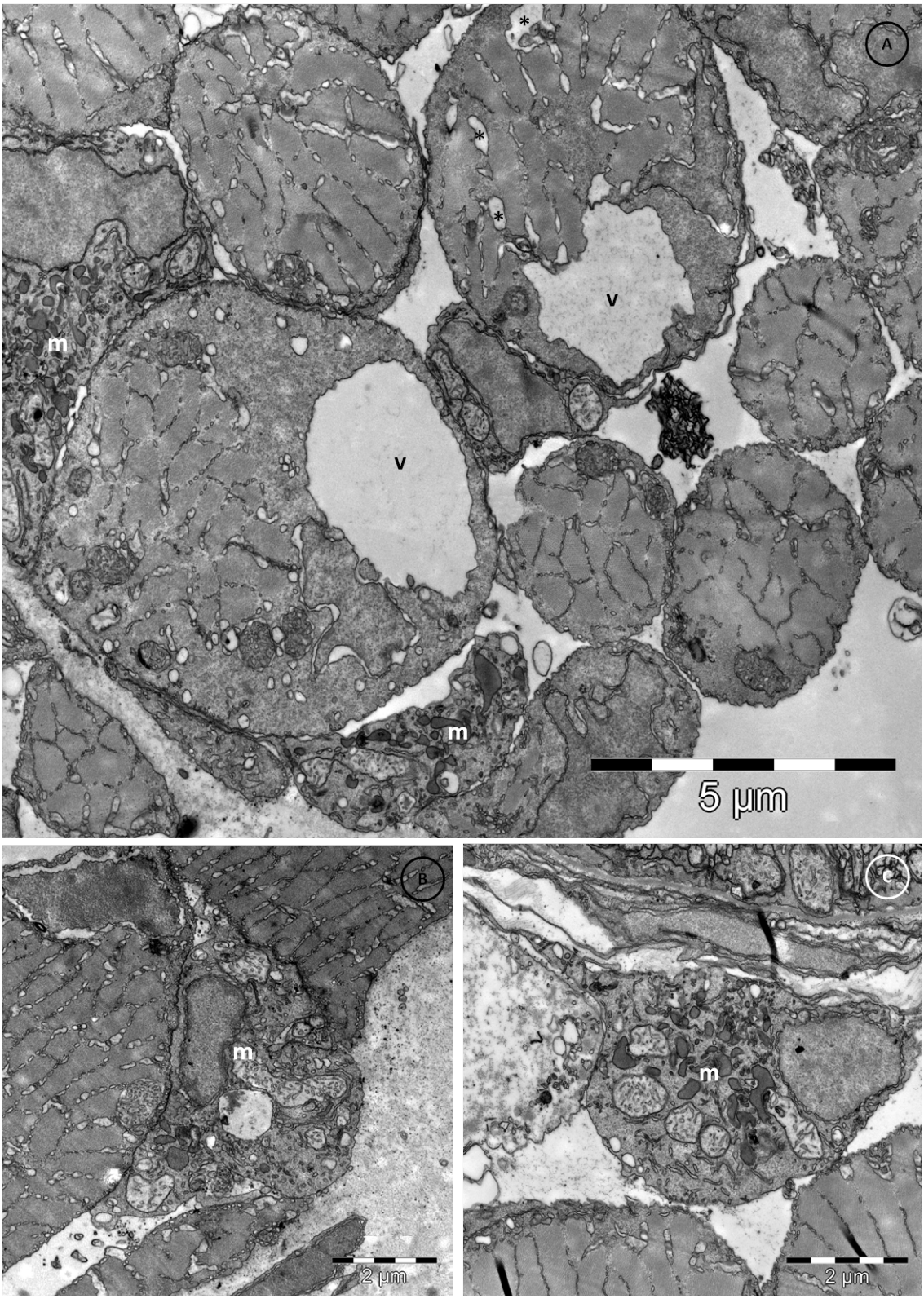

Figure 4.6 Electro micrographs of 35 dph sea bass larvae fed diets 5/150 and 5/300. (**A**) Different size between muscle fibres is evident, as well as the lost of continuity between them. Dilatation of sarcoplasmic reticulum (*) is observed, leading to the formation of vacuoles (v) within sarcoplasm. As well, two macrophages cells are present (m). (**B** and **C**) Detail of macrophages attached to different muscle fibres.

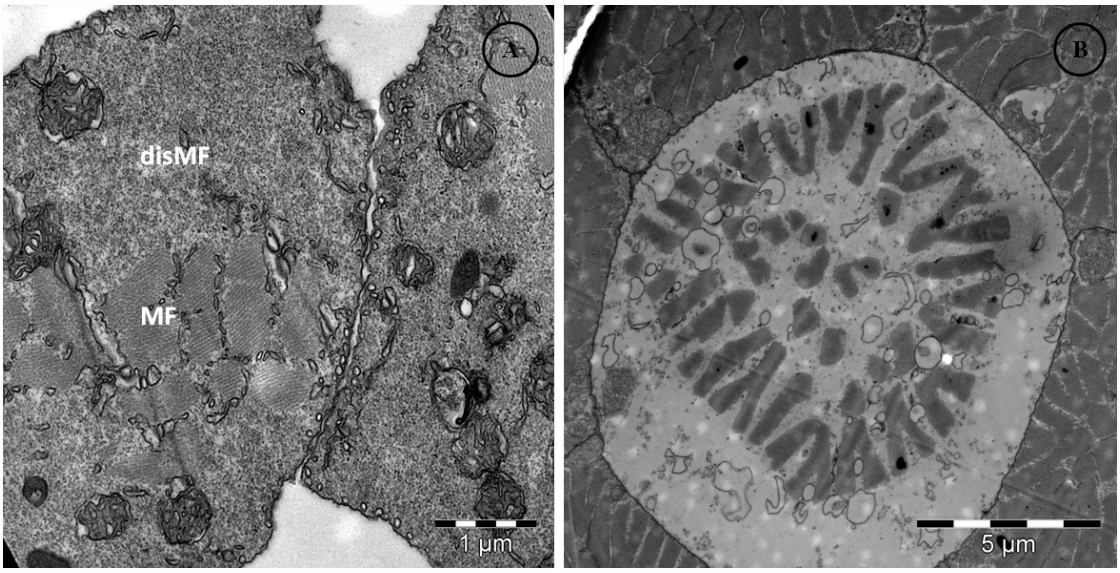

Figure 4.7 (**A**) Normal (MF) and disorganized (disMF) myofilaments inside a mildly affected muscle fibre. (**B**) Presence of a sarcoplasmic halo in the periphery of the fibre which was almost completely devoid of myofibrils and mitochondria.

Sections examined at TEM level revealed irregular size and shape of fibres and loss of continuity between them (Figure 4.6, A). It seems that one of the first alterations would be the swelling of organelles, such as sarcoplasmic reticulum (Figure 4.6, A). This change could lead to the rupture of the cisternae of endoplasmic reticulum and the formation of large translucent vacuoles within the sarcoplasm (Figure 4.6, A). This series of alterations is defined as hydropic degeneration. Macrophages containing cellular debris and numerous lysosomes were observed in severely affected fibres (Figure 4.6, A). Some fibres presented disarrangement of the myofilaments (Figure 4.6, B) as well as sarcoplasmic “halos” devoid of myofibrils and mitochondria (Figure 4.6, C). Autophagic vacuoles were frequently noticed inside affected fibres (Figure 4.7, A) just like numerous myelin figures (Figure 4.7, B and C), as a result of intracellular lipid peroxidation. Fragmentation of affected fibres was observed with subsequent degeneration of the fragments and disintegration of myofilaments (Figure 4.7, B and C). Finally, frequent satellite cells were observed in sea bass larvae fed diet 5/300 (Figure 4.7, D). No morphological signs of apoptosis were observed.

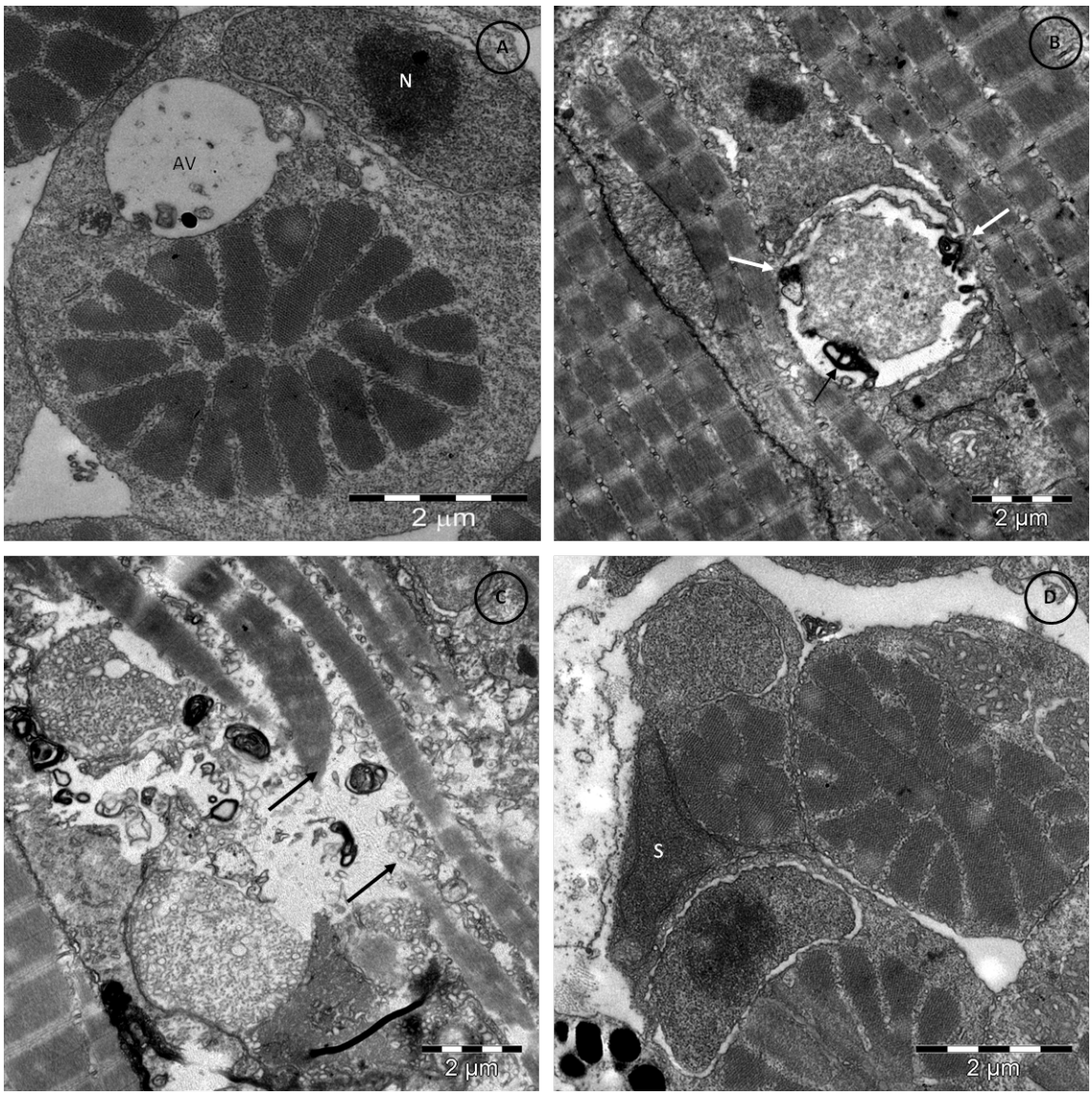

Figure 4.8 Electron micrographs of transversal (**A** and **D**) and longitudinal (**B** and **C**) sections of sea bass larvae fed diets 5/150 and 5/300. (**A**) Affected fibre presenting an autophagic vacuole (av) within its sarcoplasm, adjacent to the nucleus (N). (**B**) Presence of numerous myelin figures (arrow) in a degenerated muscular fibre. (**C**) Fragmentation of an affected muscle fibre (arrow) surrounded by myelin figures. (**D**) Presence of muscular satellite cells (S) between muscle fibres.

4.4 Discussion

The results of this study determine the muscle response to high levels of dietary DHA. As expected, the different dietary fatty acid compositions were reflected in the fatty acid compositions of larval tissues. Thus, diets high in DHA resulted in increased levels of *n*-3 LC-PUFA, particularly DHA, whereas diets with a low DHA inclusion resulted in high larval contents of oleic acid. Therefore, the potential for lipid peroxidation was theoretically higher in larvae fed diets containing 5% of DHA due to its high unsaturation. Oxidation products may potentially lead to suppressed growth (Hung *et al.*, 1981; Murai *et al*., 1988; Koshio *et al.*, 1994; Baker and Davies, 1997; Fontagné, *et al.*, 2006). Thus, growth in 5/150 larvae which did not significantly differ from that of 1/150 larvae could have been suppressed by oxidation products. However, elevation in vitamin E in 5/300 larvae, despite not completely preventing oxidation, lead to a significantly higher *n*-3 LC-PUFA content in larval tissues and allowed for growth promotion. Similarly, in juvenile gilthead sea bream, an increase in vitamin E dietary content improves growth particularly when oxidized oils are present in the diet (Tocher et al., 2003), further demonstrating the beneficial effect of vitamin E to their antioxidant properties. In this sense the inclusion of high levels of vitamin E (300mg/100g) in sea bream, *Sparus aurata*, larval microdiets improved larvae performance in terms of growth and survival, especially when supplemented with vitamin C (Atalah *et al.,* 2010).

TBARS were significantly influenced by dietary DHA levels, being significantly increased in sea bass larvae fed the highest dietary DHA content, irrespective of vitamin E content suggesting its ineffective protective role against oxidation. Moreover, TBARS values obtained in the present work are quite elevated compared to other studies in sea bass adults and juveniles exposed to different pollutants (Passi *et al*., 2004; Almeida *et al*., 2010). The fact that TBARS values were higher in the present study could be due to the poor activity of the antioxidant system in fish larvae. Mourente *et al.* (1999b) for example, found a decrease in TBARS values during early *Dentex dentex* development probably due to an enhancement of the antioxidant systems or an increased excretion rate. A reduction in TBARS value could be expected when vitamin E contents are increased in diets containing high DHA levels (Stéphan *et al*., 1995). However, there are several other antioxidants involved that could contribute to reduce aldehyde production in fish (Bell and Cowey, 1985; Nakano *et al*., 1999; Hidalgo *et al*., 2002).

In mammals, many studies have focused on the relation between oxidative stress and cell damage (Kannan and Jain, 2000; Kowaltowski *et al*., 2001; Lin and Beal, 2006), particularly aging and neurodegenerative diseases, although the effects of free radicals on muscular cells are not well studied. However, some studies have focussed on the potential role of free radicals in genetic muscular dystrophies (Rando, 2002). For example, it has been observed that vitamin E deficiency in animals leads to muscle degeneration with pathologic characteristics very similar to those of muscular dystrophies (Kakulas and Adams, 1966; Hadlow, 1973; Bradley and Fell, 1980). Many of the typical pathological features of genetic muscle disorders, such as peripheric sarcoplasmic halos (Farkas *et al*., 1974), have been observed in sea bass larvae muscle, supporting the idea that the mechanism of muscle injury is the same as in mammalian genetic muscular dystrophies.

Lesions observed by optical microscopy are similar to those found in our previous work (Chapter 3), corroborating the pathological effect of excess dietary DHA in the appearance of muscular lesions. Moreover, the increase in dietary levels of vitamin E reduced the incidence of these muscular lesions (Chapter 3). However, this beneficial effect of vitamin E was not observed in the present study when 5% of DHA was included in the diet, suggesting that the addition of vitamin E alone as an antioxidant is not enough to control lipid peroxidation when high levels of DHA are included in fish larvae diets. For example, it has been well studied that vitamin E has a sparing effect by dietary vitamin C in fish (Hamre *et al*., 1997; Shiau and Hsu, 2002; Lee and Dabrowsky, 2003) or how α-tocopherol and selenium seem to interact synergistically (Poston *et al*., 1976; Bell and Cowey, 1985).

On thick sections, hypercontracted myofilaments could be observed, probably due to the attack of free radicals on muscle proteins. Although red fibres were affected, the highest incidence of alterations was found in white fibres. It has been hypothesized that muscle fibres that are minimally affected or unaffected by the disease process may have enhanced antioxidant defences as the basis for this protection (Rando, 2002). In humans, a selective involvement of type II fibres over type I fibres has been observed in vitamin E deficiency myopathy (Lazaro *et al*., 1986; Tomasi, 1979). Although it could be assumed, a priori, that red fibres would be more affected by disorders of oxidative stress due to their metabolism being primarily oxidative, it does not take into account potential differences between red and white fibres in free radical scavenging capacity and their oxidative repair mechanisms, as happens in humans (Salminen and Vihko,

1983; Asayama *et al*., 1986). Thus, although red fibres may generate lower levels of reactive oxygen species during normal metabolism, white fibres may be more susceptible to an increase in oxidative stress as they have less robust antioxidant defences.

Ultrastructurally, one of the first changes observed in sea bass larvae muscle was the presence of large translucent vacuoles within the sarcoplasm, defined as hydropic degeneration, which is caused by the distension of the sarcoplasmic reticulum. These non-lethal lesions are related to adaptative cellular changes but, if continued in time could cause cellular lysis (Cotran *et al*., 2004). The appearance of these vacuoes is due to the failure of ion pumps and Na^+ and water influx to the endoplasmic reticulum, causing the alteration of this structure and the subsequent formation of vacuoles. This kind of alteration is a common response to free radical injury (Cotran *et al.,* 2004). Another cellular change observed was the presence of autophagic vacuoles containing cellular debris found in the intermyofibrillar spaces and sarcoplam throughout affected muscle fibres. Autophagic vacuoles are considered to be secondary lysosomes and are a frequent feature in numerous mammalian neuromuscular disorders, forming an emerging new group of conditions called autophagic vacuolar diseases (Nishino, 2006). In the tropical fish *Colossoma macropomum*, autophagic vacuoles have been described in skeletal muscle after treatment with 2-chloro-4,6-bis-ethylamine-s-tryazine, a drug employed for the control of aquatic weeds in fish cultivation ponds (Medina *et al*., 2000). This chemical drug produces skeletal muscle alterations, describing the pathology as a neurogenic atrophy because of the changes in motor nerve and end – plates. Medina *et al.* (2000) also described the finding of myelin figures that represent endogenous material produced by intracellular lipid peroxidation in mammals (DeGritz *et al*., 1994).

Numerous macrophages were observed around affected fibres ready to phagocytate cellular debris. Tissue injury can itself cause ROS generation by the activation of phagocytes, which may contribute a worsening effect on the injury (Aruoma, 1998). These cells contribute to oxidative stress in large part because they contain the potent NADPH oxidase system. Once activated the NADPH system produces large amounts of superoxide (Finkel and Holbrook, 2000).

The increase in the number of satellite cells is a common finding in several kind of genetics muscle dystrophies. These undifferentiated muscle precursor cells are stem cells located between the plasmalemma and basal lamina of the skeletal muscle fibre

(Zammit *et al*., 2006; Figeac *et al*., 2007) and are responsible for the regenerative capacity of the tissue (Phelan and Gonyean, 1997; Schultz and McCormick, 1994). Accordingly, we have hypothesized that the presumptive major presence of satellite cells could reflect a regenerative process in sea bass larvae. Satellite cells are relatively resistant to metabolic and toxic challenges compared with differentiated myofibres (Schultz and Lipton, 1978; Klein-Ogus and Harris, 1983), explaining why they are not affected by free radical attack.

Excessive oxidative stress kills cells either by necrosis or by apoptosis (Zamzami *et al*., 1995; Zamzami *et al*., 1996), however in the present study the only type of cell death observed morphologically was necrosis. Kroemer (1995) determined that the same toxin can induce apoptosis at low dose and primary necrosis at high dose, explaining why no morphological sign of apoptosis was observed at this work. Nevertheless, the fact that apoptosis usually involves only scattered individual cells, as well as the fact that the cellular destruction is very rapid (3 hours), makes its morphological analysis very difficult (Fidzianska, 2002). Therefore, apoptosis cannot be excluded from being implicated in sea bass larvae muscle cell death.

In summary, the present study indicates that an increase in dietary DHA levels was associated to muscular necrosis due to an impaired antioxidant protection, as indicated by TBARS content, suggesting the imbalance between prooxidants and antioxidants nutrients. Furthermore, the oxidative damage on muscle could not be counteracted by an increase in vitamin E dietary levels when high DHA contents were included in sea bass larvae diets. Other nutrients related to the oxidative status of fish may also be implied and are being studied at the present.

Chapter 5

Selenium inclusion decreases oxidative stress indicators and muscle injuries in sea bass larvae fed high-DHA microdiets

This report is in press at *British Journal of Nutrition* (doi:10.1017/S0007114512000311) as Betancor *et al*. (2012)

ABSTRACT

Keywords:

DHA
larvae
myopathy
oxidative stress
sea bass
vitamin E
Selenium

Sea bass larvae muscle has proved to be very sensitive to free radical injury when high levels of docosahexaenoic acid (DHA) are included in the diet. Despite of the increase of vitamin E in combination with the increase of DHA, muscular lesions were still present. Therefore, the inclusion of other nutrients with antioxidative functions, such as selenium (Se), is thought to be beneficial to prevent these injuries. The objective of the present work is to study the effect of Se inclusion in high DHA and vitamin E content sea bass larvae microdiets (5/300 and 5/300+Se) compared to a control diet (1/150) on their growth, survival, biochemical composition, MDA content, muscle morphology as well as antioxidant enzymes, IGFs and myosin expression. For a given DHA and vitamin E dietary content, Se inclusion favoured total length, SGR and reduced the incidence of muscular lesions, MDA contents and antioxidant enzymes expression. In contrast, IGFs expression was increased in 5/300 larvae suggesting an increased muscle mitogenesis that was corroborated by the increase in the mRNA copies of myosin heavy chain. The results of the present work confirm the beneficial effect of Se in preventing oxidative stress, not only as a GPX cofactor, but probably due to other unknown physiological functions.

5.1 Introduction

Free radicals and/or oxygen derivatives are continuously generated during regular cellular metabolism. At low concentrations, these reactive oxygen species (ROS) may be beneficial or even indispensable in processes such as defence against microorganisms, contributing to phagocitic bactericidal activity. However, when an imbalance between ROS generation and ROS removal occurs, oxidative stress arises (Rando, 2002). The detrimental effects include oxidative damage to molecules of great biological importance, including lipids, proteins and DNA, causing alterations that produce a range of cellular damages which can ultimately lead to cell death (Halliwell and Gutteridge, 1996).

Several effective antioxidant systems prevent oxidative damage in fish. Among them, various antioxidant enzymes (AOE) prevent the cascade of oxidation reactions, intercepting and inactivating the reactive intermediates and closing the lipid-peroxidation catalytic cycle. This defence system includes enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) (Bell et al, 1987; Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1996). In addition to these enzymes, dietary micronutrients such as vitamin E and C as well as carotenoids have been also regarded as antioxidant defences in fish (Wilhem-Filho, 1996; Lygren *et al*., 1999; Bell *et al*., 2000; Montero *et al*., 2001; Fernández-Palacios *et al*., 2005; Díaz *et al*., 2010; Hamre, 2010). During larval stages oxidation risks are particularly high, due to the increased metabolic rate, oxygen demand, water and long chain polyunsaturated fatty acid (LC-PUFA) tissue contents found in fish larvae. Therefore, adequate supplementation of marine fish larvae with antioxidant elements is important when formulating diets in order to avoid *in vivo* lipid peroxidation. However, few studies have dealt with the effects of antioxidants in early fish feeding (Mourente *et al*., 1999b; Atalah *et al*., 2008; Penglase *et al*., 2010; Chapter 3). Among the other antioxidants, selenium is an essential trace mineral in animal nutrition obtained partly from the surrounding water (Lall and Bishop, 1977), but mostly from the diet (Halver, 2002). The importance of selenium to oxidative stress involves its presence at the active site of the antioxidant enzyme GPX (Felton *et al*., 1996), which reduces hydroperoxides at the expense of reduced glutathione (Artel and Sies, 2001). In fish, studies have shown a synergistic action between vitamin E and selenium (Poston *et al.*, 1976; Bell and Cowey, 1985). Moreover, a selenium deficiency may lead to reduced levels of tissue α-tocopherol in several fish species (Poston *et al*., 1976; Bell *et al.*, 1985, 1986, 1987; Gatlin *et al*.,

1986). Additionally, a recent study in grouper (*Epinephelus malabaricus*) juveniles suggested a sparing effect between selenium and vitamin E (Lin and Shiau, 2009).

One of the most important factors that can lead to oxidative stress in fish is their high requirement for LC-PUFA, which are high in diets, and, subsequently, in fish tissues (McEvoy *et al*., 1998; Evjemo *et al*., 2003). For instance, severe dystrophic lesions in the epaxial musculature of sea bass larvae have been related to the deleterious effect of oxidative stress due to the high ingestion of LC-PUFA, particularly DHA (Chapters 3 and 4). Muscle growth is an essential process during larval stages, as a massive increment of muscle fibres takes place from hatching to maturity (Johnston and Hall, 2004) with damages to the musculature appearing to compromise larval growth (Chapters 3 and 4).

Muscle formation processes require the influence of growth factors and a sequence of cellular events that, result in the regulation of myoblasts (myosatellite cells) (Allen and Boxhorn, 1989; Johnston, 1999). Insulin-like growth factors I and II (IGF-I and IGF-II) are two myogenic regulatory factors which increase satellite cell proliferation and differentiation (Goldspink *et al*., 2001; Bower *et al*., 2008). In various species of fish it has been shown that hepatic (Duan and Plisetskaya, 1993; Matthews *et al*., 1997; Terova *et al*., 2007; Hevrøy *et al*., 2010) and muscular (Chauvignè *et al*., 2003; Terova *et al*., 2007; Hevrøy *et al*., 2010) IGF-I and IGF-II mRNA levels depend on feeding status. Differences in the regulation of myogenesis such as myosin isoform expression have also been observed during the earliest stages of development as well as during temperature acclimation (Watabe 1999; Silva *et al*., 2008). However, nutritional regulation of the various components of the IGF signalling pathways in muscle growth in fish as well as myosin expression is not well studied.

Different investigations have demonstrated the importance of vitamins for the correct skeletogenesis, showing that inadequate vitamin levels could contribute to the development of skeletal deformities (Dedi *et al*., 1997; Takeuchi *et al*., 1998; Villeneuve *et al*., 2005; Mazurais *et al*., 2008; Fernández *et al*., 2008, 2009). Besides, moderately oxidized lipids lead to a higher incidence of lordosis and scoliosis in halibut (*Hypoglossus hypoglossus*) (Lewis-McCrea and Lall, 2007; 2010) denoting the deleterious effect that free radicals can have on skeletal tissues.

The purpose of the present study was to investigate whether diets supplemented with Se (5mg kg^{-1} DW) and vitamin E can protect sea bass larvae muscle from oxidative stress when high DHA levels are included in diets. To reach this objective, growth,

survival, TBARS, fatty acid profile, α-tocopherol and selenium contents and mRNA expression levels of CAT, SOD, GPX, IGF-I, IGF-II and myosin heavy chain (MyHC) genes were determined in sea bass larvae fed diets with different LC-PUFA, Se and vitamin E contents.

5.2 Material and methods

5.2.1 Fish

The experiment was carried out at the *Instituto Canario de Ciencias Marinas* facilities (Telde, Canary Islands, Spain). Sea bass, *Dicentrarchus labrax,* larvae were obtained from natural spawnings from the *Instituto de Acuicultura de Torre de la Sal* (Castellón, Spain). Prior to the start of the feeding experiment, larvae were fed enriched (DHA Protein Selco®, INVE, Belgium) yeast-fed rotifers until they reached 14 dph. Prior to the start of the feeding experiment larvae (total length 8.58 ± 0.64 mm, dry body weight 0.36 ± 0.0 mg) were randomly distributed into the experimental tanks at a density of 1000 larvae/tank and were fed one of the experimental diets for 21 days, at a water temperature of 19.5 to 21.0ºC. All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (34 g L^{-1} salinity) at an increasing rate of 1.0 - 1.5 L/min along the feeding trials. Water entered the tank from bottom to top; water quality was tested daily and no deterioration was observed. Water was continuously aerated (125 ml/min), attaining 5-8 g L^{-1} dissolved O_2 and saturation ranged between 60 and 80%.

6.2.2 Diets

Three isonitrogenous and isolipidic experimental microdiets (pellet size<250 μm) similar in their EPA content and different in DHA, selenium and vitamin E content were formulated (Table 1) using concentrated fish oils INCROMEGA™ EPA500 and DHA500 (CRODA, East Yorkshire, UK) as sources of EPA and DHA in triglyceride form and DL-α-tocopheryl Acetate (Sigma-Aldrich, Madrid, Spain) as source of vitamin E. Diets were chosen based on previous trials (Chapters 3 and 4) and their names elected according to the level of dietary DHA and vitamin E content. A positive control diet was formulated to include 1 g DHA 100 g^{-1} DW and 150 mg vitamin E 100 g^{-1} DW (diet 1/150). The negative control diet consisted of 5 g DHA 100 g^{-1} DW and 300 mg vitamin E 100 g^{-1} DW

(diet 5/300). The third diet had identical DHA and vitamin E content to the 5/300 diet, but was supplemented with selenium in organic form (Sel-Plex® 2000, 2000 mg kg^{-1}, Alltech, Lexington, KY) (diet 5/300+Se).

Table 5.1 Formulation of experimental diets

	Experimental diets		
Ingredients	*1/150*	*5/300*	*5/300+Se*
Defatted squid powder (g $100g^{-1}$)*	69.00	68.85	68.60
INCROMEGA™ EPA 500TG g $100g^{-1}$ (DW)†	2.80	1.80	1.80
INCROMEGA™ DHA 500TG g $100g^{-1}$ (DW) †	0.20	6.70	6.70
Oleic acid (%)‡	10.00	4.50	4.50
Soy lecithin§	2.00	2.00	2.00
Gelatin**	3.00	3.00	3.00
Attractants††	3.00	3.00	3.00
Taurin‡‡	1.50	1.50	1.50
Vitamin premix§§	6.00	6.00	6.00
Mineral premix***	2.50	2.50	2.50
Sel-Plex 2000® (g $100g^{-1}$) †††	-	-	0.25
Vitamin E mg $100g^{-1}$ (DW) ‡‡‡	-	0.15	0.15

* Riber and Son, Bergen, Norway
† Croda Chemicals Europe, East Yorkshire, UK
‡Merck, Darmstadt, Germany
§ Acrofarma, Barcelona, Spain
** Panreac, Madrid, Spain
††Attractants premix supplied per 100 g diet: Inosine-5-monophosphate, 500.0 mg; betaine, 660.0 mg; L-serine, 170.0 mg; L-phenilalanine, 250.0 mg; DL-alanine, 500.0 mg; L-sodium aspartate, 330.0 mg; L-valine, 250.0 mg; glycine, 170.0 mg
‡‡ Sigma-Aldrich, Madrid, Spain
§§Vitamin premix supplied per 100 g diet: Cyanocobalamine, 0.03 mg; Astaxanthin, 5.0 mg; folic acid, 5.4 mg; pyridoxine-HCl, 17.2 mg; thiamine, 21.7 mg; riboflavin, 72.5 mg; Ca-pantothenate, 101.5 mg; p-aminobenzoic acid, 145.0 mg; nicotinic acid, 290.1 mg; *myo*-inositol, 1450.9 mg; retinol acetate, 0.2 mg; ergocalcipherol, 3.6 mg; menadione, 17.3 mg; α-tocopheryl acetate, 150.0 mg
***Mineral premix supplied per 100g diet: NaCl, 215.133 mg; $MgSO_4 \cdot 7H_2O$, 677.545 mg; $NaH_2PO_4 \cdot H_2O$, 381.453 mg; K_2HPO_4, 758.949 mg; $Ca(H_2PO_4) \cdot 2H_2O$, 671.610 mg; $FeC_6H_5O_7$, 146.884 mg; $C_3H_5O_3 \cdot 1/2Ca$, 1617.210 mg; $Al_2(SO_4)_3 \cdot 6H_2O$, 0.693 mg; $ZnSO_4 \cdot 7H_20$, 14.837 mg; $CuSO_4 \cdot 5H_2O$, 1.247 mg; $MnSO_4 \cdot H_2O$, 2.998 mg; KI, 0.742 mg; $CoSO_4 \cdot 7H_2O$, 10.706 mg
††† Sel-Plex 2000, 2000 mg Se kg^{-1}, Alltech, Lexington, KY
‡‡‡ DL-α-tocopheryl acetate, Sigma-Aldrich, Madrid, Spain

The protein source was derived from squid meal, naturally containing a 14% lipid, defatted 3 consecutive times to allow complete control of the microdiet fatty acid profile. No other ingredients were defatted due to their poor lipid content. The squid meal was defatted with a chloroform:squid meal ratio of 3:1, rinsed and dried in at oven at 37ºC

until complete solvent evaporation. EPA500 and DHA500 were added in different quantities to the defatted meal (2.4% lipid content) to obtain the desired ratios. Oleic acid (Merck, Darmstadt, Germany) was added to equalize the lipid content in each diet (Table 5.1) and soy bean lecithin (Acrofarma, Barcelona, Spain) was included as a source of phospholipids (Table 5.1). The microdiets were processed as previously described (Liu *et al*. 2002). Briefly, the squid powder and water soluble components were mixed, followed by the lipid and fat soluble vitamins and, before adding gelatine (Panreac, Barcelona, Spain) dissolved in warm water, as a binder. The paste was pelleted and oven dried at 38ºC for 24 h. Pellets were ground and sieved to obtain particles size below 250 µm. To avoid peroxidation, diets were stored under nitrogen at -20ºC until use. Diets were analyzed for proximate and fatty acid composition on a dry basis and manually supplied; fourteen times per day at 45 min intervals from 9:00-19:00. Daily feed supplied was 2, 2.5 and 3 g/tank during the first, second and third week of feeding respectively.

5.2.3 Growth and survival

Final survival was determined by counting live larvae at the beginning, middle and end of the experiment. Growth was determined by measuring dry body weight (105ºC for 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish tank^{-1} at the beginning, middle and at the end of the trial.

5.2.4 Biochemical analysis

All remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition, TBARS, selenium and vitamin E analysis after 12 hours of starvation at the end of the trial. Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and lipid (Folch *et al*., 1957) contents of larvae and diets were analyzed.

5.2.4.1 Total lipid fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipids as described by Christie (1982). FAMEs were separated by GLC, quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1992) and identified by comparison to previously characterized standards and GLC-MS.

5.2.4.2 Determination of vitamin E content

Vitamin E concentrations were determined in diets and total larvae using HPLC at the University of Stirling (Scotland, UK). Samples were weighed, homogenised in pyrogallol and saponified as described by McMurray *et al.* (1980) for diets or according to Cowey *et al.* (1981) for larval tissues. HPLC analysis was performed using 150 x 4.60 mm reverse phase Luna 5µm C18 column (Phenomenox, California, USA). The mobile phase was 98% methanol supplied at a flow rate of 1.0 ml min^{-1}, the effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with (+)-α-tocopherol (Poole, UK) as external standard.

5.2.4.3 Selenium determination

Total selenium concentration was measured in total larvae and diets. Samples were acidified in a microwave digestor (MarsXpress, CEM, Kamp-Lintfort, Germany) with 5 ml of 69% pure nitric acid, then poured after digestion into a 10 ml volumetric flask and made up to volume with distilled water. A total of 0.4 ml of this solution was then added to a 10 ml sample tube, 10 µl of the internal standard (Ga and Sc, 10 ppm) included and 0.3 ml of methanol added. The tubes were made up to volume with distilled water and total selenium measured by collision/reaction by ICP-MS (Thermo Scientific, Cheshire, UK) using argon and hydrogen as carrier gas.

5.2.4.4 Measurement of thiobarbituric acid reactive substances (TBARS)

TBARS from triplicate samples were determined using a method adapted from that used of Burk *et al.* (1980). Approximately 20-30 mg of larval tissue per sample were homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol. To this 2.95 ml of freshly prepared 50mM thiobarbituric acid solution was added before mixing and heating for 10 minutes at 100ºC. After cooling protein precipitates were removed by centrifugation (Sigma 4K15, Osterode am Harz, Germany) at 2000 x *g*, the supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA) expressed as µmol MDA per g of tissue was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

5.2.5 Histopathological sampling

5.2.5.1 Paraffin inclusion

Thirty larvae from every tank were collected each seven days from the beginning of the feeding trial, and fixed in 10% buffered formalin for 1 or 2 days, dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax. Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm, and stained with Haematoxilin and Eosin (H&E) for histopathological evaluation (Martoja and Martoja-Pearson, 1970).

5.2.5.2 Resin inclusion

Ten larvae per tank were fixed for 24 hours at 4º in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). Samples were then rinsed in phosphate buffer and post-fixed for 1 hour in 2% osmium tetraoxide in 0.2 M potassium ferrocyanide. Each larva was then embedded in an Eppon/Araldite resin block. Serial transverse and longitudinal larvae thick sections were cut at 2 µm, stained with toluidine blue and examined under light microscopy (Hoffman *et al*., 1983). Thin sections were cut at 50 nm and stained with lead citrate before observing with a ZEISS EM 910 transmission electron microscope (Germany) at the Electron Microscope Service of the University of Las Palmas de Gran Canaria.

5.2.5.3 Whole mount staining

Besides, one hundred larvae from each tank at 35 dph were fixed in 10% buffered formalin in order to perform deformities analysis. Prior to staining, larvae were measured under a Profile Projector (Mitutoyo, PJ 3000, Japan) and divided in three size classes (< 10.0 mm, 10.0-12.0 mm and >12.0 mm) and therefore stained with Alizarin red following Vandewalle *et al.* (1998) to demonstrate bone. Larvae from the different experimental group were stained simultaneously in order to prevent any technical variability. Deformities were classified in three different groups according to their localization: cranial deformities, lordosis, kyphosis and others, including deformities such as lordosis, vertebral compression or neural processes alterations. The surface corresponding to bone in whole coloured larvae was visualized and quantified using a computerized image analysis package (Image-Pro Plus®, Media Cybernetics, Maryland, USA). Selecting

ranges of pixel values in colour images allowed the pixels associated with red to be distinguished. The number of selected pixels was then quantified using a particle analysis operation and by counting the area of all bright objects (in pixels). Larval size was estimated by calculating the surface areas (in pixels) covered by whole stained larvae.

5.2.6 RNA extraction and quantitative RT-PCR

Molecular biology analysis was carried out at the University of Insubria (Varese, Italy). Total RNA was extracted from sea bass larvae (≈200 mg; pool per tank), using PureYield RNA Midiprep System (Promega, Italy). The quantity and purity of RNA was assessed by spectrophotometer. Visualization on 1% agarose gel stained with ethidium bromide showed that RNA was not degraded. After DNAse treatment (Invitrogen, Milan, Italy), 3 µg of total RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated at 65ºC for 5 minutes, chilled on ice and then 4 µl of 5X reverse transcription buffer, 2 µl 0.1M DTT, 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT) was added. After incubation at 37ºC for 50 minutes, the reaction was stopped by heating at 75ºC for 15 minutes.

PCR primer sequences used for the PCR amplification of the cDNAs of target genes were CAT, SOD, GPX, IGF-I, IGF-II and MyHC. To perform PCR, a 4 µl aliquot of cDNA was amplified using 25 µl GoTaq Green Master Mix (Promega, Italy) in 50 µl of final volume and 50 pmol of each designed primer.

A total of 31 PCR amplification cycles (eight touchdown) were performed for all primer sets, using an automated Thermal Cycler (MyCycler, BioRad, Italy). An aliquot of each sample was then subjected to electrophoresis on a 1% agarose gel in 1X TAE buffer (Bio-Rad, Italy) and bands were detected by ethidium bromide staining. Samples were run with a 100 bp-1.5 kb DNA Ladder to control the molecular weight of DNA. The negative control (a reaction mixture without cDNA) confirmed the absence of genomic contamination. The PCR products from each primer set amplification were cloned using pGEM®-T easy vector (Promega, Italy) and subsequently sequenced in both directions (T7 and SP6).

TaqMan® real time reverse transcription PCR was performed on a StepOne Real Time PCR System (Applied Biosystems, Italy) using Assays-by-Design℠ PCR primers (Applied Byosystems) and gene-specific fluorogenic probes. Primer sequences and TaqMan® probes of target genes were as follows:

Target gene: Sea bass CAT

Forward primer: 5'- ATGGTGTGGGACTTCTGGAG - 3'

Reverse primer: 5'- GCTGAACAAGAAAGACACCTGATG - 3'

TaqMan® probe. 5'- CAGACACTCAGGCCTCA - 3'

Target gene: Sea bass SOD

Forward primer: 5'- TGGAGACCTGGGAGATGTAACTG - 3'

Reverse primer: 5'- TCTTGTCCGTGATGTCGATCTTG - 3'

TaqMan® probe. 5'- CAGGAGGAGATAACATTG - 3'

Target gene: Sea bass GPX

Forward primer: 5'- AGTTAATCCGGAATTCGTGAG - 3'

Reverse primer: 5'- AGCTTAGCTGTCAGGTCGTAAAAC - 3'

TaqMan® probe. 5'- AATGGCTGGAAACGTG - 3'

Target gene: Sea bass IGF-I

Forward primer: 5'- GCAGTTTGTGTGTGGAGAGAGA- 3'

Reverse primer: 5'- GACCGCCGTGCATTGG - 3'

TaqMan® probe. 5'- CTGTAGGTTTACTGAAATAAAA - 3'

Target gene: Sea bass IGF-II

Forward primer: 5'- TGCAGAGACGCTGTGTGG - 3'

Reverse primer: 5'- GCCTA CTGAAATAGAAGCCTCTGT - 3'

TaqMan® probe. 5'- CAAACTGCAGCGCATCC - 3'

Target gene: Sea bass MyHC

Forward primer: 5'- TGGAGAAGATGTGCCGTACTCT - 3'

Reverse primer: 5'- CGTGTCATTGATTTGACGGACATTT - 3'

TaqMan® probe. 5'- AACTGAGTGAACTGAAGACC - 3'

Data from TaqMan® PCR runs were collected using ABI´s Sequence Detector Program. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA. To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

5.2.7 Calculations

Larval survival was determined by comparing the number of larvae at the beginning of the trial with the larvae number measured in individual tanks at 35 dph to which the average number of larvae sampled from tanks during the trial was added. Percentage survival could then be calculated for each tank to get a mean and SD per treatment. The incidence of muscular lesions was calculated as the percentage of injured larvae per tank compared to the total larvae observed, with SD refering to deviation among tanks. Specific growth rate (SGR) was calculated as:

$$SGR = [(\ln W_1 - \ln W_0)]*100/t_2-t_1$$

Where W_0 and W_1 are the initial and final dry weights (tank means) respectively, and t_2-t_1 is the time interval in days between the beginning and end of the experimental trial (21 days).

5.2.8 Statistical analysis

Survival, growth and molecular biology data were tested for normality and homogeneity of variances with Levene´s test. Where necessary data were log

transformed before further statistical analysis. Chi-squared test was employed for incidence of muscular lesions and TBARS content. Survival, growth and biochemical analysis data were treated using one-way ANOVA and molecular biology results were treated using a general linear model (GLM). Means were compared by Duncan's test. Results are presented as means and standard deviation. The tank was considered as the experimental unit, except for the estimation of the incidence of muscular lesions, where each individual larvae was considered as a unit. For percentage data (final survival), arcsine transformation was performed before analysis. For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ijk}$$

where Y_{ijk} is the mean value of the tank, μ is the mean population, α_i is the fixed effect of the diet and ε_{ij} is the residual error. For analysis of molecular biology data a two variables GLM was employed to analyze possible interactions between treatment and time:

$$Y_{ijk} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + \varepsilon_{ijk}$$

Where Y_{ij} is the mean value of the tank, μ is the mean population, α_i is the fixed effect of the diet, δ_j is the fixed effect of the time, $(\alpha\delta)_{ij}$ is the interaction between diet and time and e_{ij} is the residual error. Significance was accepted at $P \leq 0.05$. Statistical analysis was performed using SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005).

6.3 Results

Diet containing about 1% DHA (1/150) showed a higher monoenoic fatty acid level than diets containing 5% DHA (5/300, 5/300+Se) due to a higher oleic acid content in the former diet (Table 5.2). Elevation of dietary DHA (5/300 diets) increased *n*-3 and *n*-3 LC-PUFA fatty acids contents, as well as *n*-3/*n*-6 ratio. Vitamin E levels were more than 2 times higher in diets containing 300 mg $100g^{-1}$ compared to the control diet (1/150) (Table 5.3). Selenium contents differed among dietary treatments, with a higher level of this mineral found in the diet supplemented with selenium as compared to others (Table 5.3).

Table 5.2 Main fatty acids (% total identified fatty acids) of the experimental diets fed to European sea bass for three weeks.

	Diet		
	1/150	5/300	5/300+Se
14:0	1.54	1.26	0.78
14:1*n*-7	0.15	0.25	0.06
14:1*n*-5	0.22	0.35	0.09
15:0	0.28	0.43	0.15
15:1*n*-5	0.02	0.14	n.d.
16:0ISO	0.14	0.23	0.07
16:0	7.86	5.59	5.15
16:1*n*-7	3.59	2.26	2.00
16:1*n*-5	0.19	0.23	0.11
16:2*n*-4	0.32	0.39	0.25
17:0	1.21	0.82	0.65
16:3*n*-3	0.08	0.12	0.07
16:4*n*-3	0.09	0.13	0.10
18:0	1.29	2.29	2.21
18:1*n*-9+*n*-7	55.70	31.12	29.67
18:1*n*-5	0.72	0.46	0.45
18:2*n*-9	0.25	0.13	0.12
18:2*n*-6	7.40	6.99	6.87
18:2*n*-4	0.46	0.28	0.26
18:3*n*-6	0.11	0.11	0.10
18:3*n*-4	0.13	0.10	0.09
18:3*n*-3	0.72	0.83	0.82
18:4*n*-3	0.83	0.94	1.00
18:4*n*-1	0.08	0.08	0.09
20:0	0.10	0.31	0.29
20:1*n*-9+*n*-7	1.10	1.53	1.45
20:1*n*-5	0.05	0.12	0.11
20:2*n*-9	0.05	0.04	0.02
20:2*n*-6	0.09	0.21	0.21
20:3*n*-6	0.09	0.13	0.13
20:4*n*-6	0.71	1.57	1.50
20:3*n*-3	0.07	0.18	0.16
20:4*n*-3	0.32	0.52	0.55
20:5*n*-3	8.66	11.04	12.20
22:1*n*-11	0.17	0.51	0.50
22:1*n*-9	0.08	0.25	0.25
22:4*n*-6	0.02	0.19	0.20
22:5*n*-6	0.19	1.75	1.78
22:5*n*-3	0.32	1.29	1.43
22:6*n*-3	4.58	24.55	27.95
Saturated	12.28	10.70	9.22
Monoenoics	61.99	37.23	34.69
n-3	15.68	39.61	44.27
n-6	8.61	11.14	10.85
n-9	57.19	33.10	31.54
n-3 LC-PUFA	13.96	37.58	42.28
n-3/*n*-6	1.82	3.56	4.08

All experimental diets were well accepted by the larvae. Dietary increase of vitamin E or Se did not significantly affect larval survival (*P*= 0.158; Table 5.4). The highest larval growth, in terms of total length, was found in larvae fed the positive control diet containing the lowest vitamin E and DHA contents (1/150). However, increases in vitamin E and DHA (diet 5/300) significantly reduced larval growth (*P*= 0.001), whereas Se inclusion (diet 5/300+Se) significantly increased this parameter (*P*=0.02 Table 5.4). The average SGR was higher (*P*=0.024) in larvae fed the 1/150 diet, although no differences were found with larvae fed 5/300+Se.

In terms of fatty acid composition, larvae fed 5/300 diets resulted in higher concentration of *n*-3 and *n*-3 LC-PUFA, reflecting the higher content of these components in the diets (Table 5.5). However, larvae fed diet 1/150 showed a higher retention rate of DHA (279.26%) and EPA (68.24%) compared to larvae fed 5/300 (73.48 and 43.93% respectively) and 5/300+Se diets (77.00 and 44.75% respectively) and also a higher content of 18:1*n*-9, displaying levels similar to those found in the former microdiet. In contrast, 5/300 larvae showed a higher retention rate of arachidonic acid (around 50%) compared to 1/150 larvae (29.83%).

The level of lipid oxidation, as indicated by MDA content (µmol g^{-1} larval tissues) was significantly higher (*P*=0.001) in larvae fed diets with the highest DHA content. Nevertheless, the inclusion of selenium showed a beneficial effect preventing the formation of hydroperoxides, as denoted by the decrease in MDA levels (Table 5.4). The lowest peroxidation level was observed in larvae fed diet 1/150. Despite the increase in dietary α-tocopherol in diets 5/300 and 5/300+Se, the content of this nutrient in larvae did not significantly increase in comparison to larvae fed diet 1/150 (*P*=0.601; Table 5.4). Therefore, the retention rate of dietary vitamin E in larvae fed 5/300 (17.87%) and 5/300+Se (17.38%) diets was lower than 1/150 larvae (44.68%). Sea bass larvae fed diets supplemented with selenium showed a significantly higher (*P*=0.001) content of this mineral compared to other larvae, this level being 1.7 times higher than the larvae fed 1/150 diet and 2.4 times higher than the 5/300 larvae (Table 5.4). Thus, although Se contents in diets 1/150 and 5/300 were similar, larvae fed the latter diet showed lower Se levels together with the highest TBARS value.

Table 5.3 Gross composition, α-tocopherol and selenium content in experimental diets fed to sea bass larvae.

	Diets		
	1/150	5/300	5/300+Se
Protein (%)	66.79±0.52	67.77±0.09	66.24±0.36
Ash (%)	4.49±0.11	4.76±0.12	4.87±0.13
Moisture (%)	10.31±0.46	9.99±0.28	9.09±0.27
Lipids (% DW)	14.98±0.31	15.80±0.02	15.97±0.05
α-tocopherol (µg/g DW)	1410.12±38.77	3033.01±43.33	3217.37±14.45
Selenium (µg/mg)	1.54±0.12	1.33±0.26	6.27±0.26

Data are means ± SD

Histopathological examinations revealed the presence of lesions affecting larvae axial musculature. These lesions showed the typical features of necrotic degeneration of muscle, characterized by marked eosinophilia, loss of striations and adjacent nucleous. The incidence of muscular lesions increased with an increase in DHA dietary content (Table 5.4). However, inclusion of Se was shown to reduce this incidence to almost half.

Table 5.4 Sea bass larvae performance and levels of lipid peroxidation products (TBARS), vitamin E (α-tocopherol) and selenium content of sea bass larvae at the beginning and after eating the experimental diets

		Diets		
	Initial	1/150	5/300	5/300+Se
Results of dietary trial				
Larval total length (mm)	8.58 ± 0.64	12.60 ± 0.93^{a}	10.89 ± 1.24^{c}	11.35 ± 1.29^{b}
Larval dry weight (mg)	0.36 ± 0.00	1.46 ± 0.47	0.94 ± 0.05	1.08 ± 0.10
Survival (%)	-	60.51 ± 9.10	48.42 ± 4.00	49.03 ± 8.02
Incidence muscular lesions (%)	-	17.5 ± 14.14^{b}	52.63 ± 15.93^{a}	27.6 ± 6.94^{ab}
Vitamin E (α-tocopherol) µg g dry mass^{-1}	111.45±43.26	630.24±12.39	542.10±80.51	559.23±88.58
TBARS nMol g dry mass^{-1}	62.85±0.61	166.62±25.08^{c}	2402.15±67.91^{a}	282.29±92.48^{b}
Selenium µg mg dry mass^{-1}	1.38±0.10	1.58±0.12^{b}	1.11±0.31^{c}	2.65±0.27^{a}

Data are means ± SD

In semithin sections more detailed features of these muscular lesions could be observed. In severely damaged fibres a coagulation of the muscular proteins could be observed as a darkening of surrounding fibres due to hypercontraction (Figure 5.1A). In initial-mild stages of the condition an increase in the presence of vacuoles within fibres was observed, together with the loss of shape of muscular fibres, alteration of sarcoplasmic membranes and variation in the diameter of fibres (Figure 5.1B).

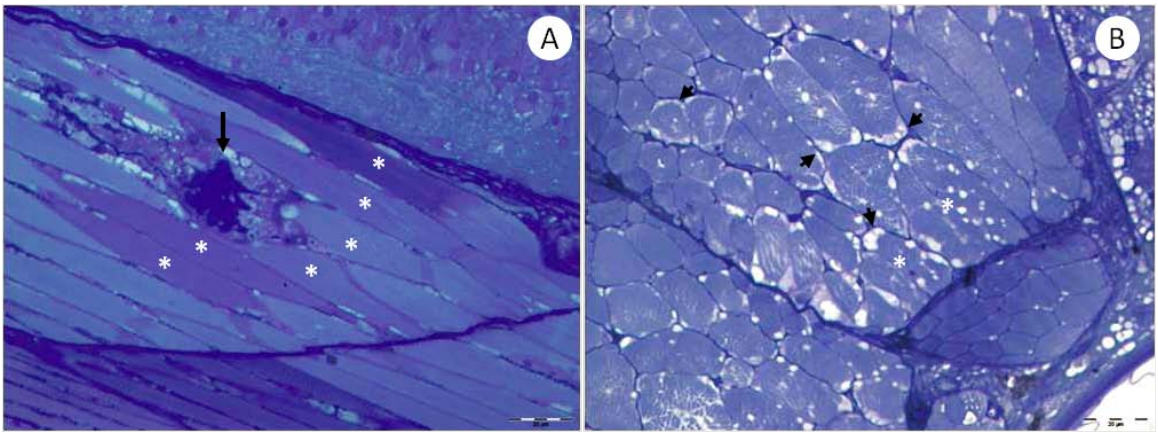

Figure 5.1 Semithin micrographs of longitudinal (**A**) and transversal sections (**B**) showing; (**A**) coagulation of muscular proteins in affected fibre (arrow) and hypercontraction of the surrounding muscular fibres (*). (**B**) Mild affected fibres showed loss of the polyedrical structure, abundant vacuoles (*) and dilatation of sarcoplasmic membranes (arrow).

TEM showed muscle degeneration with the presence of hydropic and autophagic vacuoles, considered secondary lysosomes, within some affected fibres in larvae fed 5/300 diet (Figure 5.2A). Altered mitochondria, observed as swollen double membrane organelles were seen in affected fibres and presented loss of the cristae (Figure 2.A) in contrast to normal ones (Figure 5.2B). Additionally, satellite cells were observed attached to existing damaged muscle fibres under the basal lamina (Figure 5.2C).

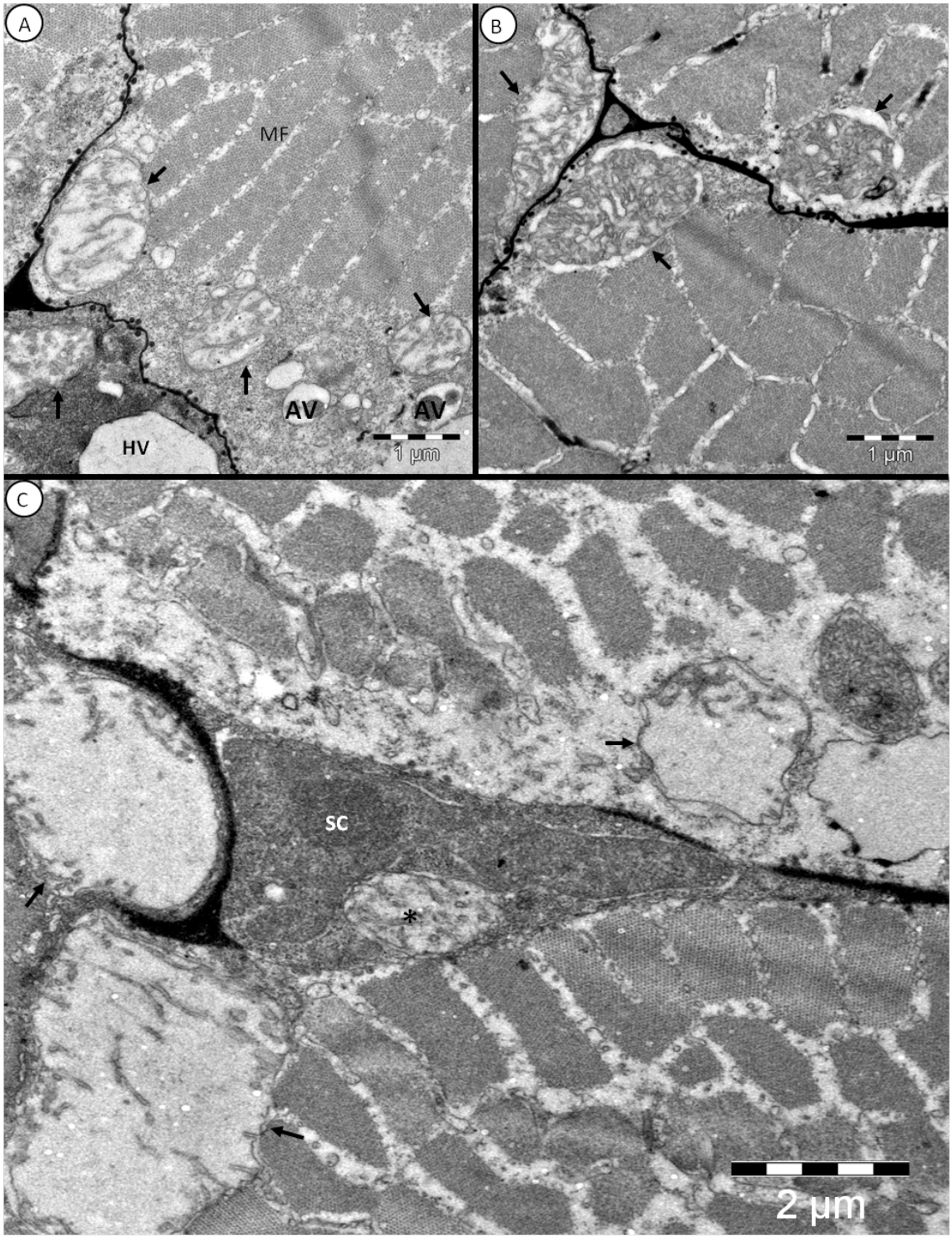

Figure 5.2. Electro micrographs of transversal sections of sea bass larvae fed 5/300 diet. (**A**) Damaged muscle fibre showing autophagic (AV) and hydropic vacuoles (HV) and swollen mitochondria (arrow). (**B**) Not affected fibre where normal mitochondria can be observed (arrow). (**C**) Presence of a satellite cell (SC) with a mitochondria (*) between two damaged muscle fibres, with the presence of vacuoles and degenerated mitochondria (arrow). Normal myofilaments (MF); disarranged myofilaments (disMF).

Table 5.5 Main fatty acid composition of total lipids from sea bass larvae after three weeks of feeding the experimental diets (% total identified fatty acids). Data are means±SD

	1/150	5/300	5/300+Se
14:0	0.93±0.06	0.76±0.03	0.71±0.03
14:1*n*-7	0.08±0.01	0.08±0.03	0.09±0.04
14:1*n*-5	0.07±0.02	0.04±0.01	0.06±0.00
15:0	0.63±0.29	0.98±1.22	0.22±0.05
15:1*n*-5	0.12±0.10	0.09±0.06	0.06±0.00
16:0ISO	n.d.	0.11±0.01	0.10±0.01
16:0	17.51±2.44	17.61±0.25	16.56±0.42
16:1*n*-7	2.02±0.14	1.60±0.06	1.56±0.00
16:1*n*-5	0.25±0.01	0.25±0.01	0.29±0.10
16:2*n*-6	0.30±0.00	0.36±0.04	0.37±0.02
16:2*n*-4	0.93±0.40	0.96±0.15	0.81±0.06
17:0	0.91±0.10	0.80±0.03	0.75±0.00
16:3*n*-3	0.12±0.01	0.14±0.03	0.12±0.03
16:3*n*-1	0.10±0.02	0.54±0.10	0.64±0.05
16:4*n*-3	0.62±0.35	0.44±0.10	0.56±0.03
16:4*n*-1	n.d.	0.17±0.01	0.21±0.02
18:0	11.66±3.41	12.29±0.36	11.37±0.39
18:1*n*-9	26.35±4.87^{a}	20.92±0.51^{b}	19.36±0.32^{b}
18:1*n*-7	4.85±0.29	4.44±0.39	3.98±0.29
18:1*n*-5	0.62±0.24	0.49±0.07	0.54±0.02
18:2*n*-9	n.d.	0.13±0.11	0.22±0.01
18:2*n*-6	4.23±0.08	3.90±0.17	3.75±0.08
18:2*n*-4	0.04±0.05	0.06±0.01	0.07±0.00
18:3*n*-6	0.43±0.01	0.38±0.04	0.32±0.02
18:3*n*-4	0.07±0.04	0.06±0.02	0.08±0.00
18:3*n*-3	0.32±0.05^{b}	0.44±0.04^{ab}	0.49±0.03^{a}
18:4*n*-3	0.29±0.06	0.29±0.13	0.37±0.04
20:0	0.38±0.19	0.47±0.01	0.47±0.08
20:1*n*-9+*n*-7	1.83±0.0	1.77±0.06	1.77±0.21
20:1*n*-5	0.26±0.15	0.13±0.01	0.16±0.00
20:2*n*-6	0.50±0.13	0.65±0.08	0.67±0.07
20:3*n*-6	0.08±0.01	0.08±0.00	0.08±0.01
20:4*n*-6	2.38±0.04^{b}	3.07±0.22^{a}	3.03±0.03^{a}
20:3*n*-3	0.12±0.09	0.15±0.04	0.14±0.01
20:4*n*-3	0.14±0.00	0.16±0.01	0.16±0.00
20:5*n*-3	5.91±1.18^{a}	4.85±0.20^{b}	5.46±0.36^{ab}
22:1*n*-11	0.46±0.30	0.19±0.08	0.23±0.01
22:1*n*-9	0.26±0.15	0.26±0.06	0.23±0.08
22:4*n*-6	n.d.	0.11±0.02	0.12±0.00
22:5*n*-6	1.09±0.09	1.26±0.07	1.40±0.03
22:5*n*-3	0.64±0.26	0.57±0.06	0.69±0.02
22:6*n*-3	12.79±0.37^{b}	18.04±1.19^{ab}	21.80±0.94^{a}
Saturated	32.01±6.29	32.91±1.54	30.08±0.76
Monoenoics	36.73±4.51^{a}	30.20±0.55^{b}	28.26±0.39^{b}
n-3	20.94±2.23^{c}	25.11±1.43^{b}	29.78±1.25^{a}
n-6	8.45±0.97	9.80±0.47	9.74±0.04
n-9	28.44±4.72^{a}	23.08±0.39^{b}	21.58±0.04^{c}
n-3 HUFA	12.92±7.45^{c}	23.78±1.42^{b}	28.25±1.30^{a}
n-3/*n*-6	2.51±0.55^{b}	2.56±0.05^{b}	3.06±0.12^{a}

Among larvae measuring 10-12mm, groups fed 1/150 diet showed 25.0±1.3% skeletal deformities, and similar values were found for sea bass provided 5/300+Se diet (31.3±7.2%). Larvae fed 5/300 diet presented 35.7±5.1% deformities, however no statistical differences were detected between groups (*P*=0.05). Skull deformities, particularly pugheadness (Figure 5.3A), were identified in all experimental groups especially in fish fed high DHA levels (*P*=0.042). Although no significant differences were found between groups, selenium supply appeared to decrease the incidence of lordosis (Figure 5.3B; *P*=0.083) and kyphosis (Figure 5.3C; *P*=0.14). Other bone anomalies, such as scoliosis and vertebral compression (Figure 5.3D) were not present in 1/150 fed sea bass. Regarding the bone mineralization of larvae, DHA seemed to have an effect on mineralization, observing the lowest amounts of red in 5/300 larvae (Figure 5.4; *P*=0.001). Nevertheless, selenium supplementation increased the mineralization rate, not existing differences with the control group (*P*=0.242). Besides, a negative correlation was found between the bone mineralization and total deformities (*y=-2.9668x+82.365; R^2=0.9715*).

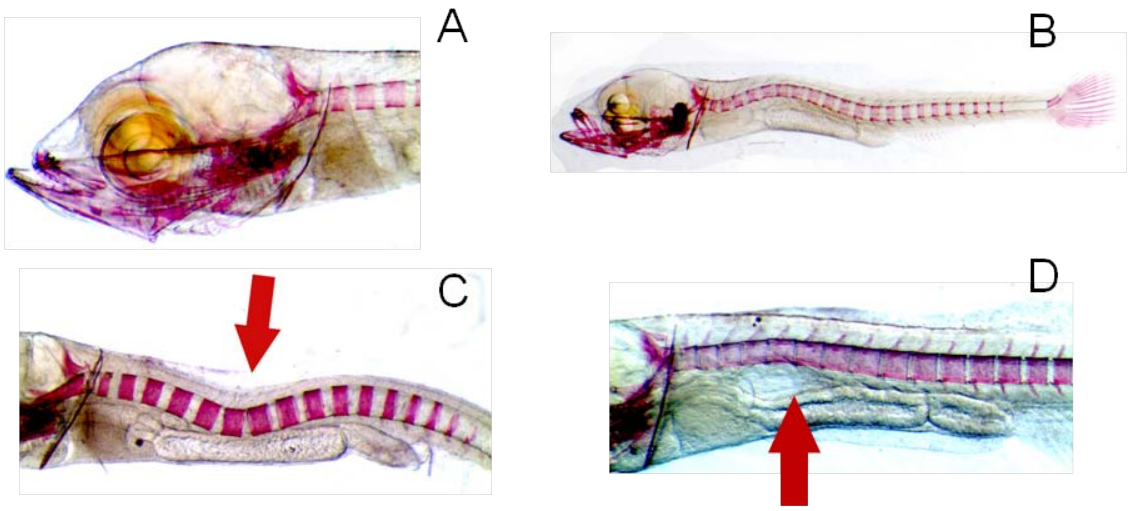

Figure 5.3 Characteristic skeletal anomalies found in sea bass larvae: pugheadness (**A**), lordosis (**B**), kyphosis (**C**), and vertebral compression (**D**).

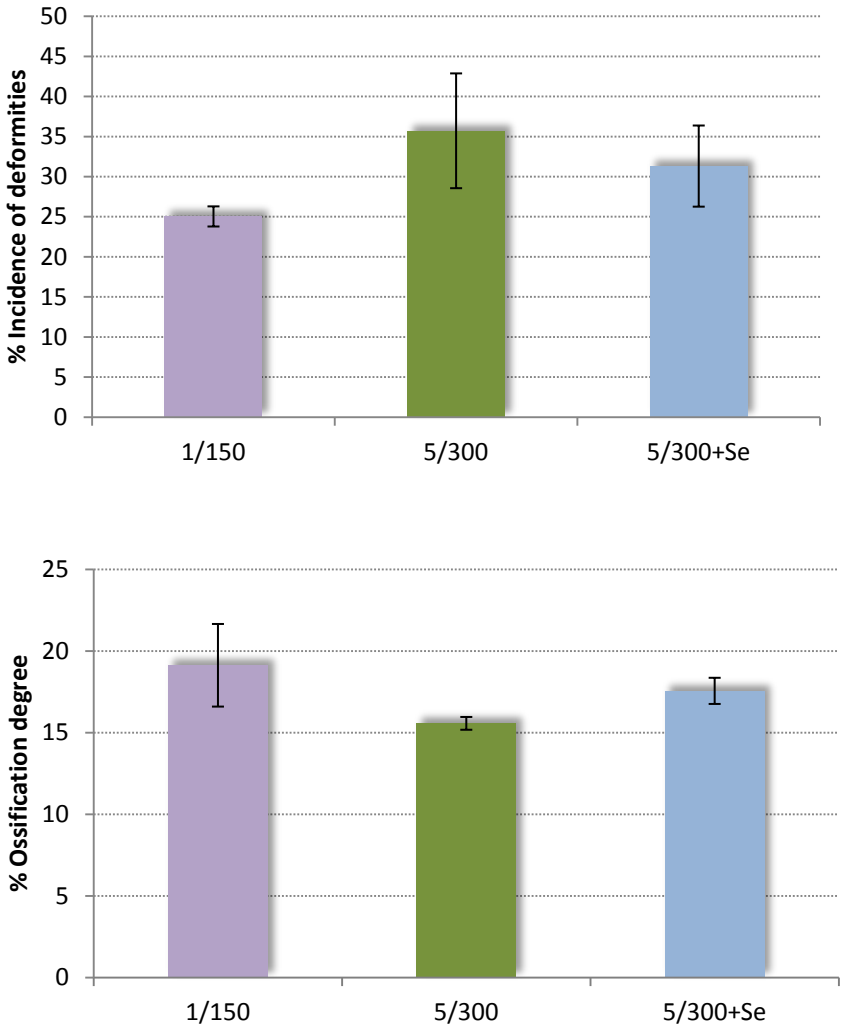

Figure 5.4 Sea bass larvae bone mineralization and deformities frequency (%) at 35 dph of fishes from the different dietary treatments.

The general pattern of antioxidant enzyme gene expression in all groups of sea bass larvae was characterised by a rapid increase between 14 and 26 dph, followed by a decrease back to levels slightly higher than those observed at 14 dph, by 29 dph (Figure 5.5A-C). The only exception to this trend was observed in GPX gene expression, where sea bass larvae fed diet 1/150 had a lower mRNA level of this enzyme at 35 dph in comparison to that at 14 dph (Figure 5.5C). CAT gene expression was higher in larvae fed diets containing a high content of DHA at all sampling points, although no statistical differences were observed (Figure 5.5A). However, GLM analysis showed differences between all the three treatments (P=0.001; Figure 5.5A; Table 6).

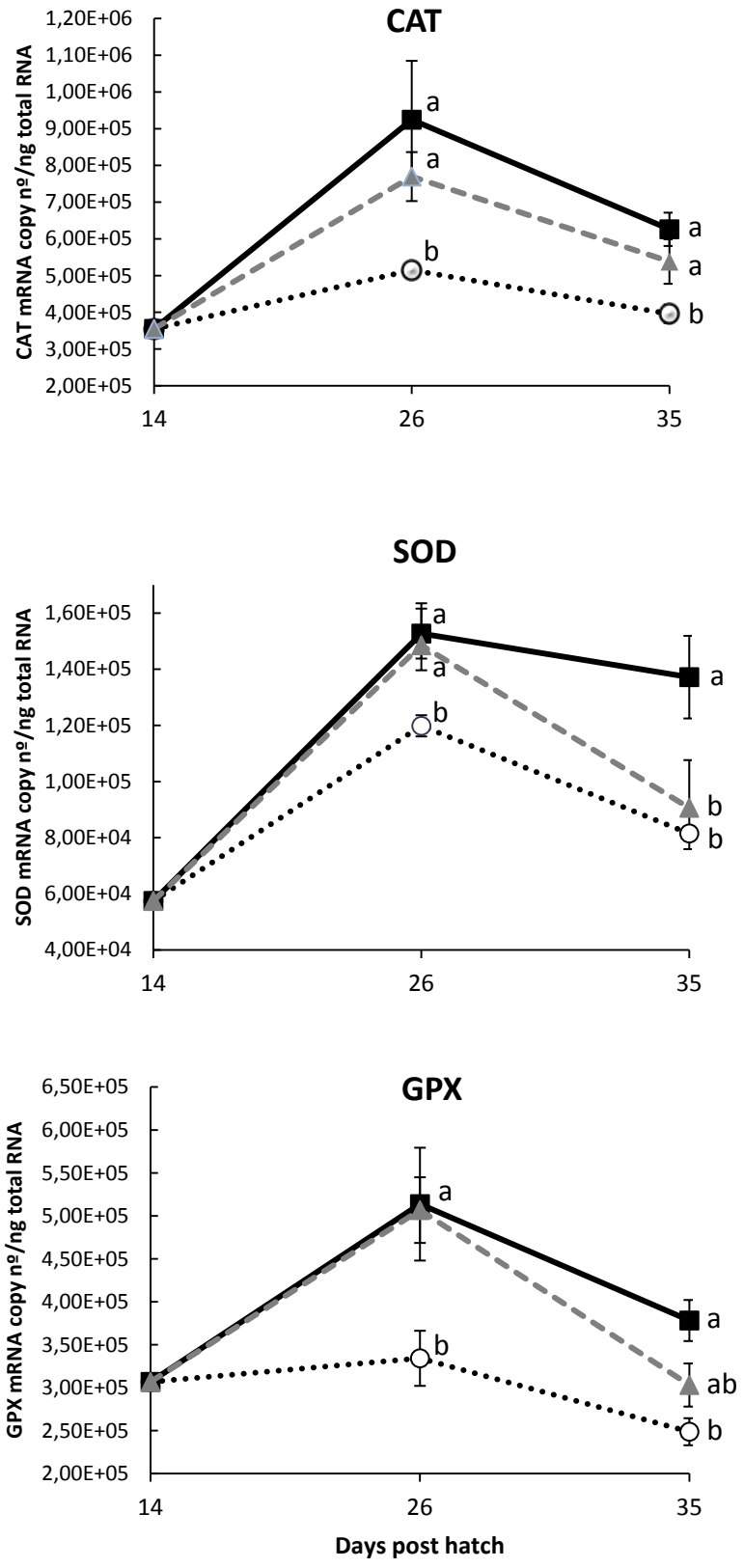

Figure 5.5 CAT, SOD and GPX expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/300 (■) or 5/300+Se (●). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA.

The SOD mRNA copy number was significantly higher (*P*=0.004) at 35 dph in 5/300 larvae (Figure 5.5B). GPX expression level was highest in larvae fed diets containing a high level of DHA compared to larvae fed low DHA levels. Nevertheless, larvae fed diets supplemented with selenium showed a lower number of GPX mRNA copies, comparable to larvae fed 1/150 diet (Figure 5.5C).

Regarding IGF genes, IGF-I mRNA copy number increased from 14 to 26 dph in all treatments, showing a decrease at day 35 in 1/150 and 5/300+Se larvae. In contrast, 5/300 larvae showed an increasing IGF-I expression levels throughout the experimental trial, with significantly higher levels at 35 dph (*P*=0.006; Figure 5.3D). No significant differences were observed among treatments taking into account the whole experimental period (Figure 5.6D; Table 6). The mRNA levels of IGF-II followed a similar increasing pattern in 5/300 and 5/300+Se larva from 14 to 26 dph, in contrast to 1/150 larvae. At 26 dph, 5/300+Se larvae showed a marked decrease in IGF-II expression, whereas in 5/300 larvae a steady increase could be observed. In larvae fed diet 1/150 a decrease in IGF-II expression could be observed at all sampling points (Figure 5.6B).

Myosin heavy chain expression levels were elevated in larvae fed the 5/300 diet, with a lower expression of this gene mRNA copy number was found 26 dph, when selenium was included in the diet (*P*=0.007). At 35 dph, no statistical differences were observed when Se was added to the 5/300 diet, with the lowest values in 1/150 larvae (Figure 5.73). GLM analysis showed significant differences between 5/300 larvae and larvae fed the other dietary treatments (*P*=0.001; Figure 5.7; Table 6).

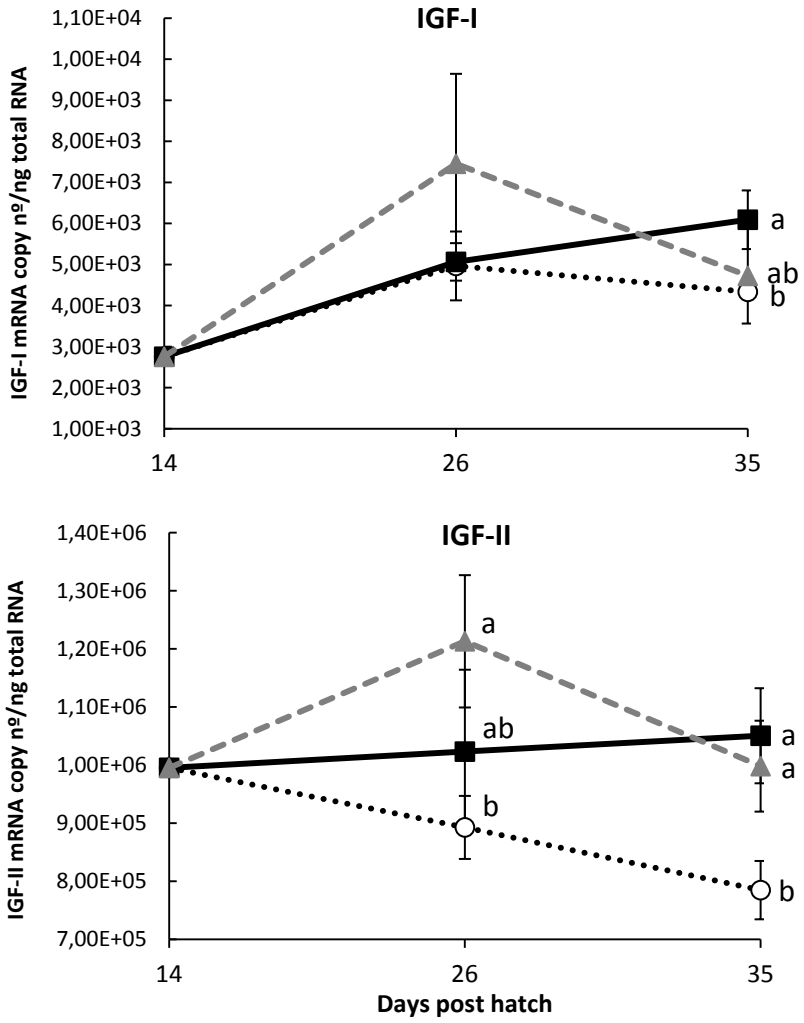

Figure 5.6 IGF-I and IGF-II expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/300 (■) or 5/300+Se (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA.

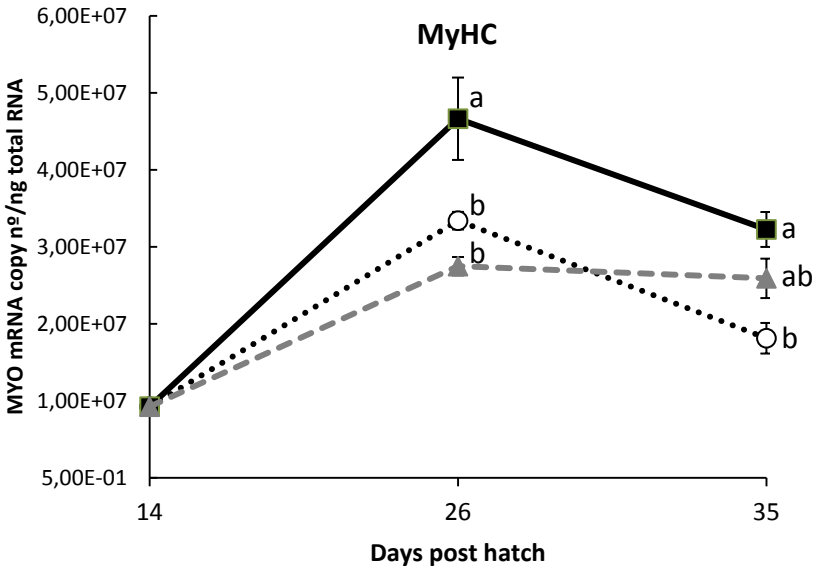

Figure 5.7 MyHC expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/300 (■) or 5/300+Se (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA.

Interactions were found in gene expression between dietary treatment and time within the experimental trial for SOD and IGF-I (Table 5.6), indicating that the increase in expression of these genes may be induced for the larval stage and the effect of the diet. However, IGF-I showed no differences in expression among treatments or during the whole experimental trial (Table 5.6).

Table 5.6 Effects of the dietary treatment, time and their interaction on the global gene expression

	DIET	TIME	D x T
CAT	**	**	n.s.
SOD	**	**	*
GPX	**	**	n.s.
IGF-I	n.s.	n.s.	*
IGF-II	**	*	n.s.
MyHC	**	**	n.s.

Asterisks indicate significant differences as **$P \leq 0.01$, *$P \leq 0.05$. n.s. indicates non-significant differences

6.4 Discussion

The aim of the present study was to evaluate the oxidative status of sea bass larvae when selenium and vitamin E were included in the diet, at high DHA levels inclusion. Previously, Chapters 3 and 4 provided evidence of the appearance of muscular dystrophy in sea bass larvae when fed high DHA, implying an excessive production of free radicals was present. Furthermore, the same authors showed that an increase of vitamin E alone could not prevent its adverse effects. Selenium and vitamin E have different but complementary biochemical functions which may allow these nutrients to interact physiologically (Combs and Scott, 1976).

Se contents in the 5/300+Se diet were adjusted according to the levels found in copepods (Hamre *et al*., 2008; van der Meeren *et al*., 2008), natural live prey of marine fish larvae, although they were higher than those recommended by National Research Council (NRC, 1993) for juveniles of other fish species. Since marine fish larvae have a rapid growth rate it is possible that they may well have a higher requirement than the juveniles which have been used in most of the requirement studies quoted by NRC. In addition, the Se level did not seem to be excessive, as larvae fed this diet did not show reduced growth in comparison to the larvae fed the same vitamin E and DHA contents. Reduced growth is one of the first symptoms occurring when excessive levels of Se are fed to fish (Hilton *et al*., 1980; Lin and Shiau, 2005; Jaramillo *et al*., 2009; Lin and Shiau, 2009; Penglase *et al*., 2010). Moreover, the Se source used in this work was derived from yeast, making it less likely to be toxic as Se toxicity is highly dependent on its speciation (Tinggi, 2003), with mineral Se being more toxic than organic Se (Hilton *et al*., 1980; Rider *et al*., 2010).

A dose-dependent effect of dietary vitamin E on larval tissues concentration was not observed in the present work, with the highest vitamin E content found in larvae fed the lowest level of vitamin E (150 mg 100 g^{-1} DW). These results are in contrast with previous reports where vitamin E concentrations in fish were linked to dietary input (Kiron *et al*., 2004; Puangkaew *et al*., 2005). It is also noteworthy that vitamin E levels were influenced by dietary DHA ratio; they were lower in larvae fed diets containing the higher amount of DHA (5/300 diets). This indicates that more vitamin E was being utilized as an antioxidant in larvae from groups 5/300 and 5/300+Se to protect tissue lipids from an increased oxidation risk. Consequently α-TOH was accumulated at lower amounts in larval tissues. These results match previous reports where vitamin E concentration in

juvenile or adult fish were lower when high contents of *n*-3 LC-PUFA were included in the diets (Puangkaew *et al*., 2005). In sea bass larvae fed 1/150 diet, which contained only 4.58% 20:5*n*-3, it was notable that this fatty acid was accumulated in larval tissues, denoting its importance for marine fish larvae. In addition, the low DHA retention rate observed in 5/300 larvae could probably be due to the high *in vivo* lipid oxidation induced by this fatty acid.

In agreement with this, a dietary DHA increase resulted in higher levels of MDA, whereas Se supplementation improved protection against peroxidation by decreasing TBARS values. Moreover, Se incorporation rate was very low in 5/300+Se larvae (42.26%) in contrast to 1/150 (100%) and 5/300 (83.46%) larvae suggesting that this mineral was being used at the active sites of the antioxidant enzyme GPX (Felton *et al*., 1996). The synergism between tocopherol and Se has previously been observed in trout and salmon using diets deficients in vitamin E, Se or both (Poston *et al*., 1976; Bell *et al*., 1985). In this study, tocopherol levels were high enough to avoid a deficiency in this nutrient, suggesting that vitamin E addition as the sole antioxidant is not sufficient enough to control lipid peroxidation when high levels of DHA are included in fish larval diets. Therefore, the role of tocopherol as an effective antioxidant depends on the extent of the oxidative stress in the fish and is thus related to the degree of unsaturation of dietary fatty acids

The availability of literature on the activities of AOE in fish are mainly focused on pollutant detoxification (Ji *et al*., 2011; Kim *et al*., 2010) or developmental aspects (Mourente *et al.*, 1999b; Otto and Moon, 1996; Peters and Livingstone, 1996). A few reports exist concerning the effect of dietary components on their activity and gene expression during early developmental stages of marine fish larvae (Tovar-Ramírez *et al*., 2010). The results from the present study demonstrate that there is an increase in expression of specific antioxidant genes in sea bass larvae exposed to oxidative stress in order to neutralize the generated ROS. Moreover, when sea bass larvae were exposed to high dietary DHA contents (5%), the induction of antioxidant enzyme genes coincided with increases in MDA levels. Accordingly, studies in Manchurian trout (*Brachymystax lenok*) larvae (Zhang *et al*., 2009), revealed that high dietary lipid levels produced elevated MDA levels, inducing an antioxidant response noticeable by an increase in the activity of AOE.

In the present research, an initial increase in the expression of each AOE was observed in all treatments at 26 dph, including the control group. Fernández-Díaz *et al*.

(2006) found that the administration of inert diets in *Solea senegalensis* larvae produced increased CAT and SOD activity compared to larvae fed with *Artemia*. Therefore, the observed initial increase in expression could be due to the use of inert food.

In an attempt to dismutate superoxide anions and to decompose hydrogen peroxide, increases in SOD, CAT and GPX expression were detected in fish larvae fed a high DHA and Se-free diet. Similarly, exposure to high DHA diets caused a significant increase in CAT and GPX in larvae fed Se-supplemented diets. Given that increases in SOD activity were less significant in Se-supplemented larvae, it can be concluded that H_2O_2 formation declined or that CAT activity was sufficient to remove H_2O_2. These results agree with Monteiro *et al*. (2009) who observed that Se supplementation had a protective effect against oxidative stress caused by methyl parathion in *Brycon cephalus*, as denoted by a decrease in CAT and SOD activity. In contrast with these authors, an increased level in GPX was not observed with Se supplementation, neither the decrease in GPX caused by methyl parathion. In contrast with these authors, Se supplementation did not increase GPX level, or decreased GPX caused by methyl parathion. In mammals, it is likely that maintaining the activity of known selenoproteins (SEP), including GPX is not the mechanism by which Se acts since it appears to be saturated at normal nutritional intakes. Thus, supranutritional levels of Se are required to reduce the incidence of human and animal diseases (Brown and Arthur, 2001). Therefore, it appears that other SEP could be implicated in tissue antioxidant defence mechanisms. Among all SEPs, SEPP seems to play an important role as an antioxidant defence in mammals (Burk *et al*., 1997) by associating with endothelial cells. Previous studies in zebrafish (*Danio rerio*) indicate that SEPP is utilized to a larger extent than in humans as it is encoded by two genes and has 17 selenocysteine residues, the largest number of selenocysteine residues found in any known protein (Tubajeva *et al*., 2000; Kryukov and Gladyshed, 2000). In consequence, an action of SEPP or any other SEP could be critical as an antioxidant defence against lipid hydroperoxides in sea bass larvae when Se requirements are covered. Thus, further studies are required to clarify the antioxidant mode of Se action in marine fish larvae.

As observed in our previous studies (Chapters 3 and 4), high dietary DHA levels caused pathological changes in sea bass larvae muscle. However, in the present study, inclusion of Se proved to be efficient in controlling the damage caused by ROS, reducing the incidence of muscle injury to almost half as compared to the 5/300 diet. Moreover, certain properties of muscle may render it especially susceptible to ROS injury (Jackson *et al*., 1993; Giulivi and Cadenas, 1994; Ostdal *et al*., 1997). For instance, muscle is

prone to oxidative injury as a result of increased electron flux due to its requirement and ability to undertake rapid and coordinated changes in energy supply and oxygen flux during contraction (Haycock *et al*., 1996). There is also a very high concentration of myoglobin in muscle, and it is known that such a heme – containing protein may confer greater sensitivity to free radical induced damage by conversion of hydrogen peroxide to a more reactive species (Ostdal *et al*., 1997). Furthermore, the requirement of skeletal muscle membranes for phospholipids containing large proportions of polyunsaturated fatty acids may render those membranes particularly susceptible to oxidative stress (Murphy and Kehrer, 1989). Finally, low Se accumulation in muscular tissues will make this tissue more susceptible to oxidative damage (Monteiro *et al*., 2009; Elia *et al*., 2011). However, interaction of LC-PUFA with other cellular components should be taken into account to complete the scenario. In this sense, studies using juvenile salmon fed with high EPA and DHA diets showed loss of the mitochondrial β-oxidation, reduced lipid deposition and apoptosis in white adipose tissue, indicating that high supplementation rates of these LC-PUFA may lead to oxidative stress (Todorčević *et al*., 2009).

IGF-I and IGF-II are polypeptides well known for promoting proliferation and differentiation in many vertebrates with nutritional status having a profound effect on the IGF system in fish (Duan, 1998). However, most nutritional studies have focused on the effects of food restriction (Moriyama *et al*., 1994; Terova *et al*., 2007; Hevrøy *et al.*, 2010), dietary protein or carbohydrate content (Pérez-Sánchez *et al*., 1995; Enes *et al*., 2010) and probiotics (Carnevali *et al*., 2006) whereas little information is known about the effect of lipids on this system. Moreover, no information exists about the effect of oxidative stress on IGFs in fish larvae. In the present study an increase in IGF-I and IGF-II in larvae fed the 5/300 diets was observed, especially when no Se was added, suggesting oxidative stress may play a role in the expression of these growth factors. Accordingly, larvae fed the highest content of DHA showed a higher incidence of muscular lesions and the presence of abundant satellite cells. Satellite cells are able to regenerate damaged muscle by forming new myofibres by fusing to existing muscle fibres or fusing together (Bischoff, 1994; Schultz and McCormick, 1994). It is known that to control the satellite cell population growth factors are required (Grounds, 1999; Seale and Rudnicki, 2000). In mammals, IGF-I appears to utilize multiple signalling pathways in the regulation of the satellite cell pool such as the mitogen-activated protein (MAP) or phosphatidylinositol-3-OH kinase (PI-3K) (Coolican *et al*., 1997; Semsarian *et al*., 1999). In agreement with this, Pozios *et al*., (2001) showed that IGF-II and IGF-I potently activate cell proliferation and DNA synthesis in embryonic zebrafish cells via MAP and PI-3K, suggesting that the increase of IGF-I mRNA copies observed in the present work

in larvae with the highest incidence of muscular lesions could be due to the regeneration process carried out by satellite cells. Similarly, the mitogenic effect of IGF in fish has also been described in cultured muscle cells from rainbow trout (Castillo *et al*., 2004).

Other of the known functions of the IGF-I is that of stimulating mature osteoblast function (Hughes *et al*., 2006), being IGF-I more potent in bone, although this might be different both between and within species (Rosen *et al*., 1997). Considering the results in the present study, IGFs expression was higher in diets containing a high level of DHA, especially when Se was not added to diet. Conversely, these larvae showed the lowest bone mineralization rate, indicating that the highest expression of this polypeptide did not favour minerals deposition. IGF-I has been found in different soft tissues in adult sea bass (Terova *et al*., 2007; Enes *et al*., 2010) and, as has been previously pointed out, it has a role in promoting proliferation and differentiation, as well as controlling satellite cell pool, thus, the elevated IGFs expression could be mainly addressed to regenerate muscle and not to potentiate bone growth. Our results diverge from those obtained by Villeneuve *et al*. (2006), who aimed that an excess amount of PUFA accelerated osteoblast differentiation in sea bass larvae. However, in that study the quantity of DHA employed was lower than ours (4.8% of EPA+DHA) and it was supplemented only as phospholipids. Therefore, the high deformities and low mineralization rate may be due to the direct effect of free radicals on bone cells. Lewis-McCrea and Lall (2007) found a reduction in bone formation, resulting in development of skeletal abnormalities in halibut fed oxidized dietary lipid. In this same report and in agreement with this study, vitamin E supplementation did not improve the frequency of abnormalities observed. Similarly, inclusion of selenium seemed to help to reduce the incidence of deformities in sea bass larvae, although only significant differences were found in skull deformities. Nevertheless, a clear protective effect of Se was observed in bone mineralization, not finding differences among 1/150 and 5/300+Se larvae. It is known that both delay and acceleration of the ossification process can induce malformations in sea bass larvae (Darias *et al*., 2010; Darias *et al*., 2011). In the present report, a negative effects between bone mineralization rate and deformities was noticed, suggesting that selenium had some protective effect on osteoblasts, promoting bone mineralization to rates equal to control larvae. Similarly, in domestic animals the combination of vitamins E and C with Se was more effective than combinations of single vitamins to prevent structural alterations in bones (Turan *et al*., 2003).

Late markers of myogenesis include the myofibrillar protein myosin heavy chain. By monitoring the expression patterns of this marker gene, the effect that nutritional

status has on muscle growth can be determined (Bower *et al*., 2008). Regeneration of fish muscle has only rarely been described. Rowlerson *et al*. (1997) demonstrated a vigorous regeneration in juvenile sea bream (*Sparus aurata*) after mechanical injury, with myosin expression in regenerating fibres resembling that seen in newly produced fibres in post-larval white muscle. In the present study, a higher expression of myosin was observed in 5/300 larvae, especially when Se was not added to diets with a positive correlation observed between the incidence of muscular lesions and myosin mRNA copies at 35 dph ($y=2E-06x-29.567$; $R^2=0.9133$).

In this study, the high levels of AOE and MDA content observed in 5/300 larvae demonstrate an adaptive response in attempting to neutralize the ROS generated. Moreover, a reactive response was observed by the increase in IGFs and MyHC expression in larval tissues, suggesting regenerative processes in injured muscle. Organic Se proved to enhance the cell antioxidant capacity, protecting muscle, as showed by the decrease in the incidence of muscular lesions, MDA content and AOE expression. Therefore, when high levels of LC-PUFA are included in sea bass larvae microdiets, an adequate combination of dietary α-TOH and Se must be included to avoid the appearance of oxidative stress in larval tissues and favour culture performance.

Chapter 6

Supplementation with vitamin C enhances the vitamin E status and reduces oxidative stress indicators in sea bass (*Dicentrarchus labrax)* larvae fed high-DHA microdiets

This manuscript has been submitted to *Lipids*(LIPIDS-12-0093)

ABSTRACT

Docosahexaenoic acid (DHA) is an essential fatty acid necessary for many biochemical, cellular and physiological functions in fish. However, high dietary levels of DHA increase free radical injury in sea bass larvae muscle, even when vitamin E (α-tocopherol, α-TOH) is increased. Therefore, the inclusion of other nutrients with complementary antioxidant functions, such as vitamin C (ascorbic acid, AA), could further contribute to prevent these lesions. The objective of the present study was to determine the effect of AA inclusion in high DHA and α-TOH microdiets (5/300 and 5/300+AA) in comparison to a control diet (1/150) on sea bass larvae growth, survival, biochemical composition, malonaldehyde (MDA) content, muscle morphology, skeletal deformities and antioxidant enzymes, insulin-like growth factors (IGFs) and myosin expression (MyHC). AA effectively controlled oxidative damages in muscle and reduced MDA content and the occurrence of skull deformities. IGFs gene expression was elevated in 5/300 larvae, suggesting an increased muscle mitogenesis that was confirmed by the increase in the mRNA copies of MyHC. The results of the present study showed the antioxidant synergism between vitamins E and C when high contents of DHA are included in sea bass larvae diets.

Keywords:

DHA
vitamin E
vitamin C
oxidative stress
muscle injury
antioxidant enzymes

7.1 Introduction

The importance of polyunsaturated fatty acids (PUFA) for marine fish larvae has been extensively studied during the last 20 years (Watanabe 1993, Watanabe and Kiron, 1994, Sargent *et al*., 1999, Izquierdo, 2005), especially in relation to eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3) (Watanabe, 1993; Takeuchi *et al*., 1996; Copeman *et al*., 2002; Rezek *et al*., 2010). The particular structure of DHA provides this fatty acid with many important functions in fish metabolism (Izquierdo, 2005), and has been demonstrated to be superior to EPA in promoting growth and conferring vitality to larvae (Watanabe *et al*., 1989; Watanabe, 1993), being preferentially incorporated into biomembranes (Rodríguez *et al*., 1997; Copeman *et al*., 2002). Due to their limited capacity to synthesize DHA and EPA from their precursors, fish require diets rich in these fatty acids (Koven *et al*., 2001). Nowadays, live prey substitution by compound diets is crucial for lowering costs and increasing quality of production to provide high and constant quality juveniles, using a formulation having high levels of DHA, similar to those of live prey (Koven *et al*., 2001). However, DHA is very susceptible to attack by reactive oxygen species (ROS) due to its high unsaturation degree (Nagaoka *et al*., 1990).

ROS are produced during normal cellular function (Matés, 2000), being beneficial or even indispensable at low concentrations in processes such as defence against microorganisms, contributing to phagocitic bactericidal activity. Fish possess enzyme systems and low-molecular-weight molecules with antioxidant functions capable of neutralizing ROS and protecting against their adverse effects (Machlin and Bendich, 1987). However, ROS generation can exceed its removal and oxidative stress can occur (Sies, 1985) with ROS attacking diverse cellular components.

One of the consequences of oxidative stress is the oxidative peroxidation of PUFA, known as lipid peroxidation. The high unsaturation content of these fatty acids renders them very susceptible to lipid oxidation. Thus, the high requirements of marine fish larvae for long chain PUFA (LC-PUFA), mainly DHA and EPA, makes them more prone to suffering peroxidative attack than are adults (Hamre *et al*., 2010). Therefore, the importance of nutrition in the pro-oxidant-anti-oxidant balance process may be highly critical for fish larvae, as their high LC-PUFA contents disposes larval tissues vulnerable

to oxidative stress and an increase in the content of antioxidant nutrients is essential. Among the antioxidant nutrients, vitamin E (tocopherols and trienols) is the major membrane-bound lipid-soluble antioxidant (Machlin and Bendich, 1987), whereas vitamin C (ascorbic acid, AA) is an important water-soluble antioxidant which protects low density lipoproteins from oxidation and is required for the correct formation of cartilage (Levine, 1986). AA is easily oxidized to the unstable dehydroascorbic acid (DHAA), which is not normally detectable in plasma but may develop transiently during oxidant stress (Padayatty and Levine, 2001). The presence of sparing mechanisms between both vitamins was first hypothesized by Tappel (1962). This hypothesis proposes that the oxidized α-tocopherol (α-TOH) is reduced by ascorbate, thereby regenerating α-TOH. In some fish species the presence of a vitamin C/E sparing mechanism has been suggested (Lovell *et al*., 1984; Sealey and Gatlin, 2002; Shiau and Hsu, 2002; Yildirim-Aksoy *et al*., 2008) reporting an influence on growth, tissue composition or immune responses. For instance, supplementation with 100 mg kg^{-1} of ascorbyl-2-polyphosphate to a α-TOH deficient diet in juvenile channel catfish (*Ictalurus punctatus*) decreased vertebral deformities and improved weight gain, feed intake and feed efficiency rate (Yildirim-Aksoy *et al*. 2008). High supplementation of ascorbate might also spare α-TOH in diets for hybrid tilapia (*Oreochromis niloticus* x *O. aureus)* as shown by the increased weight gain, feed efficiency and α-TOH concentrations. However, little is known about the effect of both vitamins in preventing oxidative stress in fish larvae, when high levels of LC-PUFA are administered.

The potentially deleterious effects of ROS are counteracted by a suite of antioxidant enzymes (AOE), including radical-scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD) or peroxidases such as glutathione peroxidase (GPX). In Manchurian trout larvae (*Brachymystax lenok*) high lipid content microdiets stimulated the activity of AOE, generally accompanied by an increase in malonaldehyde (MDA) contents (Zhang *et al*., 2009). In contrast, Mourente *et al.* (1999a) did not find a direct relationship between the activity of AOE and the level of dietary *n*-3 LC-PUFA in *Dentex dentex* larvae, but decreased α-TOH and increased MDA contents were found in larvae fed high *n*-3 LC-PUFA enriched *Artemia*. Furthermore, it seems that the level of antioxidant enzymes rises with larval development, whereas the level of antioxidant molecules falls (Rudneva, 1999). In mammals, ROS can induce changes in gene expression during normal development (Saal *et al*., 1990) causing defective embryo development and retardation of embryo growth (Agarwal *et al*., 2006). Similarly, oxidative stress causes embryonic mortality and developmental arrest in sea urchins (*Paracentrotus lividus* and *Spherechinus granularis*) larvae (Pagano *et al*., 2001).

Oxygen stress during early larval stages of fish may also alter development. Somatic growth in fish is regulated by insulin-like growth factors I and II (IGF-I and IGF-II), two single chain polypeptides that also have a function as myogenic regulatory factors which increase satellite cell proliferation and differentiation (Goldspink *et al*., 2001; Bower *et al*., 2008). In addition, IGFs stimulate cartilage growth by the incorporation of sulphate into cartilage and also affect cell differentiation, growth and proliferation (Duan 1997, 1998). Differences in myogenesis regulation, such as myosin isoform expression, have also been observed in the earliest stages and during temperature acclimation (Watabe, 2001; Johnston and Hall, 2004; Silva *et al*., 2008). However, no information is available about the effect of different dietary components on the regulation of the various components of the IGF signalling pathways, as well as their role on muscle growth in fish (Chapter 5).

In previous studies we have shown the alteration of sea bass (*Dicentrarchus labrax)* larvae oxidative status when they were fed high levels of DHA (5%), with α-TOH having a limited effect in preventing alterations such as muscular dystrophy or hepatic ceroidosis (Chapters 3 and 4). Therefore, the aim of the present study was to evaluate the combined effect of α-TOH and AA in preventing oxidative stress in sea bass larvae fed high levels of DHA in relation to larval performance or antioxidant status as well as the incidence of morphological alterations, including the expression of selected related genes.

7.2 Material and methods

7.2.1 Fish

The experiment was carried out at the *Instituto Canario de Ciencias Marinas* facilities (Telde, Canary Islands, Spain). Sea bass, *Dicentrarchus labrax,* larvae were obtained from a natural spawning from the *Instituto de Acuicultura de Torre de la Sal* (CSIC, Castellón, Spain). Prior to starting the feeding experiment, larvae were fed enriched yeast-fed rotifers (DHA Protein Selco®, INVE, Belgium) until they reached 14 days post hatching (dph). Then, larvae (total length 8.58 ± 0.64 mm, dry body weight 0.36 ± 0.00 mg) were randomly distributed in experimental tanks (n=9) at a density of 1000 larvae $tank^{-1}$ and were fed one of the experimental diets for 21 days, at a water temperature of 19.5 to 21.0ºC. All tanks (170 L light grey colour cylindrical fibreglass tanks) were supplied with filtered sea water (34 g L^{-1} salinity) at an increasing rate of 1.0 - 1.5 L min^{-1} along the feeding trial. Sea water entered the tank from bottom to top; water

quality was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min^{-1}), attaining 5-8 g L^{-1} dissolved O_2 and 60-80% saturation.

7.2.2 Diets

Three isonitrogenous and isolipidic experimental microdiets (pellet size < 250 µm) similar in their EPA content were prepared containing two levels of DHA, AA and α-TOH. A low oxidation risk diet (1/150) contained low DHA (1% DW) and α-TOH (150 mg 100 g^{-1}); a high oxidation risk diet contained the highest DHA (5% DW) and α-TOH (300 mg 100 g^{-1}) levels and a third diet contained increased AA (360 mg 100 g^{-1}). The protein source used was squid meal 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. EPA, DHA, α-TOH and AA sources used in the experimental diets were EPA500 and DHA500 (CRODA, East Yorkshire, England, UK), DL- α- Tocopheryl Acetate (Sigma-Aldrich, Madrid, Spain) and Rovimix Stay-C 35 (Roche, Paris, France). Oleic acid (Merck, Darmstadt, Germany) was added to equalize the lipid content in all diets (Table 6.1). Microdiets were prepared according to Liu *et al*. (2002) by first mixing the squid powder and water soluble components, followed by lipid- and fat- soluble vitamins and, finally, warm water dissolved gelatine. The paste was pelleted and oven dried at 38ºC 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 matter and manually supplied 14 times per day every 45 min from 9:00-19:00. Daily feed supplied was 2, 2.5 and 3 g/tank during the first, second and third week of feeding respectively. Each diet was tested in triplicates.

7.2.3 Growth and survival

Final survival was calculated by individually counting live larvae at the beginning and end of the experiment. Growth was determined by measuring dry body weight (105ºC for 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 larvae $tank^{-1}$ at the beginning, middle and end of the trial.

Table 6.1 Formulation of experimental diets

Dietary DHA/vitamin E	*1/150*	*5/300*	*5/300+AA*
Defatted squid powder (g $100g^{-1}$)*	69.00	68.85	68.32
EPA g $100g^{-1}$ (DW)†	2.80	1.80	1.80
DHA g $100g^{-1}$ (DW) †	0.20	6.70	6.70
Oleic acid (%)‡	10.00	4.50	4.50
Soy lecithin (g $100g^{-1}$)•	2.00	2.00	2.00
Gelatin (g $100g^{-1}$)	3.00	3.00	3.00
Attractants (g $100g^{-1}$)	3.00	3.00	3.00
Taurin (g $100g^{-1}$)	1.50	1.50	1.50
Vitamin premix§	6.00	6.00	6.00
Mineral premix**	2.50	2.50	2.50
Vitamin C g $100g^{-1}$ (DW) ††	0.18	0.18	0.36
Vitamin E g $100g^{-1}$ (DW) ‡‡	-	0.15	0.15

* Riber and Son, Bergen, Norway.
† Croda, East Yorkshire, UK.
‡Merck, Darmstadt, Germany.
•Acrofarma, Barcelona, Spain
§ Vitamin premix supplied per 100g diet: Cyanocobalamine, 0.030; Astaxanthin, 5.00; folic acid, 5.44; pyridoxine-HCI, 17.28; thiamine, 21.77; riboflavin, 72.53; Ca-pantothenate, 101.59; p-aminobenzoic acid, 145.00; nicotinic acid, 290.16; *myo*-inositol, 1450.9; retinol acetate, 0.180; ergocalcipherol, 3.650; menadione, 17.280; α-tocopheryl acetate, 150.000.
**Mineral premix supplied g per 100g diet: NaCl, 215.133; $MgSO_4 \cdot 7H_2O$, 677.545; $NaH_2PO_4 \cdot H_2O$, 381.453; K_2HPO_4, 758.949; $Ca(H_2PO_4) \cdot 2H_2O$, 671.610; $FeC_6H_5O_7$, 146.884; $C_3H_5O_3 \cdot 1/2Ca$, 1617.210; $Al_2(SO_4)_3 \cdot 6H_2O$, 0.693; $ZnSO_4 \cdot 7H_20$, 14.837; $CuSO_4 \cdot 5H_2O$, 1.247; $MnSO_4 \cdot H_2O$, 2.998; KI, 0.742; $CoSO_4 \cdot 7H_2O$, 10.706.
†† Rovimix Stay-C 35, Roche, Paris, France.
‡‡ DL-α-tocopheryl acetate, Sigma-Aldrich, Madrid, Spain.

7.2.4 Biochemical analysis

All remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition and TBARS analysis after 12 hours of starvation at the end of the trial. Moisture, protein (A.O.A.C., 1995) and lipid (Folch *et al.*, 1957) contents of the larvae and diets were analyzed.

7.2.4.1 Total lipid fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipids as described by Christie (1982). FAMEs were separated by GLC, quantified by FID (GC -

14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1992) and identified by comparison with previously characterized standards and GLC-MS.

7.2.4.2 Determination of vitamin E content

α-TOH concentrations were determined in diets and larvae samples using a high-pressure liquid chromatography (HPLC) with UV detection. Samples were weighed, homogenised in pyrogallol and saponified as described by McMurray *et al.* (1980) for diets or according to Cowey *et al.* (1981) for larvae. HPLC analysis was performed using a 150 x 4.60 mm, reverse-phase Luna 5µm C18 column (Phenomenox, California, USA). The mobile phase was 98% methanol pumped at 1.0 ml min^{-1}. The effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich, Madrid, Spain) as external standard.

7.2.4.3 Determination of vitamin C content

Ascorbil-2-monophosphate concentrations were determined in diets using a HPLC procedure with UV detection. The HPLC system comprised of a 150 x 4.6 mm, 5 µm particle size, Gemini C18 column fitted with a Gemini pre-column of the same material. The mobile phase consisting of phosphate buffer was delivered at a flow rate of 0.8 ml min^{-1}. Samples were dissolved with 0.4 M phosphate buffer (pH 3.0) and centrifuged at 1610 x *g* for 5 minutes at room temperature. The supernatants were kept at 4ºC until assayed. AA concentrations were determined at a wavelength of 293 nm and quantification achieved by comparison with tris(cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich, Madrid, Spain), used as a reference substance.

7.2.4.4 *Measurement of thiobarbituric acid reactive substances (TBARS)*

TBARS were measured in triplicate samples using a method adapted from that used by Burk *et al.* (1980). Approximately 20-30 mg of larval tissues per sample were homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol. To this 2.95 ml of freshly prepared 50mM thiobarbituric acid solution were added before mixing and heating for 10 minutes at 100ºC. After cooling, protein precipitates were removed by centrifugation (Sigma 4K15, Osterode am Harz, Germany) at 2000 x *g*, the supernatant was read in a spectrophotometer (Evolution 300, Thermo

Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA) expressed as µmol MDA per g of tissue was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

7.2.5 Histopathological sampling

Thirty larvae from each tank were collected every seventh day from the beginning of the feeding trial, and fixed in 10% buffered formalin for 1 or 2 days, dehydrated through graded alcohols, then xylene and finally embedded in paraffin wax. Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm and stained with Haematoxilin and Eosin (H&E) for histopathologic evaluation (Martoja and Martoja-Pearson, 1970).

Ten larvae per tank were fixed for 24 hours at 4ºC in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). Samples were then rinsed in phosphate buffer and post-fixed for 1 hour in 2% osmium tetraoxide in 0.2 M potassium ferrocyanide. Each larva was then embedded in an Eppon/Araldite resin block. Serial transverse and longitudinal larvae thick sections were cut at 1 µm, stained with toluidine blue and examined under light microscopy (Hoffman *et al*., 1983). Thin sections were cut at 50 nm and stained with lead citrate before observing with a ZEISS EM 910 transmission electron microscope (Germany) at the Electron Microscope Service of University of Las Palmas de Gran Canaria.

Besides, 10 larvae from each tank at 35 dph were fixed in 10% buffered formalin in order to perform deformity analyses. Prior to staining, larvae were measured under a Profile Projector (Mitutoyo, PJ 3000, Japan), divided into three size classes (< 10.0 mm, 10.0-12.0 mm and >12.0 mm) and stained with Alizarin red (Vandewalle *et al.* 1998) to determine bone mineralization. Larvae from the different experimental groups were stained simultaneously in order to prevent any technical variability. Deformities were classified in three different groups according to their localization: cranial deformities, lordosis, kyphosis, neural process alterations and others, including deformities such as vertebral compression or scoliosis. The surface corresponding to bone in whole coloured larvae was visualized and quantified using a computerized image analysis package (Image-Pro Plus®, Media Cybernetics, Maryland, USA). By selecting ranges of pixel values in colour images the pixels associated with red could be distinguished. The number of selected pixels was then quantified using a particle analysis operation and by

counting the area of all bright objects (in pixels). Larval size was estimated by calculating the surface areas (in pixels) covered by whole stained larvae.

7.2.6 RNA extraction and quantitative RT-PCR

Molecular biology analyses were carried out at the University of Insubria (Varese, Italy). Total RNA was extracted from sea bass larvae (≈200 mg; pool per tank), using PureYield RNA Midiprep System (Promega, Italy). The quantity and purity of RNA was assessed by spectrophotometer. Visualization on 1% agarose gel stained with ethidium bromide showed that RNA was not degraded. Three micrograms of total RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated at 65ºC for 5 minutes, chilled on ice and then 4 µl of 5X reverse transcription buffer, 2 µl 0.1M DTT, 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT) were added. After incubation at 37ºC for 50 minutes, the reaction was stopped by heating at 75ºC for 15 minutes.

PCR primers sequences used for the PCR amplification of the cDNAs of target genes were CAT, SOD, GPX, IGF-I, IGF-II and MyHC. To perform PCR, an aliquot of 4 µl of cDNA was amplified using 25 µl GoTaq Green Master Mix (Promega, Italy) in 50 µl of final volume and 50 pmol of each designed primer.

A total of 31 PCR amplification cycles (eight touchdown) were performed for all primer sets, using an automated Thermal Cycler (MyCycler, BioRad, Italy). An aliquot of each sample was then subject to electrophoresis on a 1% agarose gel in 1X TAE buffer (Bio-Rad, Italy) and bands were detected by ethidium bromide. Samples were run together with a 100 bp±1.5 kb DNA ladder to control molecular weight of the DNA. The negative control (a reaction mixture without cDNA), confirmed the absence of genomic contamination. The PCR products from each primer set amplification were then cloned using pGEM®-T easy vector (Promega, Italy) and subsequently sequenced in both directions (T7 and SP6).

TaqMan® real time reverse transcription PCR was performed on a StepOne Real Time PCR System (Applied Biosystems, Italy) using Assays-by-Design℠ PCR primers

(Applied Byosystems) and gene-specific fluorogenic probes. Primer sequences and TaqMan® probes of target genes were the following:

Target gene: Sea bass CAT

Forward primer: 5'- ATGGTGTGGGACTTCTGGAG - 3'

Reverse primer: 5'- GCTGAACAAGAAAGACACCTGATG - 3'

TaqMan® probe. 5'- CAGACACTCAGGCCTCA - 3'

Target gene: Sea bass SOD

Forward primer: 5'- TGGAGACCTGGGAGATGTAACTG - 3'

Reverse primer: 5'- TCTTGTCCGTGATGTCGATCTTG - 3'

TaqMan® probe. 5'- CAGGAGGAGATAACATTG - 3'

Target gene: Sea bass GPX

Forward primer: 5'- AGTTAATCCGGAATTCGTGAG - 3'

Reverse primer: 5'- AGCTTAGCTGTCAGGTCGTAAAAC - 3'

TaqMan® probe. 5'- AATGGCTGGAAACGTG - 3'

Target gene: Sea bass IGF-I

Forward primer: 5'- GCAGTTTGTGTGTGGAGAGAGA- 3'

Reverse primer: 5'- GACCGCCGTGCATTGG - 3'

TaqMan® probe. 5'- CTGTAGGTTTACTGAAATAAAA - 3'

Target gene: Sea bass IGF-II

Forward primer: 5'- TGCAGAGACGCTGTGTGG - 3'

Reverse primer: 5'- GCCTA CTGAAATAGAAGCCTCTGT - 3'

TaqMan® probe. 5'- CAAACTGCAGCGCATCC - 3'

Target gene: Sea bass MyHC

Forward primer: 5'- TGGAGAAGATGTGCCGTACTCT - 3'

Reverse primer: 5'- CGTGTCATTGATTTGACGGACATTT - 3'

TaqMan® probe. 5'- AACTGAGTGAACTGAAGACC - 3'

Data from TaqMan® PCR runs were collected with ABI´s Sequence Detector Program. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA. To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

7.2.7 Statistical analysis

Survival, growth and molecular biology data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation. Chi-squared test was employed for incidence of muscular lesions, deformities and TBARS content. Survival, growth, ossification degree and molecular biology data were treated by one-way ANOVA. Means were compared by Duncan's test (P<0.05) using SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005). For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = m + D_i + e_{ij}$$

where Y_{ij} is the mean value of the tank, m is the mean population, D_i is the fixed effect of the diet and e_{ij} is the residual error.

7.3 Results

The diet containing about 1% DHA (diet 1/150) showed higher amounts of monoenoic fatty acids than diets containing 5% DHA (5/300 and 5/300+AA) due to the higher oleic acid content in the former diet (Table 6.2). DHA contents in the diets varied from 4.58% in 1/150 diet, 24.55% in 5/300 diet and 27.54% in 5/300+AA diet. Elevation of dietary DHA (5/300 diets) increased *n*-3 and *n*-3 LC-PUFA fatty acids contents, as well as *n*-3/*n*-6 ratio. α-TOH levels were more than 2 times higher in diets containing 300 mg 100 g^{-1} compared to the control diet (1/150) (Table 6.3). AA contents were also higher in the diet supplemented with AA than in the others (Table 6.3).

Table 6.2 Main fatty acids (% total of fatty acids) of the experimental diets fed to European sea bass for three weeks.

	Diet		
	1/150	5/300	5/300+AA
14:0	1.54	1.26	0.78
14:1*n*-7	0.15	0.25	0.06
14:1*n*-5	0.22	0.35	0.09
15:0	0.28	0.43	0.15
16:0ISO	0.14	0.23	0.07
16:0	7.86	5.59	5.08
16:1*n*-7	3.59	2.26	2.01
16:1*n*-5	0.19	0.23	0.11
16:2*n*-6	n.d.	0.17	0.06
16:2*n*-4	0.32	0.39	0.26
17:0	1.21	0.82	0.66
16:3*n*-3	0.08	0.12	0.07
16:4*n*-3	0.09	0.13	0.10
18:0	1.29	2.29	2.18
18:1*n*-9+*n*-7	55.70	31.12	30.33
18:1*n*-5	0.72	0.46	0.36
18:2*n*-9	0.25	0.13	0.02
18:2*n*-6	7.40	6.99	6.71
18:2*n*-4	0.46	0.28	0.23
18:3*n*-6	0.11	0.11	0.10
18:3*n*-4	0.13	0.10	0.09
18:3*n*-3	0.72	0.83	0.82
18:4*n*-3	0.83	0.94	0.98
18:4*n*-1	0.08	0.08	0.09
20:0	0.10	0.31	0.30
20:1*n*-9+*n*-7	1.10	1.53	1.50
20:1*n*-5	0.05	0.12	0.11
20:2*n*-9	0.05	0.04	0.04
20:2*n*-6	0.09	0.21	0.21
20:3*n*-6	0.09	0.13	0.14
20:4*n*-6	0.71	1.57	1.62
20:3*n*-3	0.07	0.18	0.18
20:4*n*-3	0.32	0.52	0.54
20:5*n*-3	8.66	11.04	12.08
22:1*n*-11	0.17	0.51	0.53
22:1*n*-9	0.08	0.25	0.24
22:4*n*-6	0.02	0.19	0.20
22:5*n*-6	0.19	1.75	1.86
22:5*n*-3	0.32	1.29	1.42
22:6*n*-3	4.58	24.55	27.54
Saturated	12.28	10.70	9.16
Monoenoics	61.99	37.23	35.34
n-3	15.68	39.61	43.73
n-6	8.61	11.14	10.90
n-9	57.19	33.10	32.17
n-3 LC-PUFA	13.96	37.58	41.76
n-3/*n*-6	1.82	3.56	4.01

Table 6.3 Gross composition, α-tocopherol and ascorbic acid content in experimental diets fed to sea bass larvae.

	Diets		
	1/150	5/300	5/300+AA
Protein (%)	66.79±0.52	67.77±0.09	65.504±0.50
Ash (%)	4.49±0.11	4.76±0.12	4.86±0.07
Moisture (%)	10.31±0.46	9.99±0.28	9.48±0.11
Lipids (% DW)	14.98±0.31	15.80±0.02	15.94±1.05
α-tocopherol (µg/g DW)	1410.12±38.77	3033.01±43.33	3179.72.±75.69
Ascorbic acid (mg/100g DW)	149.59±5.54	147.74±3.29	299.86±7.46

Data are means ± SD.

All experimental diets were well accepted by larvae. The highest total length was found in 1/150 larvae (Table 6.4). Increasing DHA from 1 to 5% in 5/300 larvae, significantly reduced larvae total length (Table 6.4), despite the α-TOH increase. However, AA levels in 5/300+AA diet enhanced larval total length compared to 5/300 larvae (*P*=0.005). Sea bass larvae survival or dry weight was not significantly different among the treatments (Table 6.4).

Table 6.4 Sea bass larvae performance and levels of lipid peroxidation products (TBARS) and vitamin E (α-tocopherol) content of sea bass larvae at the beginning and after eating the experimental diets for three weeks.

		Diets		
	Initial	1/150	5/300	5/300+AA
Results of dietary trial				
Larval total length (mm)	8.58 ± 0.64	12.60 ± 0.93^{a}	10.89 ± 1.24^{c}	11.24 ± 1.08^{b}
Larval dry weight (mg)	0.36 ± 0.00	1.33 ± 0.46	0.94 ± 0.05	1.01 ± 0.07
Survival (%)	-	60.51 ± 9.10	48.42 ± 4.00	47.43 ± 10.50
Incidence muscular lesions (%)	-	17.5 ± 14.14^{b}	52.63 ± 15.93^{a}	20.7 ±15.62^{b}
Vitamin E (α-tocopherol)				
µg/g live mass	107.92±37.78	524.11±10.30^{ab}	463.62±82.24^{b}	646.38±23.03^{a}
µg/g dry mass	111.45±43.26	630.24±12.39^{b}	542.10±80.51^{b}	757.123±44.55^{a}
MDA				
nMol/g live mass	54.88±0.53	126.57±2.68^{c}	2062.57±192.14^{a}	659.77±87.26^{b}
nMol/g dry mass	62.85±0.61	166.62±25.08^{c}	2402.15±67.91^{a}	846.87±94.74^{b}

Data are means ± SD.

The level of lipid peroxides, as indicated by MDA content (μmol g^{-1} larval tissues) was lowest in larvae fed diet 1/150 and was significantly higher in larvae fed the higher DHA content (5/300 and 5/300+AA diets). However, the inclusion of AA efficiently prevented the formation of hydroperoxides, observed by a decrease in MDA levels (Table 6.4). Thus, in 1/150 larvae, 44.68% of the α-TOH present in the diet was preserved, in contrast to larvae fed 5/300 (17.87%). However, an increase in dietary AA contents increased α-TOH levels in larvae fed 5/300+AA diet (Table 6.4). Regarding larval α-TOH contents, the elevation of dietary vitamin E in diet 5/300, together with the increase in DHA, in comparison to diet 1/150, did not significantly affect the α-TOH contents in the larvae (Table 6.4).

Fatty acid composition of the larvae (Table 6.5) generally reflected the fatty acid composition of the diet. Accordingly, a higher content of 18:1*n*-9 was observed in 1/150 larvae. However, its retention rate regarding dietary levels was much lower in 1/150 larvae (47.30%) than in 5/300 (67.22%) or 5/300+AA larvae (67.82%), balancing the monoenoic acids content among larvae fed the different dietary treatments. Equally, EPA retention was low in all larvae, especially in those fed 5/300 (43.93%) and 5/300+AA (43.96%) diets. The highest content of total *n*-3 LC-PUFA (P=0.006) was observed in larvae fed the diet supplemented with AA probably due to a higher dietary content. However, regarding 22:6*n*-3 content, the highest retention rate was observed in 1/150 larvae. Similarly 20:4*n*-6 was highly retained in larvae fed 1/150 diet, although higher contents were found in larvae fed diets 5/300. In contrast to the differences in the *n*-3/*n*-6 ratio observed in diets, no differences were observed in larvae among the different treatments.

Histopathological examinations revealed the presence of lesions affecting larvae axial musculature, showing the typical features of necrotic degeneration of muscle. The incidence of muscular lesions increased with DHA dietary content (5/300 diet; Table 6.4). However, inclusion of AA (5/300+AA diet) proved to be effective in reducing incidences to less than half of those in 5/300 larvae. More detailed features of muscular lesions could be observed on semithin and TEM sections, where muscle degeneration with the presence of hydropic vacuoles and organelles swelling within some of the affected fibres was found in larvae fed 5/300 diet. Some fibres presented disarrangement of the myofilaments just like myelin figures, denoting intracellular lipid oxidation (Figure 6.1).

Table 6.5 Main fatty acid composition of total lipids from sea bass larvae after three weeks of feeding the experimental diets (% total fatty acid).

	1/150	5/300	5/300+AA
14:0	0.93±0.06	0.76±0.03	0.51±0.28
14:1*n*-7	0.08±0.01	0.08±0.03	0.34±0.37
14:1*n*-5	0.07±0.02	0.04±0.01	0.34±0.43
15:0	0.63±0.29	0.98±1.22	0.38±0.26
15:1*n*-5	0.12±0.10	0.09±0.06	0.79±0.23
16:0ISO	n.d.	0.11±0.01	0.28±0.25
16:0	17.51±2.44	17.61±0.25	16.32±0.26
16:1*n*-7	2.02±0.14	1.60±0.06	1.63±0.16
16:1*n*-5	0.25±0.01	0.25±0.01	0.40±0.18
16:2*n*-6	0.30±0.00	0.36±0.04	0.46±0.15
16:2*n*-4	0.93±0.40	0.96±0.15	0.89±0.12
17:0	0.91±0.10	0.80±0.03	0.81±0.08
16:3*n*-3	0.12±0.01	0.14±0.03	0.09±0.03
16:3*n*-1	0.10±0.02	0.54±0.10	0.54±0.09
16:4*n*-3	0.62±0.35	0.44±0.10	0.48±0.01
18:0	11.66±3.41	12.29±0.36	11.07±1.04
18:1*n*-9	26.35±4.87[a]	20.92±0.51[b]	20.57±0.13[b]
18:1*n*-7	4.85±0.29	4.44±0.39	3.99±0.23
18:1*n*-5	0.62±0.24	0.49±0.07	0.45±0.12
18:2*n*-6	4.23±0.08	3.90±0.17	3.85±0.14
18:2*n*-4	0.04±0.05	0.06±0.01	0.07±0.00
18:3*n*-6	0.43±0.01	0.38±0.04	0.33±0.01
18:3*n*-4	0.07±0.04	0.06±0.02	0.06±0.02
18:3*n*-3	0.32±0.05	0.44±0.04	0.46±0.05
18:4*n*-3	0.29±0.06	0.29±0.13	0.29±0.01
20:0	0.38±0.19	0.47±0.01	0.48±0.04
20:1*n*-9+*n*-7	1.83±0.0	1.77±0.06	1.80±0.02
20:1*n*-5	0.26±0.15	0.13±0.01	0.13±0.02
20:2*n*-6	0.50±0.13	0.65±0.08	0.60±0.07
20:3*n*-6	0.08±0.01	0.08±0.00	0.08±0.01
20:4*n*-6	2.38±0.04[b]	3.07±0.22[a]	3.13±0.24[a]
20:3*n*-3	0.12±0.09	0.15±0.04	0.13±0.00
20:4*n*-3	0.14±0.00	0.16±0.01	0.17±0.01
20:5*n*-3	5.91±1.18	4.85±0.20	5.31±0.33
22:1*n*-11	0.46±0.30	0.19±0.08	0.26±0.02
22:1*n*-9	0.26±0.15	0.26±0.06	0.27±0.05
22:5*n*-6	1.09±0.09	1.26±0.07	1.33±0.05
22:5*n*-3	0.64±0.26	0.57±0.06	0.64±0.08
22:6*n*-3	12.79±0.37[b]	18.04±1.19[a]	20.26±0.25[a]
Saturated	32.01±6.29	32.91±1.54	29.56±1.20
Monoenoics	36.73±4.51[a]	30.20±0.55[b]	30.56±1.44[b]
n-3	20.94±2.23[b]	25.11±1.43[a]	27.83±0.57[a]
n-6	8.45±0.97	9.80±0.47	9.91±0.09
n-9	28.44±4.72[a]	23.08±0.39[b]	22.76±0.05[b]
n-3 LC-PUFA	12.92±7.45[c]	23.78±1.42[b]	26.51±0.67[a]
n-3/*n*-6	2.51±0.55	2.56±0.05	2.81±0.03
EPA/DHA	0.33±0.07	1.16±0.10	1.11±0.17
ARA/DHA	0.18±0.01	0.88±0.07	0.80±0.04
ARA/EPA	0.54±0.04	2.56±0.05	3.00±0.33

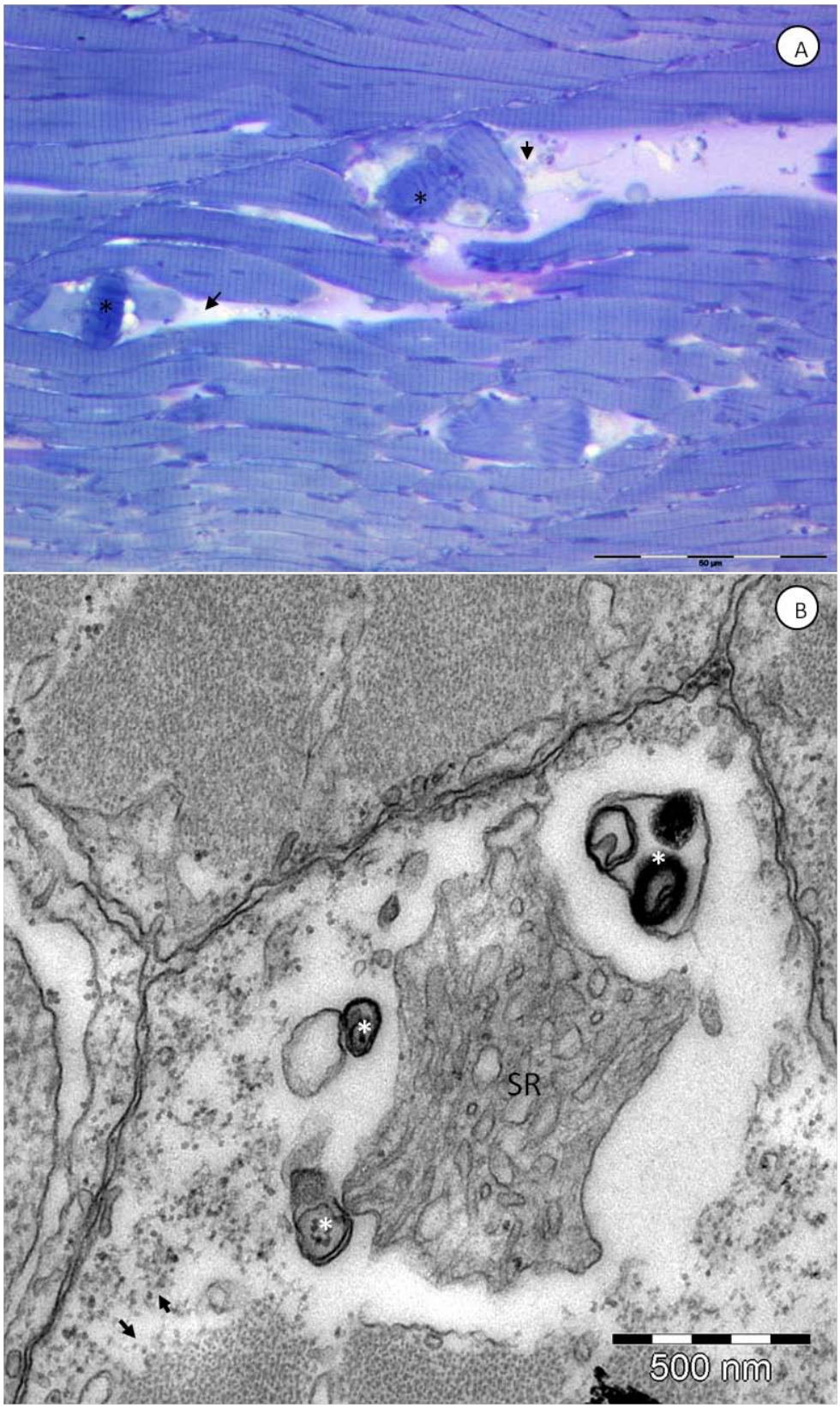

Figure 6.1 Longitudinal semithin (A) and transversal electro micrographs (B) of sea bass larvae fed 5/300 diet. (**A**) Damaged muscle fibres showing breakage (arrow) as well as darkening due to protein coagulation (*). (**B**) Affected fibre showing disarrangement of the myofilaments (arrows), swollen sarcoplasmic reticulum (SR) and myelin figures (*).

Regarding skeletal morphology, among larvae measuring 10-12mm, 28.33±1.3% of the larvae fed 1/150 diet showed skeletal deformities, and similar values were found in those fed the 5/300+AA diet (29.67±6.5%; Figure 6.2). Larvae fed the 5/300 diet presented 33.11±5.1% deformities, but no statistical differences were detected between groups (*P*=0.10; Figure 2). The ossification degree of 35 dph sea bass larvae was determined in terms of surface of mineralized bones per larval surface (Figure 6.2). High dietary levels of DHA decreased the formation of mineralized bone in larvae, whereas increase in AA did not affect mineralization.

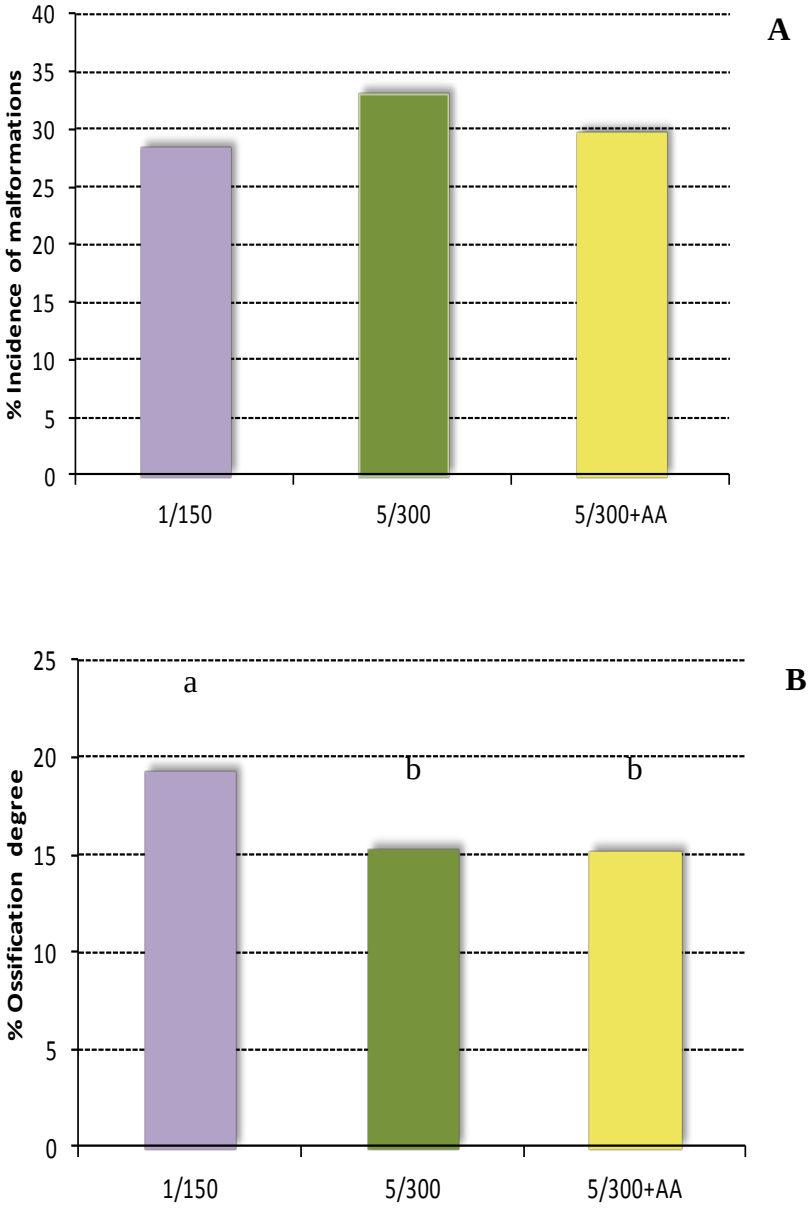

Figure 6.2 Incidence of malformations (%) (**A**) and ossification degree rate (%) (**B**) at 35 dph in sea bass fed the experimental diets.

Different types of deformities were observed at the end of the experimental period, depending on the level of dietary DHA. All of the experimental groups exhibited a statistically similar percentage of kyphosis, however, no lordosis was observed in larvae fed the 1/150 diet (Figure 6.3). Skull deformities were especially high in fish fed high DHA levels, although AA increase reduced the incidence of this deformity (*P*=0.013).

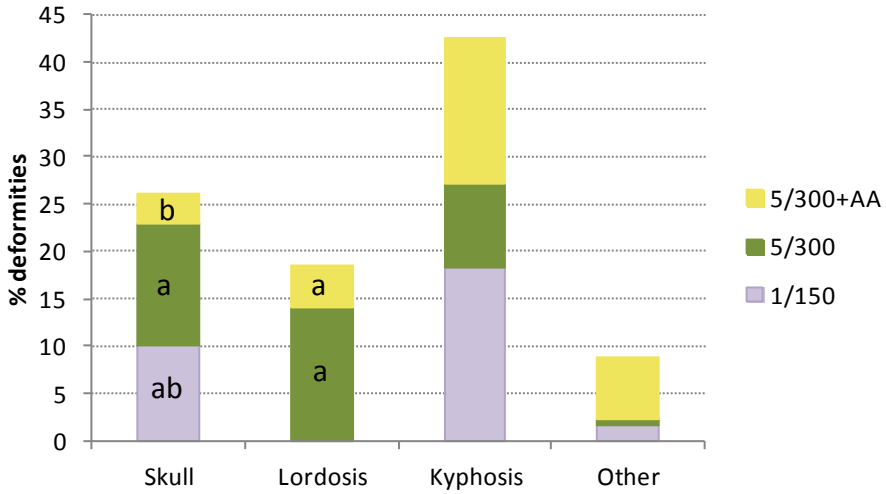

Figure 6.3 Skeletal deformities found in 35 dph larvae at the end of the experimental period. Different superscript letters mean significant differences for the same type of deformity among treatments.

CAT expression was elevated in larvae fed diets containing a high content of DHA, the highest number of mRNA copies being found in larvae fed diet 5/300+AA (*P*=0.027; Figure 6.4A). The SOD mRNA expression was also highest in 5/300 groups (*P*=0.048; Figure 6.4B). Accordingly, GPX expression was quite strong in larvae fed diets containing a high level of DHA compared to larvae fed low levels (*P*=0.039; Figure 6.4C).

The IGF-I gene expression increased from 14 to 26 dph in all treatments, at day 35 showing a decrease in 1/150 and 5/300+AA larvae and a continuous increase in 5/300 fed larvae (Figure 6.5A). Regarding IGF-II, expression was higher in larvae fed 5/300 and 5/300+AA diets than in those fed diet 1/150 throughout the trial (Figure 6.5B). MyHC expression was low in larvae fed diet 1/150 and significantly increased by the elevation of both α-TOH and DHA in diet 5/300 (Figure 6.5C). However, increase in AA in diet 5/300+AA significantly reduced MyHC expression to levels similar to those of 1/150 diet.

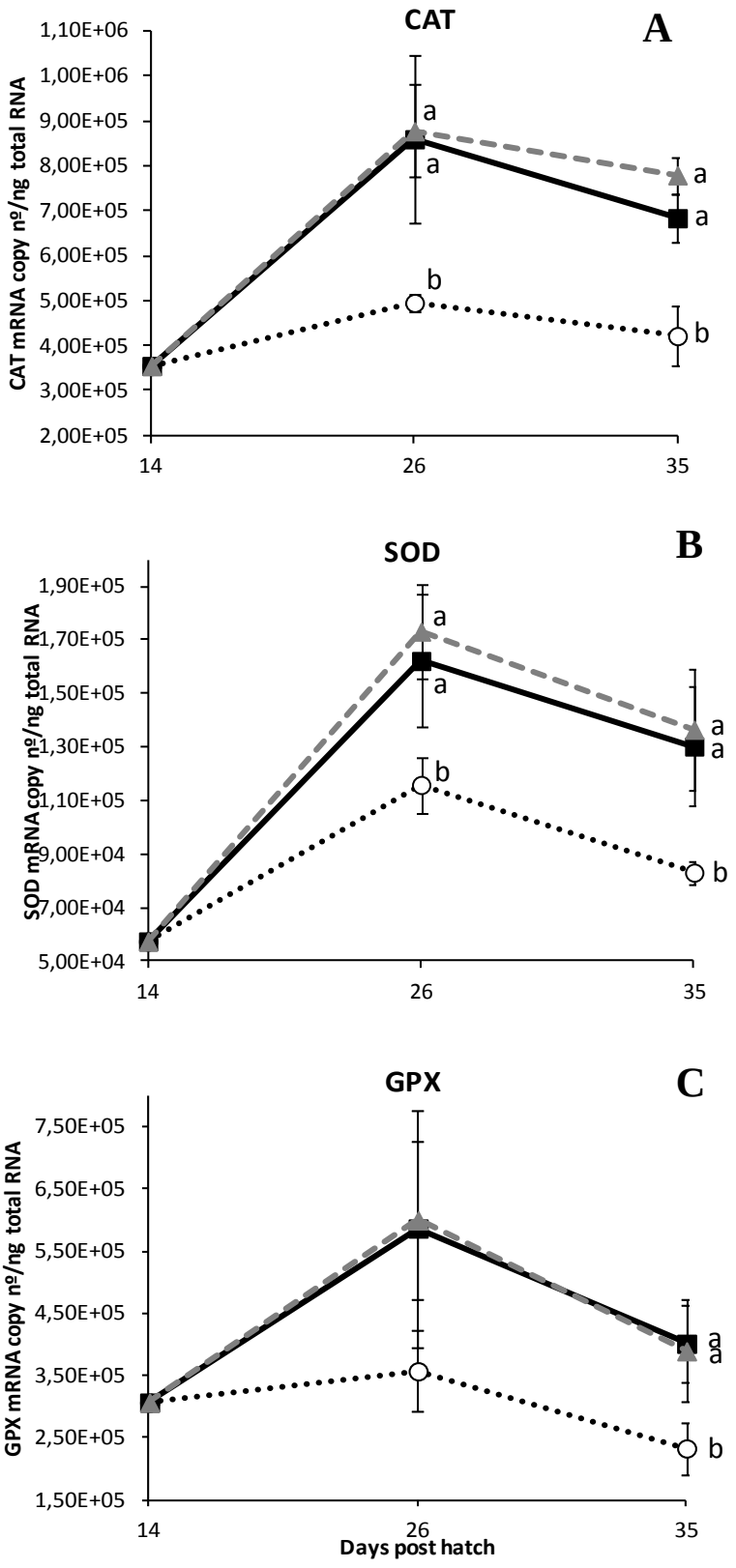

Figure 6.4 CAT, SOD and GPX expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/300 (■) or 5/300+AA (▲). Mrna copy number of each gene was normalized as a ratio to 100 ng total RNA.

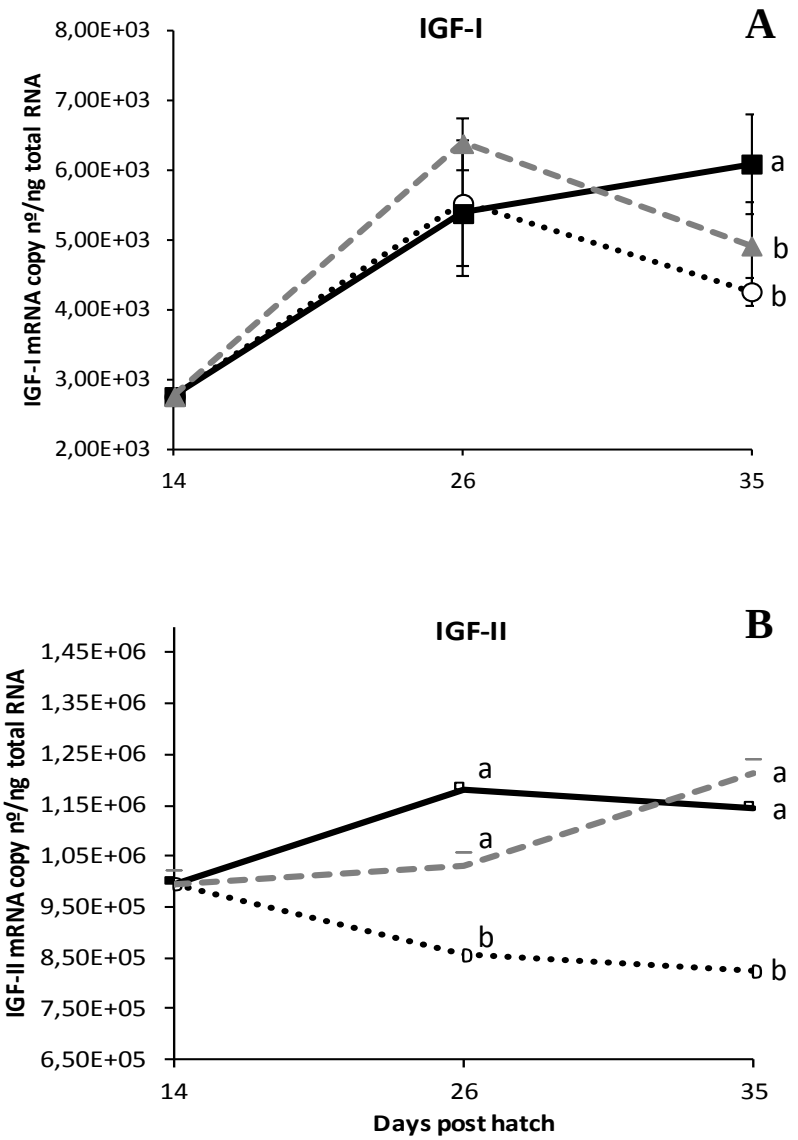

Figure 6.5 IGF-I and IGF-II expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/300 (■) or 5/300+AA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA.

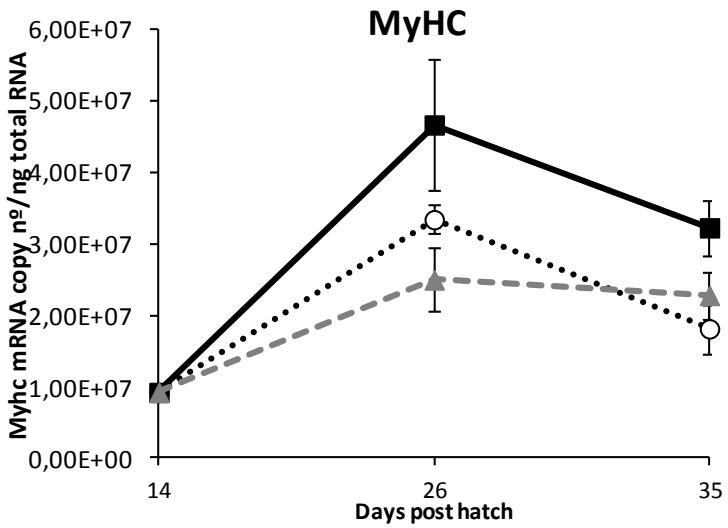

Figure 6.6 MyHC expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/300 (■) or 5/300+AA (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA.

7.4 Discussion

Marine fish larvae are subjected to high levels of oxidative stress when using inert diets due to the high content of LC-PUFA, particularly DHA, and pro-oxidants such as minerals as well as the high surface to volume ratio of these feed particles (Hamre, 2011). Therefore, inclusion of high dietary levels of LC-PUFA to match the high requirements of marine fish larvae may call for increased dietary supplementation with antioxidants such as α-TOH to prevent oxidative damage. For instance, increasing DHA by up to 5% in diets for sea bass markedly reduced larval survival and growth and increased the incidence of muscular lesions (Chapter 3). Despite an increase in dietary α-TOH from 150 to 300 mg 100 g^{-1} which partially reduced the occurrence of muscular alterations at DHA dietary levels up to 3% (Chapter 3), lesions caused by the further elevation of DHA up to 5% could not be prevented by the increase in dietary α-TOH (300 mg 100 g^{-1}) (Chapter 4). Similarly, in the present study high levels of α-TOH (300 mg 100 g^{-1}) together with high DHA (5%) were not able to counteract the adverse effects of ROS on the incidence of muscular lesions. Accordingly, these larvae showed very high levels of MDA indicating that their oxidative status is altered when they are fed high DHA levels even at such high dietary α-TOH levels, in agreement with our previous studies (Chapter 4). Moreover, AOE expression was higher in those larvae, denoting a high antioxidant response. A compensatory induction of these endogenous antioxidants is found in

animals exposed to dietary oxidative stress (Blomhoff, 2005). Indeed, α-TOH contents in these larvae were not increased by the elevation of dietary α-TOH levels, suggesting a depletion of this vitamin to neutralize ROS and its limited capacity to prevent lipid peroxidation under these conditions. Similarly, increased *n*-3 LC-PUFA did cause depletion of α-TOH contents when this vitamin was supplemented to the diet in previous studies (Hamre and Lie, 1995b; Kolkovski *et al*., 2000b).

In contrast, the increase in AA dietary supplementation from 180 to 360 mg 100 g^{-1}, markedly improved the protection against peroxidation, decreasing MDA contents to less than one third, sparing vitamin E that was significantly increased in larval tissues and effectively reducing the incidence of muscular lesions. AA supplementation has also been found to reduce TBARS formation also in hybrid striped bass (*Morone chrysops* ♀ x *M. saxatilis* ♂) fed diets deficient in α-TOH (Sealey and Gatlin, 2002). The increased α-TOH content found in the present study when larvae were fed high levels of AA, is in agreement with studies in other fish species (Shiau and Shu, 2002) and denotes the sparing effect of AA on α-TOH in sea bass larvae, the first vitamin recycling the second one. Thus, under dietary conditions of high LC-PUFA and α-TOH, AA dietary contents of 180 mg 100 g^{-1} may not be sufficient to recycle α-TOH and prevent the high rate of ROS formation and, therefore, AA requirements may be higher than under low oxidation risk dietary situations.

AA is known to be a powerful antioxidant, by efficiently trapping peroxyl radicals in the aqueous phase before they can initiate lipid peroxidation, thereby protecting the biomembranes (Sies *et al*., 1992). Thus, the decrease in the incidence of muscular lesions observed in the present study, when high levels of AA were employed, could be due to the protective effect of this antioxidant nutrient, quenching free radicals before they can attack muscular membranes, in addition to recycling α-TOH. Moreover, the species formed after the loss of one electron are relatively stable and fairly unreactive compared to tocopheroxyl radical (Padayatti *et al*., 2003), thus preventing a pro-oxidant action. The reduced incidence of muscular lesions was also accompanied by a decrease in IGF-I expression, the highest IGF mRNA copies occurring in larvae fed the highest DHA and α-TOH levels, which also showed the lowest growth and the highest incidence of muscular lesions. These results are in line with the higher expression of IGF-I found in sea bream larvae fed high DHA levels (Izquierdo *et al*., submitted). On the contrary to the present study, in sea bream, high IGF-I expression was correlated with high growth (Izquierdo *et al*., submitted), suggesting the negative effect of high DHA contents in sea bass growth in relation to the altered oxidative status. A feasible explanation for the

overexpression of IGF-I in larval groups with higher MDA values could be a compensatory mechanism in fish larvae to try to counteract the adverse effects of ROS since IGF-I interferes with activation of apoptosis in several cells and organ systems in mammals (Li *et al*., 1997). For instance, an increase in IGF-I has been found to suppress oxidative stress in atherosclerotic Apo-E deficient mice (Sukhanov *et al*., 2007). Furthermore, the IGF system can promote muscle growth and differentiation in fish, by activating cell proliferation and DNA synthesis. Thus, the increase in mRNA copies of IGF-I observed in larvae fed 5% DHA and 300 mg 100 g^{-1} α-TOH could be also due to the compensatory regeneration process carried out by satellite cells, and which was not directly reflected on a growth improvement. This is supported by the results of MyHC expression, as a high expression of myosin has been associated with regeneration processes in sea bream after mechanical injury (Rowlerson *et al*., 1997). In addition, in the present study, IGF-I and MyHC expression follows a similar pattern, indicating that their biological functions may be interrelated. In this sense, it is known that IGF-I overexpression results in greater skeletal muscle mass in fine flounder (*Paralichthys adspersus*, Fuentes et al., 2011) and in mice, in which IGF-I can activate MyHC as well as other transcriptional factors (Shanely *et al*., 2009). Thus the parallel increase in IGF-I and MyHC expression observed in the present study confirms that when sea bass larvae are subjected to oxidative stress, a compensatory overexpression of genes related to cell/muscle proliferation occurs.

In the present study, AA elevation did not reduce the expression of AOE, suggesting an antioxidant effect independently of these enzymes, but acting in parallel with them to quench ROS. AA acts as a cofactor for at least eight enzymes involved in the biosynthesis of collagen and carnitine, conversion of the neurotransmitter dopamine to noradrenaline, metabolism of tyrosine and amidation of peptide hormones. In this sense, AA acts with peptidyl-glycine alpha-amidating monooxygenase (PAM), an enzyme that adds amide groups to peptide hormones, greatly increasing their stability (Eipper *et al*., 1992; 1993). Thus, the antioxidant role of AA in fish might not only be reduced to trapping peroxyl radicals from the aqueous phase or recycling α-TOH, but also to support the formation of molecules with sound antioxidant potential. More studies are required to clarify the interrelations between the different components of antioxidant defences in marine fish.

In terms of skeletal deformities, it can be observed that a DHA increase up to 5% raised the incidence of alterations in chondroid bone, such as that of the cranium, whereas no differences were found in other deformities attaining intramembranous bone,

such as kyphosis. These results match with previous studies on sea bream larvae fed high DHA rotifers (5.2% DW; Izquierdo *et al*., submitted), as ROS are known to actively destroy cartilage tissue (Beer and Wegener, 2011), therefore affecting chondroid bones with characteristics of cartilage rather than directly affecting intramembranous bones. However, in the same study, in contrast to the present one, the incidence of cranial deformities was reduced when high contents of α-TOH were included in the rotifer enrichment media, in relation to the reduced MDA and AOE expression. In another study from our research group (Alves *et al*., 2011) inclusion of organic selenium to diets containing high DHA and α-TOH, was enough to decrease the MDA values, but not to reduce cranial deformities, suggesting that the appearance of these kind of deformity could be not only related directly to the DHA oxidation, but also to the deficit of AA caused by the production of excess ROS due to high levels of α-TOH and LC-PUFA. Similarly, in the present study, an extra dosage of AA proved to be efficient in reducing the incidence of cranial deformities when high levels of DHA are included in the diets. Apart from being a potent antioxidant, AA acts with three enzymes that participate in collagen hydroxylation by adding hydroxyl groups to the aminoacids proline or lysine in the collagen molecules, greatly increasing stability of the collagen (Padayatty *et al*., 2003). Thus, the protective effect of AA on chondroid bones could be due both to its antioxidant activity and to a higher stability in the cartilage formation. Recent works in sea bass larvae also showed a reduction in the incidence of cranial deformities when enhanced levels of AA were included in the diet (5 mg 100 g^{-1}; Darias *et al.*, 2011). However, in the same study, elevated levels of this nutrient (40 mg 100 g^{-1}) caused a similar percentage of deformities to diets with a deficiency in AA. In the present work, the levels of AA employed are much more elevated (180 mg 100 g^{-1}) but it also has to be noted that levels of AA higher than those required for growth are necessary to satisfy the demands of other nutrients, in this case, to counteract the depletion in α-TOH caused by ROS.

The appearance of lordosis in fish fed high DHA and α-TOH content could be related to the high IGF-I expression observed in these larvae caused by an imbalance in the development of the musculoskeletal system, in agreement with previous studies (Fernández *et al*., 2008). On the one hand, the high incidence of muscular lesions occurring in these larvae, may also contribute to increase the lordosis rate, as a result of the increased muscular tensions created during tissue regeneration. In this sense, Madsen and Dalsgaard (1999) showed that the rainbow trout fry syndrome, characterized by muscular dystrophy among other pathologies, was associated with an increased incidence of vertebral deformities. On the other hand, IGF-II expression

follows a different tendency within each dietary treatment and also as compared to IGF-I expression. These differences may support the idea that different hormonal signals and mechanisms of gene transcription control the regulation of expression of both IGF forms (Canalis *et al*., 1991; Fernández *et al*., 2011).

Concluding, an increased dosage of AA in microdiets for sea bass larvae containing a 5% of DHA and 300 mg $100g^{-1}$ of α-TOH proved to successful in compensating, in some manner, the effect of ROS, thereby preventing the appearance of muscular lesions, reducing cranial deformities and TBARS values, a major indicator of oxidative stress. However no counteracting effect was found on AOE expression, suggesting that other nutrients could be involved in enhancing the antioxidant defences at such levels. Moreover, a sparing effect between AA and α-TOH seems to occur in sea bass larvae. The implication of AA in regulating other antioxidant components requires further investigation.

Chapter 7

Molecular pathways involved in nutritional muscle dystrophy and healing in sea bass larvae

ABSTRACT

The effect of high dietary levels of docosahexaenoic acid (DHA) on sea bass larvae muscle has been investigated in our previous studies under the influence of different dietary contents of vitamin E (α-tocopherol, α-TOH), selenium or vitamin C, describing the structural damages in this tissue. However, the potential muscle regeneration after these lesions and, in general, the molecular pathways involved are still unknown in fish. Therefore, in order to better understand this process, an experimental trial was conducted for 3 weeks in 14 day-old sea bass larvae using microdiets containing different ratios of DHA (1 or 5%) and 150 mg 100 g^{-1} of α-TOH. After this period, part of the larvae fed 5/150 diet was switched to 1/150 diet ("wash-out") and the experiment continued for another 2 weeks. Larvae fed diet 5/150 showed altered oxidative status as indicated by the highest MDA values, AOE expression and incidence of muscular lesions. A decrease in AOE expression was observed at 49 dph, indicating that at this stage a 5% of DHA would be adequate for sea bass larvae. Accordingly, "washed-out" larvae showed lower dry weight and α-TOH content. IGF-I gene expression was elevated in 5/150 larvae at 35 dph, suggesting an increased muscle mitogenesis that was corroborated by the increase in the mRNA copies of myosin heavy chain. However, IGF-II and α-actin expression did not show this tendency. It can be concluded that high DHA contents in sea bass larvae diets alters their oxidative status and causes muscular lesions, with the morphological and molecular aspects of mammal muscular degenerative disease.

Keywords:

DHA
sea bass larvae
myopathy
oxidative stress
muscle
regeneration
wash-out

7.1 Introduction

In order to improve growth and development, marine fish larvae require high contents of long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA; 22:6*n*-3). These high requirements are due, in part, to the limited capacity of marine fish species to synthesize these fatty acids when their precursors are included in the diet (Sargent *et al*., 1995; Izquierdo, 1996). Besides, fish larvae appear to posses higher specific requirements for DHA than juveniles or adults, due to their elevated growth rate (Watanabe *et al*., 1989; Takeuchi, 1997). Therefore, high contents of LC-PUFA must be included in marine fish larvae diets. However, as DHA is highly unsaturated, the susceptibility of this fatty acid to be oxidized by reactive oxygen species (ROS) is higher than that of other fatty acids (Nagaoka *et al*., 1990). To protect DHA from reactive oxygen species attack, adequate quantities of antioxidants must be included in larval diets. Vitamin E (α-tocopherol; α-TOH) is a powerful antioxidant that also stabilizes biological membranes (Wang and Quinn, 2000). An interaction exists between α-TOH and the dietary levels of highly unsaturated fatty acids in marine fish larvae (Chapter 3; Hamre, 2011).

Apart from low weight antioxidant molecules, an array of antioxidant enzymes (AOE) helps to protect organisms from ROS attack. The AOE comprises a series of enzyme scavengers of oxyradicals and other free radicals, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX). SOD catalyzes dismutation of superoxide radicals to hydrogen peroxide and oxygen; CAT catalyzes the breakdown of hydrogen peroxide to water and molecular oxygen, and GPX decomposes peroxides (Halliwell, 2006). Most of the studies of the activities of AOE regard pollutant detoxification (Ji *et al*., 2011; Kim *et al*., 2010) or developmental aspects (Peters and Livingstone, 1996; Mourente *et al*., 1999b), existing limited information concerning the effect of dietary components on their activity and AOE gene expression during early developmental stages in sea bass larvae (Tovar-Ramírez *et al*., 2010).

Whenever there is an imbalance between the generation and removal of ROS by cellular defences, a state of oxidative stress is initiated. This status may lead to the oxidation of various cellular constituents like lipids, proteins or DNA. For instance, sea bass larvae muscle seems to be very sensitive to ROS attack, as severe dystrophic lesions in the epaxial musculature have been reported due to *in vivo* lipid peroxidation

(Chapters 3, 4, 5 and 6). The term regeneration refers to a process that allows an organism to regain the function of an organ or structure damaged by injury or disease (Stoick-Cooper *et al*., 2007). In adult zebrafish (*Danio rerio*), an exceptionally high capability for regeneration has been reported (Lien *et al*., 2006; Yoshinari *et al*., 2009). Therefore, it can be hypothesized that at younger stages an activation of muscle repair process in a situation of oxidative stress will take place. However, information about muscle regeneration in fish has been rarely described and only related to mechanical injury or bacterial infection (Rowlerson *et al*., 1997; Ingerslev *et al*., 2010) and there is a complete lack of studies describing muscle regeneration process in marine fish larvae or following regeneration after a nutritional dystrophy.

Myosin and actin are the major muscle proteins, although striated muscle tissues have myosin as the major structural component. Both myosin chains, the heavy (MyHC) and the light (MyLC), exist as multiple isoforms that are tissue and/or developmental stage-specific (Funkenstein *et al*., 2007; Ikeda *et al*., 2007). MyHC expression has been highly correlated with protein accretion (Hevrøy *et al*., 2006). Besides, the effect that nutritional status has on muscle growth can be determined by monitoring the expression patterns of this marker gene (Bower *et al*., 2008). On the other hand, a higher immunolocalization of this protein has been observed during regeneration processes in sea bream (*Sparus aurata*) juveniles muscle after a mechanical injury (Rowlerson *et al*., 1997). Actins are highly conserved proteins that play a key role in maintaining the cytoskeletal structure, cell motility and division, as well as intracellular movements and contractile processes. Different isoforms of actins exist in fish, being α-actin expressed after MyHC during the period of somite formation in carp (Watabe, 2001).

Besides, cellular proliferation is an important event necessary for muscle regeneration (Chargé and Rudnicki, 2004), growth factors expected to be upregulated during this process. The insulin-like growth factors I and II are two myogenic regulatory factors capable of inducing satellite cell proliferation and differentiation in fish (Goldspink *et al*., 2001; Bower *et al*., 2008). Moreover, IGF-II was upregulated during zebrafish heart regeneration, denoting an increase of DNA synthesis (Lien *et al*., 2006).

Calpains are Ca^{2+}-dependent cytoplasmic cysteine proteases that can be expressed ubiquitously or in a tissue-specific way. In mammals, the calpains have received a great deal of attention due to their role in muscle protein turnover and growth, as well as *post mortem* proteolysis. However, in fish, these enzymes have been mainly focussed on *post mortem* muscle tenderization and texture studies (Chéret *et al*., 2007;

Caballero *et al*., 2009; Cleveland *et al*., 2009; Terova *et al*., 2011), while limited information is available about the regulatory roles of calpains in fish larvae and the effect of nutrition of its expression (Nemova, 1996).

In previous studies (Chapters 3 and 4), when sea bass larvae were fed with high contents of DHA, α-TOH alone did not seem to be able to counteract ROS, leading to the appearance of muscular lesions affecting the axial musculature. To better understand the molecular pathways involved in fish larvae muscle dystrophy and healing, in the present study, muscular lesions were generated in sea bass larvae by feeding them diets containing a 5% DHA during 3 weeks (negative control diet), followed by a "wash-out" period of two weeks where larvae were switched to a diet containing only a 1% DHA (positive control diet). Growth, survival, TBARS, fatty acid profile, α-tocopherol contents and mRNA expression levels of CAT, SOD, GPX, IGF-I, IGF-II, MyHC, α-actin and μ-calpain (Capn1) genes were studied in order to achieve this objective.

7.2 Materials and methods

7.2.1 Fish and diets

The experiment was carried out at the *Instituto Canario de Ciencias Marinas* (ICCM) facilities (Telde, Canary Islands, Spain). Sea bass (*Dicentrarchus labrax*) larvae were obtained from natural spawnings from the *Instituto de Acuicultura de Torre de la Sal* (Castellón, Spain). Prior to the start of the feeding experiment, larvae were fed enriched yeast-fed rotifers until they reached 14 days post hatching (dph; total length 8.58 ± 0.64 mm, dry body weight 0.36 ± 0.0 mg). Afterwards, larvae were randomly distributed into the experimental tanks at a density of 1000 larvae $tank^{-1}$ and fed one of the experimental diets for 35 days, at a water temperature of 19.5 to 21.0ºC. Two experimental groups were created, consisting of four tanks for the positive control diet (1/150) and eight tanks for the negative control diet (5/150). After three weeks of trial (35 dph), larvae from each tank were individually counted, 200 larvae per tank sampled for different analytical procedures and the rest placed in three tanks per treatment. Besides, larvae fed 5/150 diets were divided into two groups (3 tanks per treatment), one was fed with the same diet and the other one was switched to 1/150 (5+1/150; "wash-out") diet for two weeks, until the end of the experimental trial (49 dph).

All tanks (170 L light grey colour cylinder fibreglass tanks) were supplied with filtered sea water (34 g L^{-1} salinity) at an increasing rate of 1.0 - 1.5 L min^{-1} during the feeding trials. Water entered the tank from bottom to top; water quality was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min^{-1}), attaining 5-8 g L^{-1} dissolved O_2 and saturation ranged between 60 and 80%.

Two isonitrogenous and isolipidic experimental microdiets (pellet size<250 μm) similar in their EPA content and different in DHA content were formulated (Table 7.1) using concentrated fish oils EPA500 and DHA500 (CRODA, East Yorkshire, England, UK) as sources of EPA and DHA and DL- α- Tocopheryl Acetate (Sigma-Aldrich, Madrid, Spain) as source of α-TOH. Diets were chosen based on previous trials (Chapters 3 and 4). A positive control diet was formulated including 1 g DHA 100 g^{-1} DW and 150 mg α-TOH 100 g^{-1} DW (diet 1/150). The negative control diet consisted of 5 g DHA 100 g^{-1} DW and 150 mg α-TOH 100 g^{-1} DW (diet 5/150). The protein source used, squid meal, was defatted 3 consecutive times with a chloroform:squid meal ratio of 3:1 to allow complete control of the fatty acid profile of the microdiet. The microdiet was based on defatted squid meal (2.4% lipid content) and EPA500 and DHA500 were added in different quantities to obtain the desired ratios. Oleic acid (Merck, Darmstadt, Germany) was added to equalize the lipid content in each diet (Table 7.1).

The microdiets were prepared according to Liu *et al*. (2002) by first mixing the squid powder and water soluble components followed by lipid and fat soluble vitamins and, finally, warm water dissolved gelatine. The paste was pelleted and oven dried at 38ºC for 24 h. Pellets were ground and sieved to obtain a particle size below 250 μm. Diets were analyzed for proximate and fatty acid composition on dry basis and manually supplied; fourteen times per day each 45 min from 9:00-19:00. Daily feed supplied was 2, 2.5 and 3 g $tank^{-1}$ during the first, second and third week of feeding respectively.

Table 7.1 Formulation of experimental diets

Dietary DHA/vitamin E	*1/150*	*5/150*
Defatted squid powder (g/100g)[*]	69.00	69.00
EPA 500 g $100g^{-1}$ (DW)[†]	2.80	1.80
DHA 500 g $100g^{-1}$ (DW) [†]	0.20	6.70
Oleic acid (%)[‡]	10.00	4.50
Soy lecithin[§]	2.00	2.00
Gelatin	3.00	3.00
Attractants	3.00	3.00
Taurin	1.50	1.50
Vitamin premix[**]	6.00	6.00
Mineral premix[††]	2.50	2.50

[*] Riber and Son, Bergen, Norway
[†] Croda, East Yorkshire, UK
[‡]Merck, Darmstadt, Germany
[§] Acrofarma, Barcelona, Spain
[**] Vitamin premix supplied per 100g diet: Cyanocobalamine, 0.030; Astaxanthin, 5.00; folic acid, 5.44; pyridoxine-HCI, 17.28; thiamine, 21.77; riboflavin, 72.53; Ca-pantothenate, 101.59; p-aminobenzoic acid, 145.00; nicotinic acid, 290.16; *myo*-inositol, 1450.90; retinol acetate, 0.180; ergocalcipherol, 3.650; menadione, 17.280; α-tocopheryl acetate, 150.000
[††]Mineral premix supplied g per 100g diet: NaCl, 215.133; $MgSO_4 \cdot 7H_2O$, 677.545; $NaH_2PO_4 \cdot H_2O$, 381.453; K_2HPO_4, 758.949; $Ca(H_2PO_4) \cdot 2H_2O$, 671.610; $FeC_6H_5O_7$, 146.884; $C_3H_5O_3 \cdot 1/2Ca$, 1617.210; $Al_2(SO_4)_3 \cdot 6H_2O$, 0.693; $ZnSO_4 \cdot 7H_20$, 14.837; $CuSO_4 \cdot 5H_2O$, 1.247; $MnSO_4 \cdot H_2O$, 2.998; KI, 0.742; $CoSO_4 \cdot 7H_2O$, 10.706

7.2.2 Growth and survival

Final survival was calculated by individually counting live larvae at the beginning, middle and end of the experiment. Growth was determined by measuring dry body weight (105ºC for 24 hours) and total length (Profile Projector V-12A Tokyo, Nikon) of 30 fish $tank^{-1}$ at 14, 35 and 49 dph.

7.2.3 Biochemical analysis

At 35 dph, larvae from the two treatments were manually counted and 150 larvae removed from each tank, washed with distilled water and kept at -80ºC for biochemical analysis. Similarly, at 49 dph, all remaining larvae in each tank were washed with distilled water, sampled and kept at -80ºC for biochemical composition, fatty acid methyl esters (FAMEs), α-TOH and TBARS analysis after 12 hours of starvation at the end of the trial. Moisture, protein (A.O.A.C., 1995) and lipid (Folch *et al.*, 1957) contents of larvae and diets were analyzed.

7.2.3.1 Total lipid fatty acid analysis

Fatty acid methyl esters (FAMEs) were obtained by transmethylation of total lipids as described by Christie (1982). FAMEs were separated by GLC and quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) under the conditions described in Izquierdo *et al*. (1992) and identified by comparison to previously characterized standards and GLC-MS.

7.2.3.2 Determination of α-TOH *content*

α-TOH concentrations were determined in diets and total larvae using HPLC. Samples were weighed, homogenised in pyrogallol and saponified as described by McMurray *et al*. (1980) for diets or according to Cowey *et al*. (1981) for larval tissues. HPLC analysis was performed using 150 x 4.60 mm reverse phase Luna 5µm C18 column (Phenomenox, California, USA). The mobile phase was 98% methanol supplied at a flow rate of 1.0 ml min^{-1}, the effluent from the column was monitored at a wavelength of 293 nm and quantification achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich, Madrid, Spain) as external standard.

7.2.3.3 Measurement of thiobarbituric acid reactive substances (TBARS)

The measurement of TBARS in triplicate samples was performed using a method adapted from that used by Burk *et al*. (1980). Approximately 20-30 mg of larval tissue per sample were homogenized in 1.5 ml of 20% trichloroacetic acid (w/v) containing 0.05 ml of 1% BHT in methanol. To this 2.95 ml of freshly prepared 50mM thiobarbituric acid solution were added before mixing and heating for 10 minutes at 100ºC. After cooling protein precipitates were removed by centrifugation (Sigma 4K15, Osterode am Harz, Germany) at 2000 x *g*, the supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm. The absorbance was recorded against a blank at the same wavelength. The concentration of TBA-malondialdehyde (MDA) expressed as µmol MDA per g of tissue was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

7.2.4 Histopathological sampling

7.2.4.1 Paraffin inclusion

Thirty larvae from each tank were collected every seven days from the beginning of the feeding trial, and fixed in 10% buffered formalin for 1 or 2 days, dehydrated through graded alcohols, then xylene, and finally embedded in paraffin wax. Six paraffin blocks containing 5 larvae per tank were sectioned at 3 µm, and stained with Haematoxilin and Eosin (H&E) for histopathologic evaluation (Martoja and Martoja-Pearson, 1970).

7.2.4.2 Resin inclusion

Ten larvae per tank were fixed for 24 hours at 4ºC in 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) every seven days. Samples were then rinsed in phosphate buffer and post-fixed for 1 hour in 2% osmium tetraoxide in 0.2 M potassium ferrocyanide. Each larva was then embedded in an Eppon/Araldite resin block. Serial transverse and longitudinal larvae thick sections were cut at 1 µm, stained with toluidine blue and examined under light microscopy (Hoffman *et al*., 1983).

7.2.5 RNA extraction and quantitative RT-PCR

Molecular biology analysis were carried out at the University of Insubria (Varese, Italy) at 14, 26, 35 and 49 dph. Total RNA was extracted from sea bass larvae (≈200 mg; pool per tank), using PureYield RNA Midiprep System (Promega, Italy). The quantity and purity of RNA was assessed by spectrophotometer. Visualization on 1% agarose gel stained with ethidium bromide showed that RNA was not degraded. After DNAse treatment (Invitrogen, Milan, Italy), 3 µg of total RNA was reverse transcribed into complementary DNA (cDNA) in a volume of 12 µl, including 1 µl of oligo dT16 primer (50 pmol) and 1 µl of 10 mM deoxynucleotide triphosphates (dNTPS). This mix was heated at 65ºC for 5 minutes, chilled on ice and then 4µl of 5X reverse transcription buffer, 2 µl 0.1M DTT, 1 µl RNAse out and 1 µl of Moloney murine leukemia virus (M-MLVRT). After incubation at 37ºC for 50 minutes, the reaction was stopped by heating at 75ºC for 15 minutes.

PCR primers sequences used for the PCR amplification of the cDNAs of the target genes were CAT, SOD, GPX, IGF-I, IGF-II, α-actin, MyHC and Capn1. To perform PCR, an aliquot of 4 µl of cDNA was amplified using 25 µl GoTaq Green Master Mix (Promega, Italy) in 50 µl of final volume and 50 pmol of each designed primer.

A total of 31 PCR amplification cycles (eight touchdown) were performed for all primer sets, using an automated Thermal Cycler (MyCycler, BioRad, Italy). An aliquot of each sample was then subjected to electrophoresis on a 1% agarose gel in 1X TAE buffer (Bio-Rad, Italy) and bands were detected by ethidium bromide staining. Samples were run with a 100 bp-1.5 kb DNA Ladder to control molecular weight of DNA. The negative control (a reaction mixture without cDNA) confirmed the absence of genomic contamination. The PCR products from each primer set amplification were cloned using pGEM®-T easy vector (Promega, Italy) and subsequently sequenced in both directions (T7 and SP6).

TaqMan® real time reverse transcription PCR was performed on a StepOne Real Time PCR System (Applied Biosystems, Italy) using Assays-by-Design℠ PCR primers (Applied Byosystems) and gene-specific fluorogenic probes. Primer sequences and TaqMan® probes of target genes were as follows:

Target gene: Sea bass CAT

Forward primer: 5'- ATGGTGTGGGACTTCTGGAG - 3'

Reverse primer: 5'- GCTGAACAAGAAAGACACCTGATG - 3'

TaqMan® probe. 5'- CAGACACTCAGGCCTCA - 3'

Target gene: Sea bass SOD

Forward primer: 5'- TGGAGACCTGGGAGATGTAACTG - 3'

Reverse primer: 5'- TCTTGTCCGTGATGTCGATCTTG - 3'

TaqMan® probe. 5'- CAGGAGGAGATAACATTG - 3'

Target gene: Sea bass GPX

Forward primer: 5'- AGTTAATCCGGAATTCGTGAG - 3'

Reverse primer: 5'- AGCTTAGCTGTCAGGTCGTAAAAC - 3'

TaqMan® probe. 5'- AATGGCTGGAAACGTG - 3'

Target gene: Sea bass IGF-I

Forward primer: 5'- GCAGTTTGTGTGTGGAGAGAGA- 3'

Reverse primer: 5'- GACCGCCGTGCATTGG - 3'

TaqMan® probe. 5'- CTGTAGGTTTACTGAAATAAAA - 3'

Target gene: Sea bass IGF-II

Forward primer: 5'- TGCAGAGACGCTGTGTGG - 3'

Reverse primer: 5'- GCCTA CTGAAATAGAAGCCTCTGT - 3'

TaqMan® probe. 5'- CAAACTGCAGCGCATCC - 3'

Target gene: Sea bass MyHC

Forward primer: 5'- TGGAGAAGATGTGCCGTACTCT - 3'

Reverse primer: 5'- CGTGTCATTGATTTGACGGACATTT - 3'

TaqMan® probe. 5'- AACTGAGTGAACTGAAGACC - 3'

Target gene: Sea bass alpha-actin

Forward primer: 5'- CCTCTTCCAGCCTTCCTTCA - 3'

Reverse primer: 5'- TGTTGTAGGCGGTCTCATGGATA - 3'

TaqMan® probe. 5'- CCAGCAGACTCCATACCGA - 3'

Target gene: Sea bass Capn1

Forward primer: 5'- ACTTTACAGGCGGCGTGA - 3'

Reverse primer: 5'- GGCTCTGCTGATGATGTTGTAGA - 3'

TaqMan® probe. 5'- TCAGATCGTACATTTCCG - 3'

Data from TaqMan® PCR runs were collected with ABI´s Sequence Detector Program. Cycle threshold (Ct) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. The Ct values were used to create standard curves to serve as a basis for calculating the absolute amounts of mRNA in total RNA. To reduce pipetting errors, master mixes were prepared to set up duplicate reactions (2 x 30 µl) for each sample.

7.2.6 Calculations

Larval survival was determined by comparing the number of larvae at the beginning of the trial with the larvae number measured in individual tanks at 35 dph to which the average number of larvae sampled from tanks during the trial was added. Percentage survival could then be calculated for each tank to get a mean and standard deviation (SD) per treatment. The incidence of muscular lesions was calculated as the percentage of injured larvae per tank compared to the total larvae observed, with SD referring to deviation among tanks.

7.2.7 Statistical analysis

Survival, growth and molecular biology data were tested for normality and homogeneity of variances with Levene´s test. Where necessary data were log transformed before further statistical analysis. Chi-squared test was employed for incidence of muscular lesions and TBARS content. Survival, growth and biochemical analysis data were treated using one-way ANOVA and molecular biology results were treated using a general linear model (GLM). Means were compared by Duncan's test. Results are presented as means and standard deviation. The tank was considered as the experimental unit, except for the estimation of the incidence of muscular lesions, where each individual larvae was considered as a unit. For percentage data (final survival), arcsine transformation was performed before analysis. For analysis of one-way ANOVA the following general linear model was used:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ijk}$$

where Y_{ijk} is the mean value of the tank, μ is the mean population, α_i is the fixed effect of the diet and ε_{ij} is the residual error. For analysis of molecular biology data a two variables GLM was employed to analyze possible interactions between treatment and time:

$$Y_{ijk} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + \varepsilon_{ijk}$$

Where Y_{ij} is the mean value of the tank, μ is the mean population, α_i is the fixed effect of the diet, δ_j is the fixed effect of the time, $(\alpha\delta)_{ij}$ is the interaction between diet and time and e_{ij} is the residual error. Significance was accepted at $P \leq 0.05$. Statistical analysis was performed using SPSS software (SPSS for Windows 14.0; SPSS Inc., Chicago, IL, USA, 2005).

7.3 Results

7.3.1 Growth and survival

All experimental diets were well accepted by larvae. Dietary increase of DHA did not have any effect on growth at 35 dph, however, at 49 dph, a higher dry weight was observed in 5/150 larvae (Table 7.2). No significant differences were observed in either the total length or survival during any of the sampling points (Table 7.2).

7.3.2 Biochemical analysis

Gross composition in experimental diets was similar among treatments (Table 7.3). The fatty acids analysis revealed a higher percentage of *n*-3 and *n*-3 LC-PUFA in 5/150 diet due to the elevation of dietary DHA, whereas higher content of monounsaturated and *n*-9 was observed in 1/150 diet due to the higher oleic acid content in the former diet (Table 7.3).

At 35 dph larval FA composition closely reflected that of the microdiets fed. Thus, the levels of 22:6*n*-3, *n*-3 and *n*-3 LC-PUFA were significantly lower ($P<0.05$) in larvae fed 1/150 diet (Table 7.4). At 49 dph, 2 weeks after starting the "washing-out" period, 5+1/150 larvae were similar in 18:4*n*-3, 18:1*n*-9, 22:6*n*-3, monoenoics, *n*-6 and *n*-9 contents compared to larvae fed 1/150 diet (Table 7.4). However, total content of *n*-3 and *n*-3 LC-PUFA resembled similar to those of larvae fed both 1/150 and 5/150 diets.

The highest MDA contents (μmol g^{-1} larval tissues), an indicator of lipid peroxidation, were detected with an increase in DHA level at 35 dph ($P=0.001$; Table 7.2). MDA contents increased with time from 35 to 49 dph in all treatments, finding again a higher value in 5/150 larvae ($P=0.025$; Table 7.2). Although larvae fed 5+1/150 larvae showed higher MDA contents than larvae fed 1/150 diet, there were no statistical differences ($P=0.539$; Table 7.2). α-tocopherol content increased along the experimental period in all larvae, increasing around five times from 14 to 49 dph in all treatments. By 35 dph, larvae fed diet 5/150 did not reflect dietary content of α-TOH, but showed a lower value compared to 1/150 larvae ($P<0.05$; Table 7.2) by 35 dph. However, at 49 dph, no differences in α-tocopherol content were observed between 1/150 and 5/150 larvae in dry weight ($P>0.05$), the lowest α-tocopherol value being in larvae fed 5+1/150 diet.

Table 7.2 Sea bass larvae performance, levels of lipid peroxidation products (TBARS) and vitamin E (α-tocopherol) content of sea bass larvae at the beginning, middle and at the end of the experimental trial

	Diets					
	Initial	35 dph		49 dph		
		1/150	5/150	1/150	5/150	5+1/150
Results of dietary trial						
Larval total length (mm)	8.58 ± 0.64	12.60 ± 0.93	12.06±1.55	12.68±1.24	12.99±1.37	13.12±1.53
Larval dry weight (mg)	0.36 ± 0.00	1.46 ± 0.47	1.26±0.12	1.76±0.25^{b}	2.72±0.40^{a}	1.95±0.05^{b}
Survival (%)	-	60.51 ± 9.10	51.30±1.99	44.42±6.94	57.42±15.42	39.26±10.26
Incidence muscular lesions (%)	-	13.33 ± 5.77	29.16±6.31	6.60±1.30	20.00±8.71	13.33±8.16
Vitamin E (α-tocopherol)						
µg g dry mass^{-1}	111.45±43.26	630.24±12.39^{a}	473.67±18.40^{b}	626.47±34.45^{a}	655.39±43.25^{a}	580.25±39.04^{b}
MDA						
nMol g dry mass^{-1}	62.85±0.61	166.62±25.08^{b}	527.08±14.71^{a}	536.35±68.57^{b}	1038.87±152.08^{a}	657.76±85.87^{b}

Data are means±SD. Values within the same row bearing different superscript letter are significantly different (P<0.05)

Table 7.3 Gross composition and principal fatty acid methyl esters (% of total fatty acids) of the experimental diets fed to sea bass larvae.

	Diets	
	1/150	5/150
Gross composition		
Protein (%)	66.79	65.99
Ash (%)	4.49	4.73
Moisture (%)	10.31	9.99
Lipids (% DW)	14.98	15.06
α-tocopherol (μg g^{-1} DW)	1410.12	1449.80
Selenium (μg mg^{-1})	1.54	1.48
Main fatty acids		
Total Saturated	12.28	10.09
Total Monoenoics	61.99	39.05
18:3*n*-3 ALA	0.72	0.80
18:4*n*-3 SDA	0.83	0.97
20:5*n*-3 EPA	8.66	12.05
22:5*n*-3 DPA *n*-3	0.32	1.23
22:6*n*-3 DHA	4.58	23.64
Total *n*-3	15.68	39.51
Total *n*-3 LC-PUFA	13.96	37.58
18:2*n*-6 LA	7.40	6.80
20:4*n*-6 ARA	0.71	1.34
22:5*n*-6 DPA	0.19	1.48
Total *n*-6	8.61	10.26
18:1*n*-9 OLA	55.70	33.84
22:1*n*-9	0.08	0.24
Total *n*-9	57.19	35.66

ALA, α-linolenic acid; SDA, stearidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; LC-PUFA, long chain polyunsaturated fatty acid; LA, linoleic acid; ARA, arachidonic acid; OLA, oleic acid.

7.3.3 Histopathological evaluation

Histopathological examinations revealed the presence of lesions affecting sea bass larvae axial musculature. These lesions showed the typical features of necrotic degeneration of muscle, characterized by marked eosinophilia, loss of striations and adjacent nucleous. The incidence of muscular lesions increased along with DHA dietary content (Table 7.2), being two times-folded in 5/150 larvae in contrast to 1/150 larvae by day 35. The change of diet decreased the incidence of muscular lesions by 49 dph, however the lowest incidence of muscular lesions was found in larvae fed the low DHA diet (1/150). On the other hand, incidence of muscular lesions decreased from 35 to 49 dph.

Table 7.4 Main fatty acid composition of total lipids from sea bass larvae at the beginning (14 dph), after three weeks (35 dph) and after five weeks (49 dph) of feeding the experimental diets (% total identified fatty acids)

	Diets					
	14 dph	35 dph		49 dph		
		1/150	5/150	1/150	5/150	5+1/150
Total Saturated	26.17±5.43	32.01±6.29	35.62±3.85	25.90±2.20	25.20±0.49	25.53±1.06
Total Monoenoics	36.62±3.61	36.73±4.51	36.80±4.09	36.28±3.80^{a}	26.06±3.09^{b}	33.84±1.86^{a}
18:3n-3 ALA	0.80±0.27	0.32±0.05^{b}	0.85±0.07^{a}	0.36±0.04	0.34±0.04	0.27±0.06
18:4n-3 SDA	0.22±0.09	0.29±0.06^{b}	0.89±0.12^{a}	0.30±0.13	0.42±0.07	0.31±0.09
20:5n-3 EPA	7.60±0.33	5.91±1.18	5.82±0.07	8.39±1.34	6.76±0.04	8.57±0.57
22:5n-3 DPA	1.58±0.33	0.64±0.26	0.92±0.16	0.97±0.15	0.76±0.01	0.76±0.02
22:6n-3 DHA	14.22±4.36	12.79±0.37^{b}	25.12±4.24^{a}	16.30±3.32^{b}	28.36±3.38^{a}	19.58±1.48^{b}
Total n-3	25.39±2.48	20.94±2.23^{b}	34.60±3.57^{a}	27.59±5.24^{b}	37.61±3.67^{a}	30.67±0.88^{ab}
Total n-3 LC-PUFA	23.74±5.94	12.92±7.45^{b}	32.27±3.84^{a}	25.91±4.89^{b}	36.21±3.65^{a}	29.16±0.97^{ab}
18:2n-6 LNA	3.75±1.14	4.23±0.08	4.27±0.35	4.49±0.50	3.62±0.46	3.67±0.28
20:4n-6 ARA	3.34±0.72	2.38±0.04	2.34±0.35	2.46±0.11	2.99±0.31	2.76±0.14
22:5n-6 DPA	0.58±0.28	1.09±0.09^{b}	0.37±0.05^{a}	0.36±0.02	1.52±0.04	0.58±0.03
Total n-6	9.70±1.22	8.45±0.97	8.18±1.27	8.24±0.73^{b}	9.26±0.26^{a}	7.92±0.18^{b}
18:1n-9 OLA	16.14±2.01	26.35±4.87	25.43±1.69	26.84±2.58^{a}	18.31±1.91^{b}	25.22±1.92^{a}
22:1n-9	0.33±0.17	0.26±0.15	0.26±0.03	0.14±0.03	0.21±0.13	0.13±0.07
Total n-9	18.81±4.97	28.44±4.72	27.52±2.77	28.93±2.79^{a}	20.25±1.98^{b}	27.10±1.85^{a}

Data are means±SD. Values within the same row bearing different superscript letter are significantly different (P<0.05)

In semithin sections, more detailed features of these muscular lesions could be observed. In severely damaged fibres a coagulation of the muscular proteins could be observed just as a darkening of affected fibres due to hypercontraction (Figure 7.1.A). In initial-mild stages of the condition an increase in the presence of vacuoles within fibres was observed, together with the loss of shape of muscular fibres, alteration of sarcoplasmic membranes and variation in the diameter of fibres (Figure 7.1.A), especially if compared to not injured muscular fibres (Figure 7.1.B).

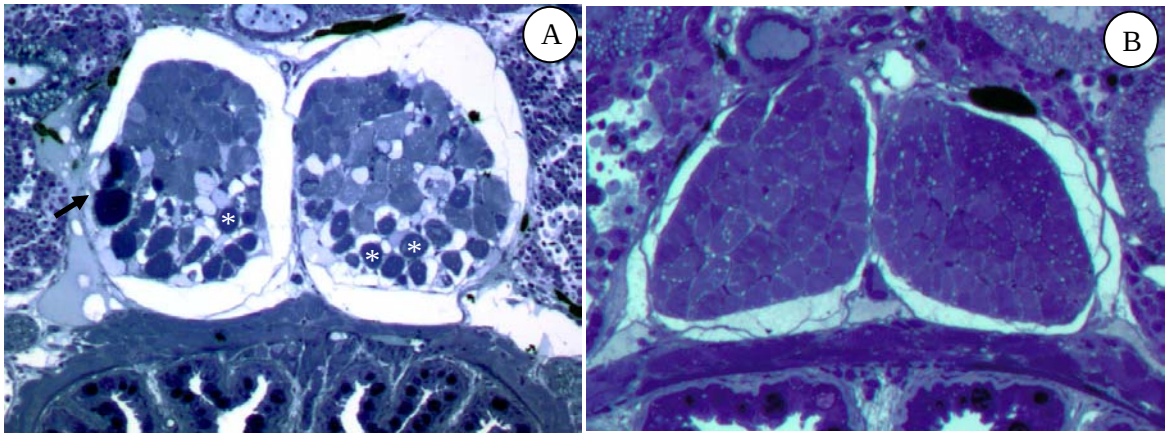

Figure 7.1 Transversal semithin sections on the same selected morphological area of 49 dph sea bass larvae fed 5/150 (A) or 1/150 (B) diets. (**A**) Damaged muscular fibres showing hypercontraction of the myofilaments (*) and coagulation of proteins, observed as darkening of the fibre (arrow). Besides, loss of the polyedrical structure can be observed, especially if compared to normal muscle (**B**).

7.3.4 Gene expression analysis

The general pattern of antioxidant enzymes gene expression in all groups of sea bass larvae was characterised by a rapid increase between 14 and 26 dph, followed by a decrease back to levels slightly higher than those observed at 14 dph, by 35 dph (Figure 7.2). CAT gene expression was higher in larvae fed 5/150 diet at 26 and 35 dph (Figure 7.2.A), as well as SOD mRNA copy number compared to 1/150 larvae (P<0.05; Figure 7.2.B). GPX expression level varied through the experimental period. At 26 dph, no differences in expression were observed (P=0.551; Figure 7.2.C), but a rapid decrease was detected in 5/150 larvae at 35 dph (P=0.012). “Wash-out” had an effect on AOE expression in sea bass larvae, expression of SOD and GPX being increased in 5+1/150 (P<0.05) larvae. Conversely, CAT expression remained similar in “washed-out” larvae compared to larvae fed 1/150 or 5/150 diet (P=0.166; Figure 7.2.A).

IGF-I expression levels showed an initial increase in both 1/150 and 5/150 larvae from 14 to 26 dph, being significantly higher in larvae fed diet 5/150 (P=0.008). At 35 dph, larvae fed diet 5/150 showed a sudden increase (Figure 7.3.A), whereas larvae fed 1/150 diet IGF-I expression decreased, a higher expression level being found in 5/150 larvae (P=0.018). Conversely, at 49 dph, a higher absolute mRNA level was found at 1/150 larvae (P=0.002). At the same sampling point, "washed-out" larvae showed an expression pattern similar to that of 5/150 larvae. Regarding IGF-II expression, 1/150 and 5/150 larvae showed a similar pattern from 14 to 35 dph, decreasing its expression with age, although not statistically differences were detected (P>0.5; Figure 7.3.B). However, there was at least a two-fold increase in IGF-II expression at 49 dph in 1/150 larvae compared with 35 dph, showing a higher expression than any other dietary treatment (P=0.001). "Washed-out" larvae IGF-II expression displayed the same pattern than that showed by 1/150 larvae.

With respect to the expression patterns of genes encoding muscle structure, all of them increased between 14 and 26 dph, and decreased by day 35 in both 1/150 and 5/150 larvae (Figure 7.4). α-actin expression was characterized by a decrease by day 35 to levels lower than those observed at 14 dph and a final slight increase by day 49 (P>0.05; Figure 7.4.A). Conversely, one-way ANOVA of α-actin at day 49 indicated that the "washed-out" larvae exhibited the lowest expression of this gene (P=0.005). MyHC expression levels showed a higher value in larvae fed diet 1/150 by 26 dph (P=0.049; Figure 7.4.B), however no differences were found neither at 35 (P=0.083), nor 49 dph (P=0.166) between 1/150 and 5/150 larvae. Similarly to α-actin expression, MyHC mRNA levels in "washed-out" larvae were the lowest by 49 dph (P=0.0017). In contrast, "wash-out" period increased the Capn1 levels of expression, making its levels comparable to larvae fed 1/150 larvae (Figure 7.4.C). No differences were appreciated between 1/150 and 5/150 larvae at 26 dph, however a higher expression (P=0.045) was found by day 35 in larvae fed 5/150 diet.

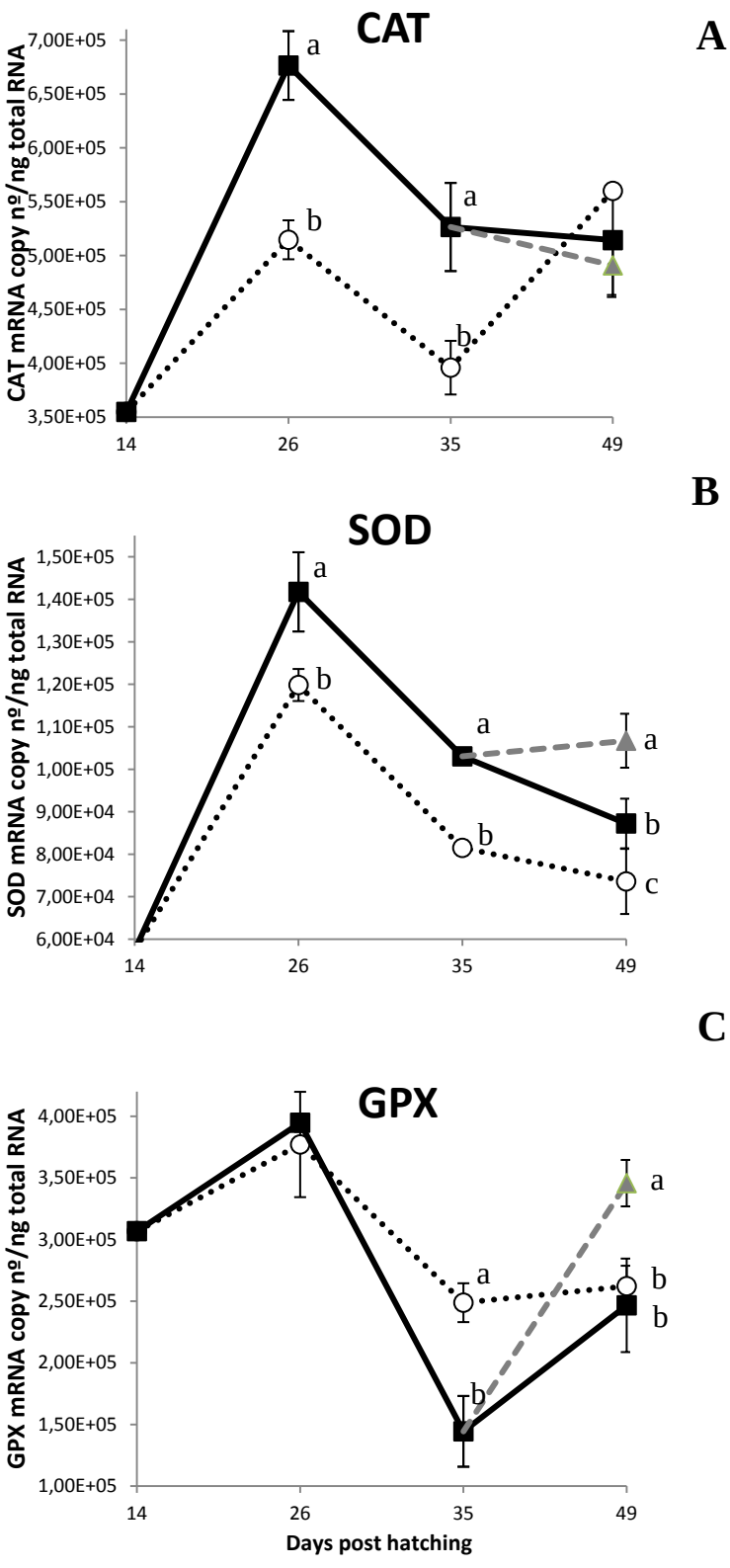

Figure 7.2 CAT, SOD and GPX expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/150 (■) or 5+1/150 (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Different letters denote significant differences between treatments within a sampling point (P<0.05).

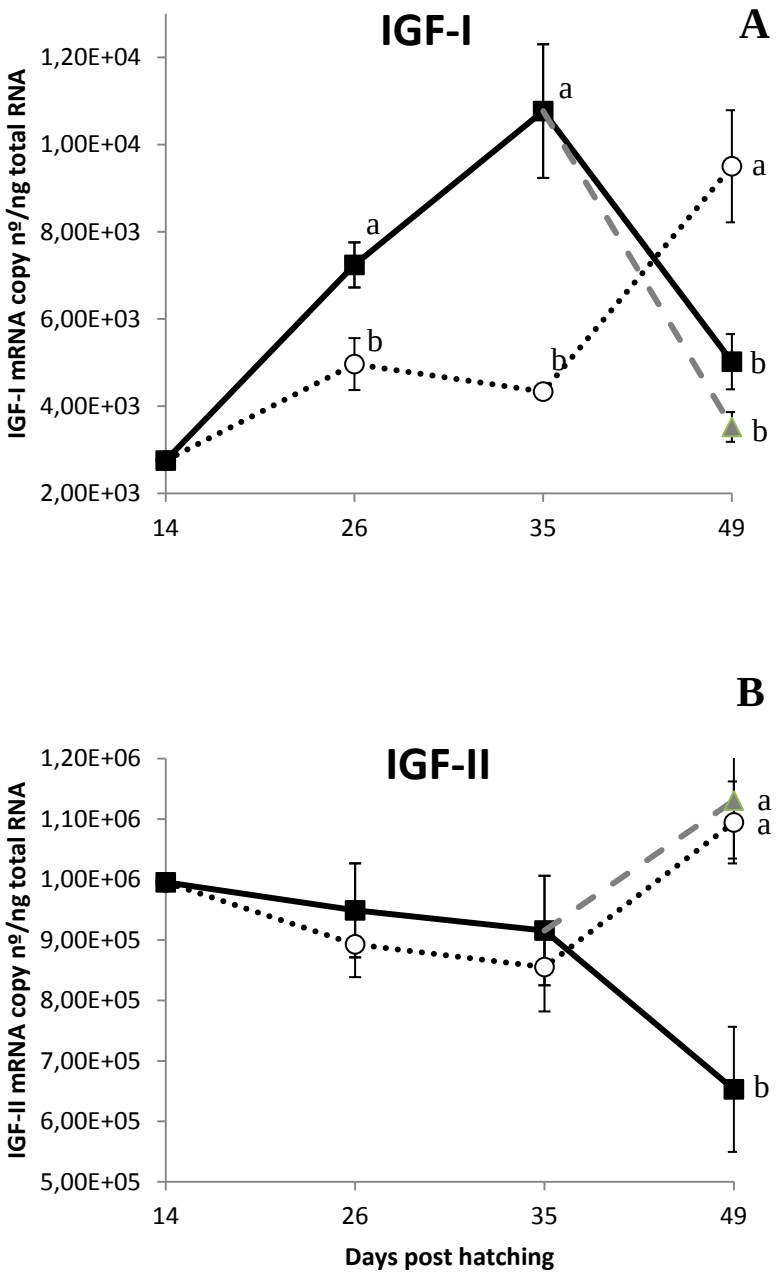

Figure 7.3 IGF-I and IGF-II expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/150 (■) or 5+1/150 (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Different letters denote significant differences between treatments within a sampling point (P<0.05).

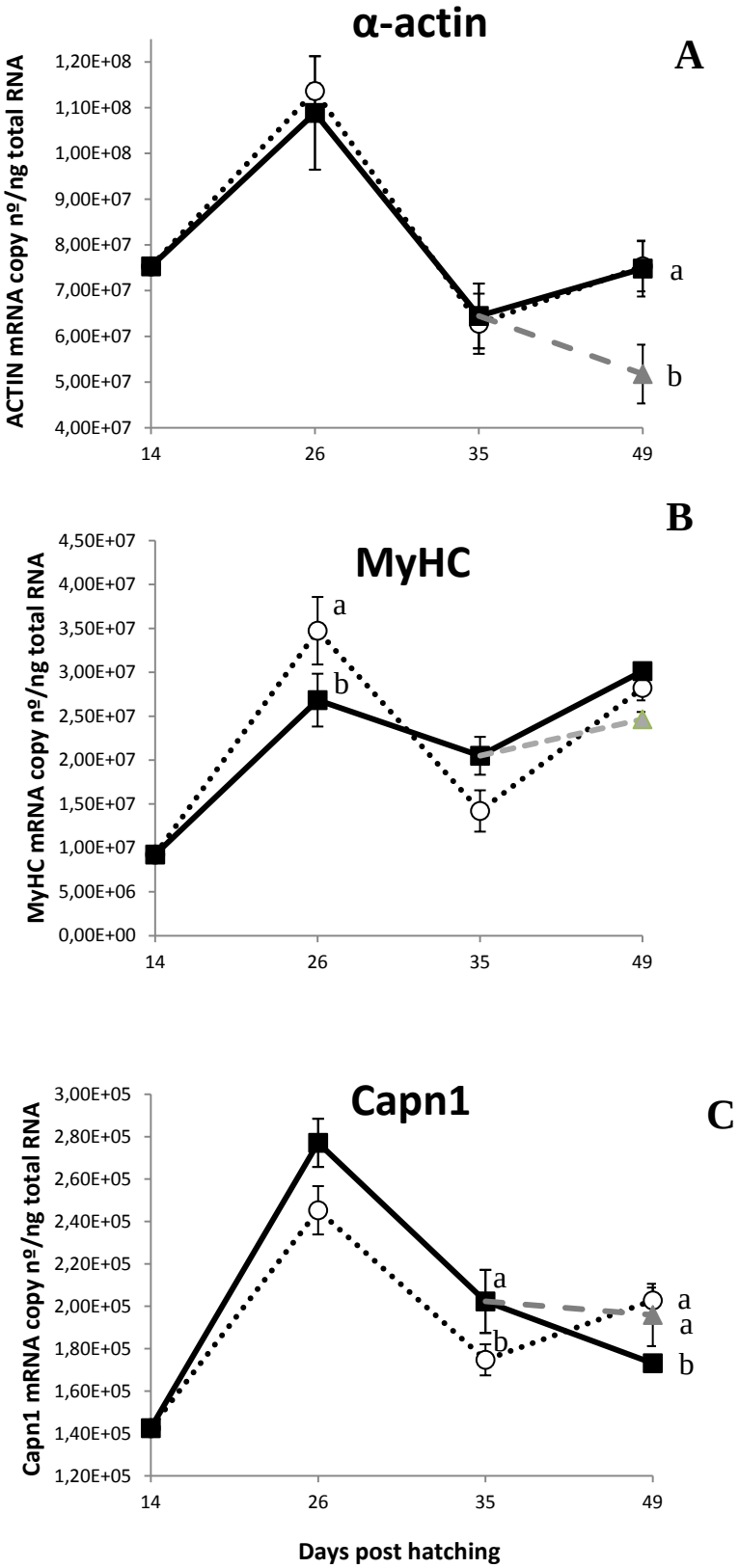

Figure 7.4. α-actin, MyHC and μ-calpain expression levels measured by real-time PCR in *Dicentrarchus labrax* larvae when were fed diets 1/150 (○), 5/150 (■) or 5+1/150 (▲). mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Different letters denote significant differences between treatments within a sampling point (P<0.05).

GLM analysis denoted a marked interaction between dietary treatments and sampling points ($P \leq 0.01$) for all the genes studied, except for SOD and α-actin, which, conversely, did show differences among diets and sampling points.

Table 7.5 Effects of the dietary treatment, time and their interaction on the global gene expression.

	DIET	TIME	D x T
CAT	**	**	**
SOD	**	**	n.s.
GPX	**	**	**
IGF-I	**	*	**
IGF-II	**	n.s.	**
MyHC	*	**	**
α-actin	**	**	n.s.
Capn1	*	**	**

Asterisks indicate significant differences as **$P \leq 0.01$, *$P \leq 0.05$; n.s. indicates non-significant differences.

7.4 Discussion

Feeding sea bass larvae with high dietary DHA contents, without the adequate combination of antioxidants, has deleterious effects on axial musculature, leading to the appearance of muscle dystrophy, as it has been previously proved (Chapters 3, 4, 5 and 6). However, limited information exists about the molecular response of the larval muscle to this pathological alteration. In the present study, the interrelations between the expression of MyHC and α-actin, the most abundant proteins in skeletal muscle, μ-calpain, a Ca^{+2}-activated proteolytic enzyme and IGFs expression was examined together with AOE gene expression.

Even if the α-TOH contents were the same for all diets (150 mg 100 g^{-1} DW), at 35 dph, the highest α-TOH was found in larvae fed the lowest level of DHA, indicating that α-TOH levels were influenced by dietary DHA ratio. This indicates that more α-TOH was being utilized as an antioxidant in larvae fed higher DHA levels to protect tissue lipids from the increased oxidation risk. These results match previous studies where α-TOH concentration in juvenile or adult fish were lower when high contents of *n*-3 LC-

PUFA were included in the diets (Puangkaew *et al*., 2005). However, at 49 dph, α-TOH contents in sea bass larvae fed diets 1/150 and 5/150 showed similar α-TOH levels. This could be due to the dependency of young larvae on low molecular weight antioxidants, such as α-tocopherol, to defend from free radicals, as their AOE systems are still poorly developed (Rudneva, 1999). Therefore, from 49 dph, an enhancement in AOE activity could efficiently quench ROS, without the need of oxidizing α-TOH.

As expected, the different dietary fatty acid compositions were reflected in the fatty acid compositions of larvae. Thus, at 35 dph, larvae fed high DHA diet resulted in increased levels of total *n*-3 LC-PUFA, particularly DHA, whereas the low DHA larvae resulted in lower levels of total *n*-3 LC-PUFA and higher levels of 18:1*n*-9. Therefore, the potential for lipid peroxidation was theoretically higher in the larvae fed diet with a 5% of DHA. This is in agreement with the high MDA values found in larvae fed high DHA diets, being folded more than 3 times at 35 dph.

The second phase of the experiment involved feeding larvae, previously fed with 5/150 diet, with the diet containing 1% of DHA for a period of two weeks. This action lowered 22:6*n*-3, *n*-6 and *n*-3 LC-PUFA levels, and increased 18:1*n*-9, monoenoics and *n*-9 levels resembling to values similar to larvae fed 1/150 diet. This result proves for first time that in larvae a "wash-out" period of only two weeks was enough to reflect the diet dietary content when changing from 5 to 1% DHA. This short "washing-out" period contrasts with previous studies in juveniles where "washing-out" periods of 12-18 weeks were necessary to restore fatty acid compositions in Atlantic salmon (Bell *et al*., 2003a; Bell *et al*., 2003b), 8-12 weeks in sea bream (Izquierdo *et al*., 2004) or longer than 8 weeks in sunshine bass (*Morone chrysops* ♀ x *M. saxatilis* ♂; Trushenki *et al*., 2008).

On the other hand, at 49 dph, although MDA values were still higher in 5/150 larvae, the difference with larvae fed a 1% DHA was less than 2 times and α-TOH content did not show differences between larvae fed both diets. Similarly, incidence of muscular lesions decreased from 35 to 49 dph in 5/150 larvae. It must be noted that, in contrast to our previous studies, in the present report larvae were fed from 14 to 49 dph on an experimental dry basis diet only, so these levels were not previously tested for such a long period of time. It has been shown that time or duration of feeding is an important additional factor to consider in relation to determine the biochemical responses to oxidative stress (Mourente *et al*., 2002). In the view of the present results, it seems that a diet containing a 5% of DHA from 35 dph would be suitable for sea bass larvae weaning. In agreement with this, larvae switched to 1/150 diet showed a lower dry weight

and α-TOH content compared to 5/150 diets, indicating that it would be appropriate to feed larvae on DHA contents of 1% during the first five to six weeks after hatching, and increase DHA content to 5% after this period, as diminished negative effects associated to an altered oxidative status were observed in 5/150 larvae by day 49 after hatching. Similarly, Villeneuve *et al.* (2006) indicated that the earlier sea bass larvae were fed with a high marine phospholipid content microdiet, the greater its negative affects upon larval growth, being larvae more resistant to this treatment at 40 dph. Accordingly, in 5/150 larvae CAT and GPX expression decreased to levels comparable to larvae fed diet 1/150 by day 49.

From 14 to 35 dph, when sea bass larvae were exposed to high DHA dietary contents (5%), the induction of AOE genes coincided with increases in MDA contents. In agreement with previous studies in this species (Chapters 5 and 6) and Manchurian trout (*Brachymystax lenok*) (Zhang *et al*., 2009) where young fish fed high lipid levels showed elevated MDA contents, inducing an antioxidant response noticeable as an increase in the activity of AOE. Besides, in the present study, a marked increase in the expression of each AOE can be observed from 14 to 26 dph, whereas exposure to high DHA content diets caused a significant increase in CAT and SOD in larvae fed diet 5/150 both at 26 and 35 dph. Similarly, juvenile rainbow trout fed with high PUFA contents, displayed significantly higher SOD activities (Trenzado *et al*., 2009). However, after 49 dph, the effects of CAT and SOD were less apparent, suggesting the adaptation of these genes expression in the latest sampling point, after the supra-induced state found in the former sampling. This would also explain why the “washed-out” larvae showed the highest mRNA copies of SOD and GPX, as larvae may be still adapting to the new dietary treatment. On the other hand, the activities of CAT and SOD would be expected to parallel each other based on the known mechanisms of the enzymes and the fact that superoxide anions are known to be efficiently scavenged by α-TOH in biological systems (Cay and King, 1980). Thus the increase in SOD expression observed at 5+1/150 larvae by 49 dph, accompanied by a decrease in α-tocopherol content, could indicate an increase in the production of superoxide anion radical and the attempt of these two antioxidant mechanisms to quench such ROS. Conversely, GPX expression differed from the others AOE, being significantly higher in larvae fed 1/150 diet by 35 dph. Both CAT and GPX, have the capability to remove hydrogen peroxide, thus it would be expectable to see a certain relationship between these two enzymes as has been observed in *Dentex dentex* larvae (Mourente *et al*., 1999b). This lack of correlation may indicate a different mechanism for regulation of gene expression for GPX when high levels of hydrogen peroxide are being generated.

The role of IGF-I and IGF–II in regulating growth and the profound effect of the nutritional status on the IGF system in fish has received much attention in the last years (Carnevali *et al*., 2006; Terova *et al*., 2007; Mazurais *et al*., 2008; Enes *et al*., 2010; Hevrøy *et al*., 2011; Darias *et al*., 2011; Fernández *et al*., 2011). However, there are no studies on the implication of IGFs in the healing of musculoskeletal tissue in fish, particularly in marine fish larvae. On one hand, in the present study, the progressive increase of IGF-I expression in 1/150 larvae was in agreement with the high cell proliferation rate, and/or the increase in specific cell activity in different tissues during the larval morphogenesis (Perrot *et al*., 1999; Patruno *et al*., 2008; Fernández *et al.*, 2011). On other hand, an abrupt increase in the expression of IGF-I was observed in larvae fed 5/150 diet in the present study, which also showed the highest incidence of muscular lesions. In mammals, IGF-I levels are upregulated in skeletal muscle undergoing regeneration (Chargé and Rudnicki, 2004), suggesting that the increase of this peptide could be associated to regenerative processes in sea bass larvae. Indeed, in a previous study (Chapter 4) abundant satellite cells, which are increased during the first phase of muscle regeneration, were appreciated in injured muscle of sea bass larvae. Besides, in fine flounder (*Paralichthys adspersus*) (Fuentes *et al*., 2011), an overexpression of IGF-I, results in greater skeletal mass, and thus, the increase in the expression of IGF-I observed in sea bass larvae could be related to a compensatory muscle growth in response to the injuries suffered from ROS.

On the other hand, an increase in MyHC expression has been described in mammals muscle undergoing regeneration process (Järva *et al*., 1997). Initially, muscular regeneration is dominated by a proliferative phase during which satellite cells and fibroblasts increase in number (Schultz *et al*., 1994). Satellite cells then fuse to form myotubes in the regenerating muscle and transcription of muscle-specific genes, such as myosin and actin, takes place in the new muscle fibres. Therefore, the higher MyHC expression in 5/150 larvae from 26 to 49 dph indicates that a regeneration process is taking place. However, the highest number of MyHC mRNA copies was found in 1/150 larvae at 26 dph, reflecting an initial stage of the regeneration process. This hypothesis was confirmed by the high IGF-I expression found in 5/150 larvae from day 26, as this peptide potently activates cell proliferation and DNA synthesis via mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3 kinase) in zebrafish (Pozios *et al*., 2001). Besides, IGF-I has been showed to increase MyHC protein in denervated skeletal muscle in mouse (Shanely *et al*., 2009).

In contrast to IGF-I, IGF-II expression was constant and decreasing in larvae fed both dietary treatments until day 35. Transcript levels of this peptide were much higher than IGF-I ones, coinciding with previous work in sea bream larvae (Radaelli *et al*., 2003) and suggesting that IGF-II acts earlier than IGF-I in myogenesis. IGFs expression in "washed-out" larvae showed irregular patterns, being decreased for IGF-I and increased for IGF-II, supporting the idea that different hormonal signals and mechanisms of gene transcription control the regulation of expression of both IGF forms (Canalis *et al*., 1991). From 35 dph on there is a marked decrease in both IGF expression in larvae fed diet 5/150, where a high expression of this peptide would be expected due to regeneration processes. No information is available about the molecular process of regeneration in fish larvae after a chronic insult, like ROS attack. It must be noticed that IGFs exert their effects on cells through binding to the IGF receptors (IGF-R) and that an increase in IGF-1R receptors has been described in cultured trout muscle cells in response to cell differentiation (Castillo *et al*., 2004). Besides, a critical element in the action of IGF and their receptors is the role of IGF binding proteins (IGFBP), as they influence IGF function by enhancing or inhibiting their action (Reinecke *et al*., 2005). On the other hand, in mammals, it is known that certain cytokines, such as tumour necrosis factor (TNF), produced as a result of any inflammatory process, like muscle injury, can inhibit the action of IGF and this has been demonstrated in chronic muscular diseases (Grounds *et al*., 2008; Gebski, 2009). A full understanding of IGFs function in fish muscle regeneration requires more work.

Expression of myosin, the most abundant protein of muscle, has been previously investigated in juvenile fish, as the myosin transcripts contents were reported to be a potential biochemical marker for growth in rainbow trout on varied nutrient intake levels (*Oncorhynchus mykiss*; Overturf and Hardy, 2001) and spotted wolffish grown at different temperatures (*Anarhichas minor*; Imsland *et al*., 2006). In contrast to these studies, in the present research no direct correlation was found between growth and MyHC expression in sea bass larvae. A recent study in Atlantic cod (*Gadus morhua*) juveniles reared at different salinity did not neither find any correlation between growth rate and myosin mRNA content, suggesting that the content of the transcript considered is not a valuable indicator of growth at this stage of development (Koedijk *et al*., 2010). Besides, histochemical and immunohistochemical studies have revealed developmental transitions in myosin composition in the sea bass (Scapolo *et al*., 1988). Different myosin isoforms appear in early and late larvae, and these are different from those found in adult white or red muscle fibres, suggesting that a different MyHC could be expressed at these larval stages and are not being detected by us.

Regarding the α-actin expression, it did not show differences between 1/150 and 5/150 larvae and was neither correlated to MyHC or IGF-I, therefore, it did not reflect any regeneration process. Although it has been stated that IGF-I induces sarcomeric actin filament formation in mammals (Takano *et al.*, 2010), a relationship between both molecules could not be proved in the present study. However, it must be noticed that several α-actin isoforms have been described in fish (Morita, 2000), thus, the reduction of the transcript content observed may reflect the switch to the expression of a different isoform that could not be detected due to the specificity of the probes designed. "Wash-out" had a negative effect on myofibrillar proteins expression, observing decreased transcripts levels of both MyHC and α-actin.

One of the first alterations observed in sea bass larvae muscle is due to the attack of ROS against the sarcoplasmic membrane, causing dysregulation of cell volume and massive intracellular increase in Ca^{2+} (Cotran *et al*., 2004). Similarly, elevated intracellular Ca^{2+} concentration has been found in the muscular dystrophies and other muscle pathologies in mammals, with this elevated Ca^{2+} concentration stimulating calpain activity (Mongini *et al*., 1988; Hopf *et al*., 1996). Accordingly, a higher expression level of μ-calpain was found in larvae fed 5/150 diet, which also showed the highest incidence of muscular lesions. In agreement with this, elevated levels of calpain were found in rapidly atrophying muscles in rabbits fed diets deficient in α-TOH (Dayton *et al*., 1979). Besides, some authors indicate that calpain activity is required for myoblasts to progress in the mitotic cycle (Zhang *et al*., 1997) as well as for myoblast fusion (Kwak *et al*., 1993; Temm-Grove *et al*., 1999), suggesting that decreased calpain activity during muscle development may be associated with an increased number of myoblasts.

In conclusion, an increased oxidative stress in sea bass larvae fed high dietary DHA contents may account for the high occurrence of muscular lesions observed in 5/150 larvae. Supplementation with 150 mg $100g^{-1}$ of α-TOH did not allow to counteract the negative effects of oxidative stress when 5 g 100 g^{-1} of DHA were included in the microdiets. Sea bass larval antioxidant defence enzymes appeared to respond strongly to high DHA contents, as proved by the high expression of CAT and SOD, however, elevated lipid oxidation products were observed. High MyHC and IGF-I expression might indicate signs of muscle regeneration, however α-actin and IGF-II expression did not support these results, suggesting the implication of different mechanisms of gene transcription. Calpn1 transcripts level was elevated in 5/150 larvae, as has been pointed out in muscle dystrophy in mammals. Thus, we can conclude that high DHA contents in

sea bass larvae diets leads to an alteration of the oxidative status and to the appearance of muscular lesions, with the morphological and molecular aspects of mammal muscular degenerative diseases. Besides, “wash-out” period indicated that contents of 5% of DHA would suit sea bass larvae requirements from 35 days on. More studies are needed in order to better understand the molecular pathways underneath regeneration processes in marine fish larvae.

Chapter 8

General Discussion

8.1 Meeting the objectives

1.- In the present Thesis, the effect of the inclusion of high dietary levels of DHA (1, 3 and 5% DW) was tested in sea bass larvae for first time. It was corroborated that too high levels of this FA may not be beneficial for sea bass larvae culture.

2.- The antioxidant protective effect of α-tocopherol when larvae were fed with high dietary levels of DHA (1, 3 and 5%) was also evaluated. Up to a determined level of DHA (5%), vitamin E seems to be ineffective in protecting larval tissues from free radicals. Suggesting that other nutrients might be also required for an effective antioxidant protection.

3.- Based on the previous results, a new experiment employing vitamin E and selenium inclusion was performed, to investigate the role of this mineral in antioxidant defences in sea bass larvae, proving that both antioxidants were effective to counteract ROS adverse effects, acting at different physiological levels.

4.- Similarly, high content of vitamin C was also included in sea bass larvae diets together with high levels of vitamin E and DHA to study its specific antioxidant role when an altered oxidative status was established.

Do high levels of DHA improve sea bass larvae culture performance?

Marine fish larvae appear to posses higher specific requirements for DHA than juveniles and pre-adults, thus formulation of feeds for marine fish larvae traditionally includes high quantities of this FA. Many studies have pointed out the negative effects associated to a deficiency of DHA in fish larvae (Watanabe *et al*., 1989; Masuda *et al.*, 1998; Brandsen *et al*., 2005; Benítez-Santana *et al*., 2007) or how an increase of this FA can improve culture performances (McEvoy *et al*., 1998; Cahu *et al*., 2003; Brandsen *et al*., 2005; Jalali *et al*., 2008; Roo *et al*., 2009; Satoh and Takeuchi, 2009a). However, in the present Thesis, it has been proved that too elevated DHA dietary levels can lead to an altered oxidative status, with negative effects to culture performance in sea bass. Growth and survival, which are often key indicators of larval performance, were not always negatively affected by DHA levels of a 5%. In Chapter 3, the lowest growth and survival was found in 5/150 larvae and in Chapters 5 and 6, 5/300 larvae showed the lowest total length, whereas in Chapters 4 and 7, these parameters were unaffected by DHA dietary content (5%). These differences could be attributable to the different age of larvae, as well as, to the quality of the spawn or the nutrition of the broodstock, as required amount of nutrient in first feeding larvae strongly depends on the previous maternal supply. In this sense, larvae from Chapter 3 were obtained from Ecloserie Marine de Gravelines (France), whereas those from the other Chapters were provided by Instituto de Acuicultura de Torre la Sal (Castellón, Spain), thus broodstock followed different feeding regimes which may affect their spawn. Additionally, larvae employed at Chapter 3 were previously fed a commercial diet, which may render more resistant to ROS than those employed in the other Chapters which were only fed with rotifers. Besides, as it has been previously stated before, as larvae grow their antioxidant defences rely more on the AOE activity than in low molecular weight antioxidants, thus the response of different age larvae to oxidative stress is expected to be different.

A common finding in all the experiments was the elevated incidence of lesions in the axial musculature of sea bass larvae when they were fed a 5% of DHA. Larvae fed a 5% of DHA developed a type of lesions known as muscular dystrophy, which are characterized by a segmental necrosis affecting both white and red muscles. These lesions are the result of an increased ROS production, which attack PUFA from the muscle membrane, leading to an increase in cell permeability and to the consequent necrosis. The imbalanced oxidative status has been corroborated through TBARS content (Chapters 4, 5, 6 and 7), as well as the expression of AOE genes (Chapters 5, 6 and 7). The significance of these muscular lesions does not only lie in the low culture

performance obtained, but in the prospective consequences, such as increased deformity rate due to increased tensions during wound healing (Cotran *et al*., 2004). Besides, the remodeling of the tissue comprises the final phase of a normal injury healing process, where the repaired tissue is organized into the correct structure by cross linking of fibrillar collagen and the formation of a scar takes place. Scarring, also termed fibrosis, is due to excessive accumulation of collagen and is also known to occur in fish following infections of the skin and the musculature by bacteria or mechanical injuries (Lunder *et al*., 1995; Ingerslev *et al*., 2010), decreasing the fillet quality. For instance, Atlantic salmon infected by *Moritella viscosus* are downgraded in quality due to a high deposition of scarring in the fillets (Lunder *et al*., 1995).

Another morphological alteration observed due to the use of high DHA dietary contents, was the presence of ceroid pigment within hepatocytes (Chapter 3). This alteration has been previously related to fish fed diets deficient in vitamin E or with oxidized lipids (Moccia *et al*., 1984; Miyazaki, 1995; Sakai *et al*., 1998) and has been used as an estimation of lipid peroxidation. However, in Chapter 3, a mild ceroidosis was observed, suggesting muscle is the first and main tissue to be attacked by free radicals in sea bass larvae. This hypothesis is reinforced by the absence of ceroid pigment occurrence in the other experiments.

DHA levels had also an effect on skeletal malformations and ossification degree, finding a higher number of deformities in larvae fed 5% of DHA, as well as lower mineralization rates (Chapters 5 and 6). Thus, free radicals may exert their adverse effects on bone by direct and indirect mechanisms; directly altering the mineralization process and indirectly by causing muscular lesions that could lead to future deformities after repairing in the juvenile fish.

Is vitamin E an effective antioxidant when sea bass larvae are fed on high DHA?

Vitamin E has been proved to be an effective antioxidant protecting sea bass larvae musculature from free radicals injury. In Chapter 3, for a given DHA content, increase of vitamin E from 150 to 300 mg 100 g^{-1} reduced the incidence of muscular lesions, increased growth and survival after a stress. However, when DHA level was up to 5%, the increase in α-tocopherol did not seem to be enough to counteract free radicals effect, observing elevated incidence of muscular lesions, as well as TBARS content and AOE expression (Chapters 4, 5 and 6). The tocopheroxyl radical, resultant of the donation of one electron to the lipid peroxyl radical, reacts slowly with PUFA, however, if

they are not regenerated, tocopheroxyl radicals abstract hydrogen atoms from PUFA, thereby initiating lipid peroxidation (Ingold *et al*., 1993). Thus, it seems that when a 5% of DHA is included in larval microdiets, a great generation of ROS happens, being α-tocopherol not capable of neutralizing them. Besides, vitamin E is not adequately regenerated, thus the tocopheroxyl radicals continue the chain reaction of *in viv*o lipid peroxidation, with α-tocopherol acting as a pro-oxidant. To sum up, vitamin E is an effective antioxidant, but other antioxidants nutrients may be necessary to counteract ROS when levels of DHA of 5% are included in the larval feeds.

What is more effective to prevent muscular dystrophy caused by oxidative stress? To increase dietary selenium or vitamin C?

Increase in both selenium and vitamin C promoted larval growth, and reduced muscular lesions and MDA contents compared to larvae fed an unsupplemented diet. However, differences were observed in AOE expression, suggesting that both antioxidant nutrients act at different levels. Se reduced the mRNA copy numbers of SOD and GPX, to levels comparable to the control group (1/150), whereas vitamin C had no effect on the expression of any AOE.

Selenium proved to be effective by exerting a certain effect on AOE expression. In addition, selenium is incorporated as selenocysteine at the active sites of a wide range of proteins, giving place to several selenoproteins, some of them with a marked antioxidant capacity. Therefore, to date, 18 selenoproteins have been purified or cloned in fish (Tubajeva *et al*., 2000), including four GPX, with different cellular and organic localizations, which represent a major class of functionally important selenoproteins.

One of the main roles of vitamin C as an antioxidant is to regenerate tocopheroxyl radicals, thus it would be logical to think that the antioxidant protection observed in larvae fed the vitamin C supplemented diet, may be due in part to vitamin E. In this sense, higher contents of vitamin E were observed in those larvae fed the diet supplemented with vitamin C, suggesting that vitamin E was being recycled by vitamin C or that the last nutrient was being preferentially employed as an antioxidant, saving in this way α-tocopherol. Besides, vitamin C is considered the most important antioxidant in extracellular fluids (Stocker and Frei, 1991) and is able to inhibit lipid peroxidation initiated by a peroxyl radical in plasma lipids (Frei *et al*., 1989). Moreover, vitamin C is an electron donor for eight different enzymes. Some of the enzymes participate in collagen hydroxylation, adding hydroxyl groups to the aminoacids proline or lysine in the collagen molecule. Finally, although vitamin C levels employed may seem quite elevated, it has

been demonstrated that levels of ascorbic acid higher than those required for growth are necessary to satisfy the demands of larvae in the case of lack of other nutrients, such as vitamin E (Hamre *et al*., 1997).

Thus, as observed in several juvenile and adult fish species, a synergism between vitamin E and selenium (Poston *et al*., 1976; Bell *et al*., 1985) and a sparing mechanism between vitamin E and C (Lovell *et al*., 1984; Sealey and Gatlin, 2002; Shiau and Hsu, 2002; Yildirim-Aksoy *et al*., 2008) was observed. It must be noticed, that it is unknown which type of radical is generated endogenously in a given disease states, therefore, these nutrients may be suitable when high levels of DHA are included in the diet, but may not counteract free radicals properly when other pro-oxidants are employed.

What is the adequate DHA inclusion level?

Foremost, it must be taken into account that the contents of DHA must be always related to the antioxidant content. Thus, increased levels of dietary and tissue DHA require increased dietary supplementation with antioxidants to prevent the occurrence of oxidative damage.

According to the results obtained in the present Thesis, in the first stages (14 to 35 dph) a content of 1% of DHA seems to be adequate, as the best results in terms of growth, survival, incidence of muscular lesions and TBARS content (Chapters 4, 5, 6 and 7). This content seems low if compared to requirement of other larval fish species, such as sea bream (3%; Izquierdo, 2005), turbot (3.2%; LeMilinaire, 1984) or common dentex (*Dentex dentex*) (4%; Mourente *et al*., 1999a). Diet 5/300 supplemented with both selenium and vitamin C were able to reduce the incidence of muscular lesions and improve culture performance, however more studies are needed in this sense to meet the exact combination of each nutrient as well as the form of supplementation, so higher contents of DHA could be employed by young larvae to reach a higher growth rate.

At later stages (35 dph and on), it seems that a 5% of DHA may be suitable to reach a better growth, as has been observed at Chapter 7, although the incidence of muscular lesions was still higher than 1/150 larvae. Maybe at these stages the inclusion of 300 mg 100 g^{-1} of vitamin E would be appropriate to avoid the incidence of muscular lesions, however, this diet was not tested in larvae older than 35 dph and these larvae were fed with a diet containing a 5% of DHA from 14 dph. Attending to the results obtained in Chapter 3, a diet containing a 3% of DHA and 300 mg 100 g^{-1} of vitamin E was the more appropriate to reach a better culture performance and a low incidence of

muscular lesions. Lacking other parameters, from 35 dph on, diet 3/300 seems to be suitable. Thus, an increase in DHA is necessary as larvae are growing. This finding indicates that although larvae have higher metabolic rate than juveniles, the amount of every nutrient in larval diets does not necessarily have to be higher than for juveniles to satisfy the larval physiological demands.

Which pathological changes are involved in nutritional muscle dystrophy in sea bass larvae?

Microscopic lesions observed in the skeletal muscle showed the typical features of nutritional muscle dystrophy, characterized by a segmental necrosis affecting both the red and white fibres. A muscle dystrophy related to deficiencies in vitamin E has been previously described in juveniles and adults from fish species (Lovell *et al*., 1984; Gatlin *et al*., 1986; Frischknecht *et al*., 1994; Bowater and Burren, 2007). However, no information is available in larvae or which are the pathological changes involved in the appearance of these muscular lesions. Semithin sections provided more information about the chronology of nutritional muscle dystrophy (Chapters 4, 5, 6 and 7). One of the most notorious changes was the presence of large clear vacuoles within the cytoplasm, displacing the other elements of the sarcoplasm. Besides, variations in the diameter of the fibres could be observed in transversal sections, as well as a marked oedema between muscular fibres, observing the loss of the normal architecture. Moreover, sarcoplasmic condensation was observed as dark, shrunken muscle fibres that were still surrounded by an intact cell membrane.

TEM sections revealed swelling of the organelles, specially sarcoplasmic reticulum and mitochondria. A well-known consequence of ROS attack is oxidative modification of membrane bound lipids resulting in membrane bilayer damage (Figure 8.1; Farooqui and Horrocks, 1998) together with the inhibition of the bound enzymes responsible for the regulation of cellular metabolism, like Na/K-ATPase. Alteration to these enzymes may cause a massive Ca^{2+}, Na^{+} and water influx to the endoplasmic reticulum, leading to the alteration of this structure and the subsequent formation of vacuoles. Besides the increase in sarcoplasmic Ca^{2+}, may lead to the opening of the mitochondrial permeability transition pore, resulting in loss of matrix components, impairment of mithocondrial functionality and substantial swelling of the organelle with consequent outer membrane rupture and cytochrome *c* release (Green and Reed, 1998; Kolkatowski and Vercesi, 1999). The increase in Ca^{2+} was corroborated by the increased gene expression of μ-calpain (Chapter 7), as intracellular Ca^{2+} concentration stimulates calpains activity (Mongini *et al*., 1988; Hopf *et al*., 1996).

Cythochrome *c* is an apoptosis inducer factor and its release to the sarcoplasm can trigger apoptosis. However, in the present study, no morphological signs of apoptosis were observed. Due to the rapidness of the cellular destruction in apoptosis and that usually involves only scattered individual cells, its morphological analysis is very difficult (Fidzianska, 2002). Thus, the only type of cell death observed in our studies was necrosis, although, apoptosis cannot be excluded from being implicated in sea bass larvae muscle cell death.

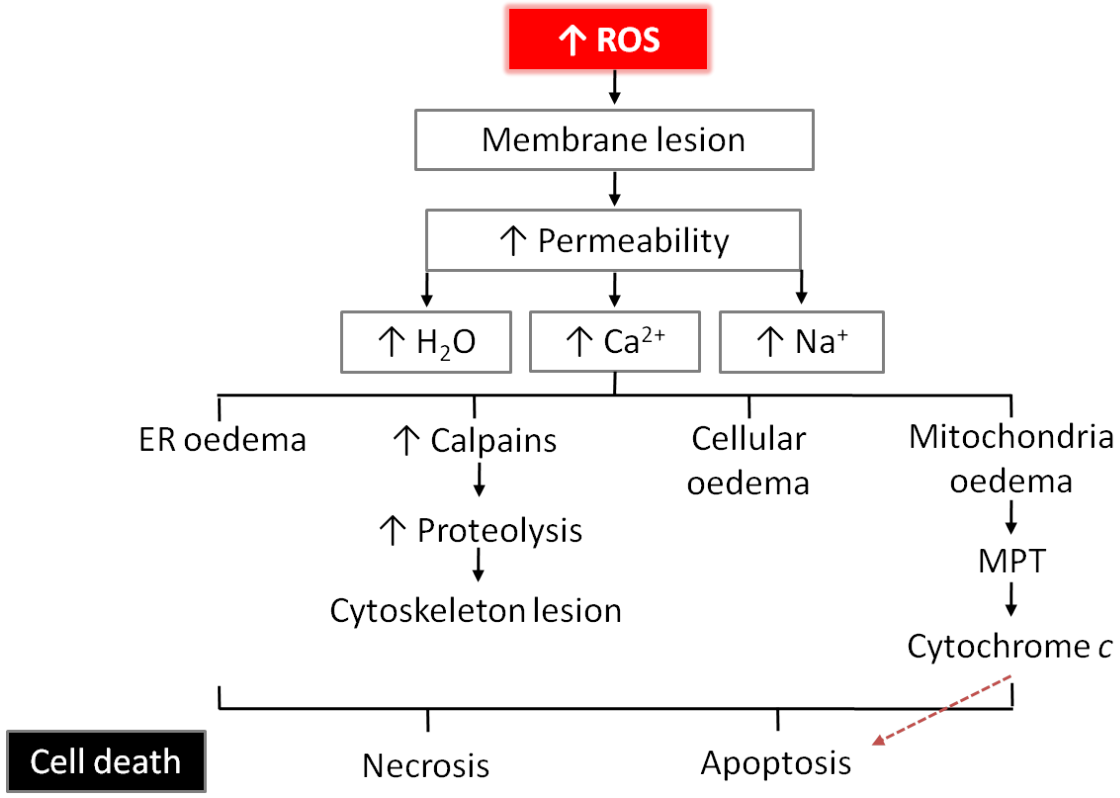

Figure 8.1 Proposed pathological mechanisms on muscle during oxidative stress in sea bass larvae. ROS exert their adverse effects on bilayer membrane phospholipids, altering the fibre permeability and favoring the massive entrance of water, Na^+ and Ca^{2+} and the decrease of K^+. Increase of sarcoplasmic Ca^{2+} causes oedema in several organelles such as endoplasmic reticulum (ER) or mitochondria, apart from whole cell swelling. Increased calcium may activate calpains, which will promote protein degradation, therefore affecting the cytoskeleton integrity. The entrance of water and Ca^{2+} into the mitochondria will lead to the opening of the mitochondrial permeability transition (MPT) pore and the consequent release of cytochrome *c*. If the muscle fibre is not able to counteract all these damages, the cell will die through necrosis or apoptosis, which can also happen due to the direct effect of cytochrome *c*. Free radicals can also attack directly the membranes of the organelles, for instance, the attack to lysosomes membranes may allow the release of enzymes that could digest different cellular components.

In general terms, TEM findings were similar to those found in many mammalian neuromuscular disorders, such as Duchene muscular dystrophy, ischemia or

denervation atrophy. For instance, myelin figures represent endogenous material produced by intracellular lipid peroxidation in mammals (DeGritz *et al*., 1994) and autophagic vacuoles are considered to be secondary lysosomes and are frequent in numerous mammalian neuromuscular disorders (Nishino, 2006). Finally, sarcoplasmic halos are also a common feature of human genetic muscle disorders (Farkas *et al*., 1974). To sum up, an excess in the production of ROS due to the high tissue content of DHA is blamed of causing muscle dystrophy, with features that resemble those observed in mammalian genetic muscle dystrophies.

Is there a muscle regeneration process in fish?

Little is known concerning tissue regeneration in fish, but it seems logical to believe that certain regenerative processes may take place in fish larvae, as they are fast growing organisms and may have the potential to form new cells. One of the studies on muscle regeneration in fish (Rowlerson *et al*., 1997) showed a vigorous regeneration in juvenile sea bream after mechanical injury, with a myosin expression in regenerating fibres resembling that seen in new fibres produced in post-larval white muscle. Similarly, a higher MyHC expression was observed in the present Thesis in larvae fed 5% DHA diets regardless of the vitamin E content (Chapters 5, 6 and 7). In this sense, a positive correlation was found between the incidence of muscular lesions and the number of copies of MyHC mRNA (Chapters 5 and 6). However, α-actin expression did not reflect any regeneration process, in contrast to what has been stated in mammals (Takano *et al.*, 2010). Another indicator of muscle regeneration in mammals is the increase in IGF-I expression (Chargé and Rudnicki, 2004). In Chapters 5, 6 and 7, an increase in IGF-I was related to larvae with the highest incidence of muscular lesions, confirming that these larvae are undergoing muscle regeneration. Besides, IGF-I appears to regulate the satellite cells pool, which are responsible for the regenerative capability of the muscular tissues. Although no quantification of the number or diameter of the satellite cells was performed in the present Thesis, a higher number of these cells seemed to be related with the highest incidence of muscular lesions (personal observation; Chapter 4). In addition, it has been shown that IGFs potently activate cell proliferation and DNA synthesis in zebrafish embryonic cells suggesting that the increase of mRNA copies of IGF-I observed in the present Thesis in larvae with the highest incidence of muscular lesions could be due to the regeneration process carried out by satellite cells.

Do high levels of DHA affect sea bass larvae mineralization?

In the light of the results obtained in the present Thesis the answer is absolutely yes. In contrast to previous reports in which DHA seemed to have a positive effect on mineralization and incidence of deformities on milk fish (*Chanos chanos*; Gapasin and Duray, 2001) and red porgy (*Pagrus pagrus*; Roo et al., 2009), in the present Thesis, increased peroxidation due to elevated levels of DHA lead to reduced mineralization rates and increased percentage of deformities, as has been observed in sea bream larvae (Izquierdo *et al*., submitted). Thus, adequate amounts of dietary DHA are necessary reduce the deformities and favour a right bone mineralization.

On the other hand, the inclusion of organic selenium to diets containing a 5% of DHA did not show to reduce the incidence of skeletal deformities (Chapter 5) in contrast to the addition of vitamin C (Chapter 6). These results are not surprising as they indicate that vitamin C plays a more active role on bone development than selenium. In this sense, a previous work studying the effect of selenium on cod (*Gadus morhua*) larvae bone development showed no effect of this mineral on deformities rates (Penglase *et al*., 2010).

8.3 Future studies

This Thesis demonstrates that excessive doses of DHA have detrimental effects on sea bass larvae, due to an alteration of the oxidative status. Apart from reducing culture performance, the most notorious adverse effect is the appearance of muscular lesions, which can be counteracted by including sufficient amounts of antioxidants, such as vitamin E, C or selenium. However, more studies are needed to elucidate the right combination of these antioxidant nutrients to completely avoid an altered oxidative status in larvae. In this way, higher contents of DHA adequately protected could promote a faster larval growth faster, without the risk of suffering oxidative stress. We proved that the inclusion of 300 mg 100 g^{-1} of α-tocopherol alone was not enough to quench all free radicals when a 5% of DHA was included, however, inclusion of 360 mg 100 g^{-1} of ascorbic acid helped to maintain a balanced oxidative status. The same happened when selenium was employed, suggesting that the combination of these three antioxidants could completely counteract adverse effects caused by free radicals in sea bass larvae. It would also be interesting to study the antioxidant effects of other nutrients such as carotenoids or ubiquinones when high levels of DHA are used. For instance, β-carotene is the most efficient quencher of singlet oxygen known in nature.

In the present Thesis, TBARS were used as an indicator of peroxidative stress in larval tissues. TBARS assay has been extensively used since the 1950s to estimate peroxidation of lipids in membrane and biological systems, as showed by the broad literature that uses TBARS as the only indicator of lipid peroxidation products in human, mammals, fish and food chemistry. However, this method has been criticized for its lack of specificity, especially when used on body fluid and tissue samples. This is due, firstly, to the capacity of aldehydes to form chromogens other than MDA with some absorbance at 532 nm; and secondly, TBARS test usually measure the MDA generated by decomposition of lipid peroxides during the acid-heating stages of the test. Besides, TBA can also react with many other substances such as sugars, aminoacids or bilirrubin. Thus, a more specific method for determining lipid peroxidation should be developed. Some studies in fish have used, as indicators of PUFA oxidation, the 8-isoprostane levels (Mourente *et al*., 2000; Fontagné *et al*., 2006; 2008) or lipid-soluble fluorescent products (Fontagné *et al*., 2008). However these techniques have some limitations as well, for instance, the determination of single 8-isoprostane may seriously underestimate the concentration of substances produced as consequence of oxidative stress, as a myriad of eicosanoids are generated under these conditions (Meagher and Fitzgerald, 2000). In future works, it would be interesting to indicate lipid peroxidation products with a combination of these biochemical techniques together with others, such as determination of 4-hydroxynenal or conjugated dienes. Besides the development of western blot methods to detect MDA or 4-hydroxynenal, natural byproducts of lipid peroxidation seems helpful to indicate alterations of the oxidative status. Moreover, it would be interesting to evaluate the cellular distribution of MDA or 4-hydroxynenal by immunohistochemistry.

Another interesting objective for the future would be to study the role of apoptosis in nutritional muscle dystrophy. Due to the difficulty of detecting apoptosis through TEM, alternative techniques are needed. To aim this, it would be interesting to study the expression of genes implicated in apoptosis, such as caspases or tumour necrosis factor (TNF). Similarly, the study of molecules involved in the inflammatory response in fish larvae to oxidative stress and muscular injuries would be very interesting. For instance the study of several interleukins, inducible nitric oxide synthase (iNOS), transforming growth factor-β or some toll-like receptors could indicate us the role of these molecules in such a young larvae as well as their relationship to oxidative stress damage. The use of immunohistochemistry techniques with antibodies against inflammatory cells could be useful too.

On the other hand, the study of several markers by TEM immunohistochemical (IHC) studies would be interesting to determine the possible role of caveolae in fish muscular membrane lipid oxidation. In the present Thesis (Chapters 5, 6 and 7) the presence of caveolae was evident in larvae fed diets 5/300 (Figure 8.2). Caveolae are membrane specialization found on the surface of cells used to internalize substances. The oxidation of cholesterol due to ROS may cause the release of caveolin, an integral membrane protein, altering the structure of caveolae (Smart *et al*., 1994). With the use of antibodies designed against caveolin, the role of this protein and caveolae in fish muscle membrane oxidation could be elucidated.

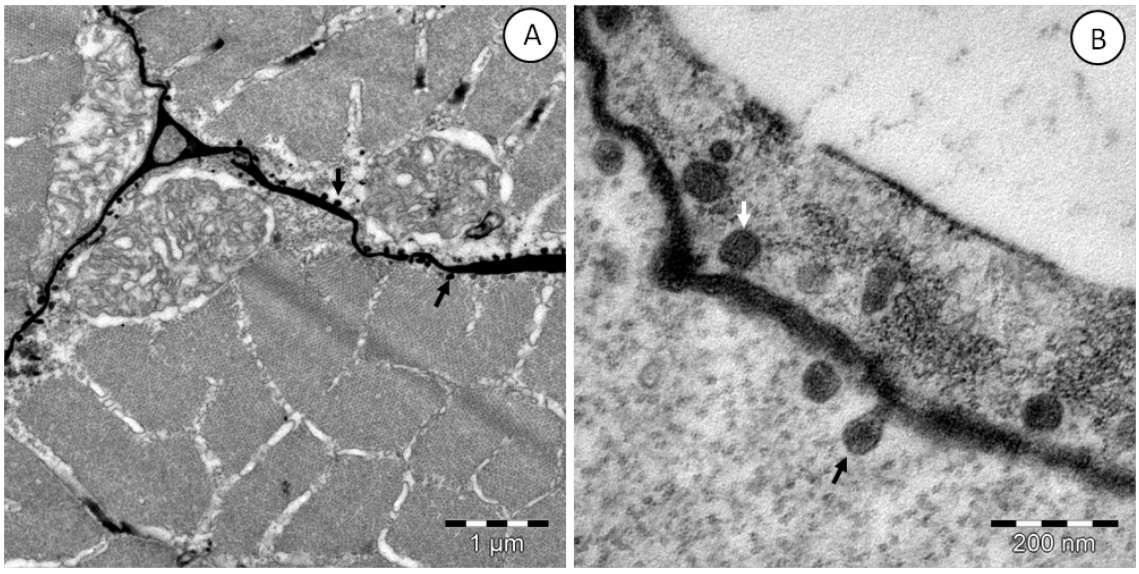

Figure 8.2 Electro micrographs of transversal sections of muscle of sea bass larvae fed 5/300 diet observing the presence of marked caveolae (arrows).

Regarding muscular tissue, it would be very interesting to study in depth the regenerative process that may take place. Along this Thesis, it has been hypothesized that a regenerative process may take place in larvae showing muscular lesions, as indicated by the highest expression of genes related to muscle cell proliferation (IGF-I and II or MyHC). However, no morphological or morphometric results support this hypothesis, only the personal observation of an increased number of satellite cells in larvae with muscular lesions. To reach this, it would be attractive to study the immunohistochemical localization of muscular proteins (actin, myosin, dystrophin, desmin) as well as catalytic enzymes (µ-calpain, m-calpain, cathepsins) or even to perform RT-PCRs or *in situ* hybridization. It would also be very interesting to carry on morphometric analysis of the damaged muscle to study if hypertrophic or hyperplastic

processes are taking place in the injured larvae. The cloning of genes related to muscle myogenesis (MyoD, myf-5, myogenin, MRF) would be remarkable for the study of the presumptive regenerative muscular process.

Dietary ascorbate has been found to have an effect on lipid metabolism in several fish species (Halver, 1989; Ji *et al*., 2003; Ji *et al*., 2010). In Chapter 6, larvae fed a diet supplemented with 360 mg/100g of ascorbil polyphosphate showed a highest content of *n*-3 LC-PUFA, which could indicate that vitamin C supplementation affects the fatty acid metabolism. However, this is only a hypothesis and the study of enzymes related to the lipogenesis and lipolysis would be necessary to confirm it. The study of the content of L-carnitine would be interesting too, as it has been proved that vitamin C can activate the lipid metabolism through carnitine metabolism (Nakagawa *et al*., 2000). On the other hand, the transport of ascorbic acid from the lumen of the intestine and to the interior of the cells thanks to the sodium-dependent transporters 1 and 2 (SCVT1 and 2) is an interesting matter to study.

Results obtained from the present Thesis open up new horizons in selenium research in fish larvae. This Thesis suggests that this mineral may be implicated in other molecular pathways different from being part of GPX enzymes and a great deal of research could be done in this sense. For instance, it would be interesting to study the activity of the membrane proteins that transport selenium into cells, to make clear if it is effectively assimilated by the fish or is partially expelled through the feces. Another interesting study would be the characterization of SEP in fish larvae (sea bass and sea bream), studying their expression to check which one is implicated in antioxidant protection. Similarly, inclusion of different sources of selenium could be used to determine the more suitable type of selenoaminoacid for fish. In this sense, ideally selenium should be supplemented in the form or forms in which it occurs in major fish staple foods (Schrauzer, 2001). XXXX MIRAR FISH NUTRITION!!

Chapter 9

General Conclusions

1. High DHA microdiets lead to reduced growth and survival, increased skeletal deformities and favoured the appearance of muscular lesions in sea bass larvae.

2. Muscular dystrophies appear as a consequence of *in vivo* lipid peroxidation caused by too elevated levels of DHA as indicated by the high TBARS values and the overexpression of the AOE.

3. Microscopical evaluation of the lesions showed cell swelling, flocculated cytoplasm or interfibrillar oedema. This indicates that one of the first alterations takes place at the muscle membrane, probably due to the direct attack of the free radicals to membrane phospholipids, causing alteration of its permeability and leading to cell and organelles swelling. Massive influx of calcium occurs and a consequent activation of μ-calpains causes protein lysis.

4. Ultrathin sections showed diffuse dilatation of sarcoplasmic reticulum, disorganized myofilaments and autophagic vacuoles eventually containing myelin figures and dense bodies, as well as sarcoplasmic halos. No signs of apoptosis were found suggesting that necrosis rather than apoptosis is the cellular death that takes place in fish muscle nutritional dystrophy.

5. When 1 or 3 % of DHA is included into the sea bass larvae diet, increase in vitamin E from 150 to 300 mg 100 g^{-1} enhanced growth and survival. However, levels of DHA of 5% lead to the appearance of nutritional muscular dystrophy in sea bass larvae, with the increase of vitamin E from 150 to 300 mg 100 g^{-1} does not prevent its appearance.

6. Levels of DHA of 5% and 300 mg 100 g^{-1} of vitamin E strongly induced the expression of the antioxidant enzymes, in an attempt to dismutate reactive oxygen species and defend cells from their attack.

7. Inclusion of organic selenium (5 mg kg^{-1}) to diets with a 5% of DHA and 300 mg 100 g^{-1} of vitamin E controlled *in vivo* lipid peroxidation, decreased the incidence of muscular lesions and increased growth when compared to the unsupplemented diets, but had no effect of skeletal deformities. No direct effect was observed of Se supplementation on the expression of glutathione peroxidase.

8. Addition of 180 mg 100 g^{-1} of vitamin C to a high oxidation risk diet helped to reduce oxidative stress in sea bass larvae by enhancing growth and reducing the incidence of muscular lesions and cranial deformities. The lack of effects on antioxidant enzyme expression indicated that vitamin C acts as an antioxidant through another mechanism.

9. A compensatory muscle growth has been found in larvae fed high dietary contents of DHA, as showed by the increase in the expression of insulin-like growth factors and myosin heavy chain, as well as the abundant satellite cells observed around the damaged muscular fibres.

De acuerdo con lo establecido en el artículo 2 del Acuerdo del Consejo de Gobierno de la Universidad de Las Palmas de Gran Canaria, del 13 de Octubre del 2008, por el que se aprueba el reglamento para la elaboración, tribunal, defensa y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, al tratarse de una tesis escrita en una lengua distinta a la castellana, se añade un apartado en castellano en el que se incluyen los objetivos, planteamiento y metodología, aportaciones originales, así como conclusiones obtenidas.

Capítulo 10

Resumen ampliado en español

Estrés oxidativo en larvas de lubina (*Dicentrarchus labrax*) alimentadas con niveles altos de DHA. Implicación de varios nutrientes antioxidantes.

10.1 Introducción General

10.1.1 Producción acuícola

La sobreexplotación de áreas pesqueras tradicionales y las restricciones impuestas por los cambios en las políticas y acuerdos de las áreas de pesca internacionales, han causado una reducción del aporte de productos de origen marino, causando un incremento en las importaciones de pescado. De acuerdo con las previsiones de la FAO, la acuicultura se considera un complemento ideal a la pesca extractiva, pudiendo satisfacer de este modo la alta demanda de productos de origen marino. De hecho, la acuicultura supuso un 47,9% del total del aporte de alimentos en el año 2008, representando un incremento continuado desde el 2006 (FAO, 2011). En este sentido, la acuicultura continúa siendo un importante sector que ofrece alimento con un alto nivel de proteína, cuya producción global ha aumentado desde los 7,3 millones de Tm en 1980 a los 55,1 millones de Tm producidas en 2009 (FAO, 2011). Este incremento exponencial en su producción se ha conseguido gracias a la mejora del control de los reproductores, al desarrollo de dietas óptimas e innovaciones tecnológicas, lo cual ha permitido el rápido desarrollo de las instalaciones tanto en tierra como en el mar. Sin embargo, las tasas de crecimiento de la producción acuícola han disminuido, variando ampliamente entre regiones. Por ejemplo, países que una vez lideraron el desarrollo de la acuicultura, como Francia, Japón o España, han visto disminuida su producción en las últimas décadas (FAO, 2011). Así mismo, la media

mundial de producción en acuicultura en Europa y en el Norte de América ha disminuido desde el año 2000 un 1,7% y un 1,2% respectivamente.

En el año 2008, la acuicultura europea produjo 1,2 millones de Tm (FAO, 2011), con un valor de mercado de 3,8 millones de euros. Sin embargo, la tasa de producción de la acuicultura ha ido disminuyendo desde 1988 y no ha sido capaz de cubrir la reducción de las capturas pesqueras. España es el país europeo con mayor producción en toneladas (249,070 Tm; 19.5%), seguido por Francia (237,870 Tm; 18.6%) e Italia (181,470 Tm; 14.2%). Las principales especies marinas producidas en el sur de Europa son la dorada (*Sparus aurata*) y la lubina (*Dicentrarchus labrax*) (Figura 10.1), con una producción de 89,3 y 58,5 Tm respectivamente, siendo las Islas Canarias el mayor productor español (32%; APROMAR, 2010). Para mantener esta alta tasa de producción, las plantas dedicadas a la cría larvaria deben proveer alevines de calidad. En el 2009 en España había 16 plantas de producción de larvas que producían 69,3 millones de alevines de lubina y dorada, suponiendo una disminución de la producción del 26,5% respecto a años anteriores. Además, esta cantidad no puede cubrir la demanda de las granjas, por lo que se hace necesario importar alevines de otros países europeos como Francia, Italia o Grecia. Hay que mencionar que la acuicultura de las Islas Canarias depende completamente de las importaciones, puesto que no existe ninguna planta local comercial de cría larvaria.

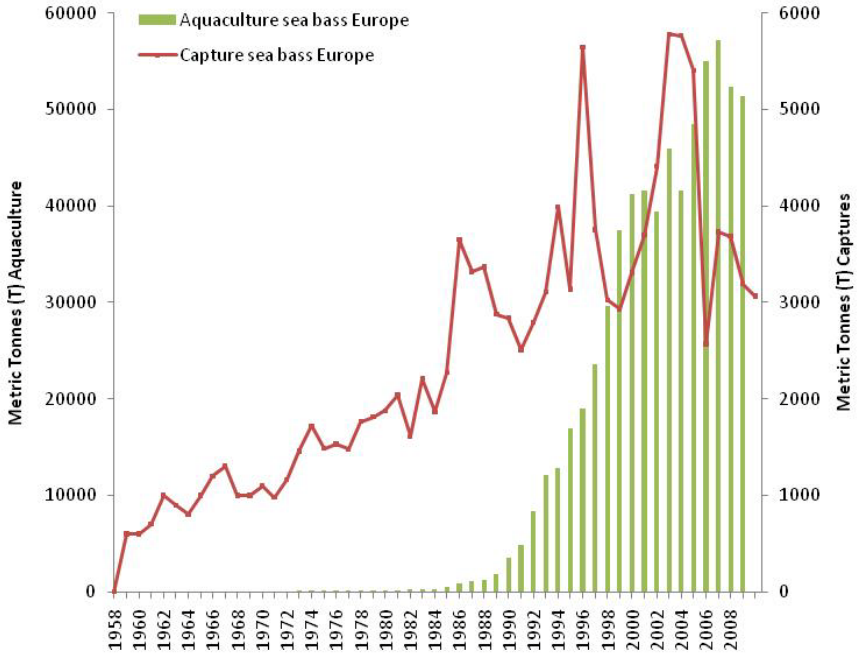

Figura 10.1 La producción de la acuicultura frente a las capturas de lubina en Europa desde 1958 hasta el 2009 (datos obtenidos de FAO FISHSTAT Plus 2.30).

10.1.2 Técnicas de cultivo y consideraciones de producción

Hoy en día la producción de larvas sigue siendo uno de los cuellos de botella en la acuicultura. Esto es debido a que las larvas de peces marinos no están completamente desarrolladas cuando eclosionan, siendo muy susceptibles de padecer altas tasas de mortalidad (más del 75%) durante los primeros días de vida. La eclosión de los huevos de peces marinos acontece tras algunos días, dependiendo de la temperatura del agua, dando paso a un segundo estadio embrionario (Figura 10.2). Las reservas vitelinas están constituidas principalmente por lipoproteínas y, en algunas especies, como la lubina, por una gota lipídica (Sargent *et al.*, 2002). Estos componentes se usan básicamente en procesos de formación de tejidos o para acometer el gasto energetico derivado de la natación. La fase embrionaria termina cuando se abre el esófago, comenzando así la fase larvaria en la que el animal tiene capacidad para ingerir alimento exógeno (Figura 10.2). La apertura de la boca es una de las etapas más críticas en la cría larvaria y ocasiona una mortandad importante. En el cultivo de la mayoría de especies marinas, se administran presas vivas durante las primeras semanas de vida, para posteriormente introducir gradualmente una dieta inerte (Fernández-Diaz y Yufera, 1997; Kolkovski *et al*., 1997). Las presas vivas constituyen un conjunto heterogéneo de organismos perteneciente a distintos grupos de fitoplancton y zooplancton, tal como crustáceos, moluscos o protozoos. Los rotíferos (*Brachionus* sp.) y la *Artemia* sp, se usan de manera común como alimento en los estadios larvarios de especies de peces. Los rotíferos se suelen suministrar durante los primeros días de alimentación exógena, mientras que los nauplios de *Artemia* se emplean cuando las larvas alcanzan una talla mayor. El uso de diferentes tipos de rotíferos se debe a la sencillez de su cultivo masivo, su adecuado tamaño para adaptarse a las dimensiones de la boca de la larva, sus hábitos planctónicos, lentos movimientos y la posibilidad de controlar su aporte nutritivo. Sin embargo, el valor nutritivo de esta presa viva varía notablemente en función de su enriquecimiento y condiciones medioambientales como la intensidad lumínica, la temperatura o la salinidad. Como ha sido mencionado previamente, los rotíferos se reemplazan a partir de los 9 días post eclosión (dph) en adelante por los nauplios de *Artemia* sp. y posteriormente por dietas secas de diferentes dimensiones (Barnabé, 1974). Debido al gran tamaño de su boca, las larvas de lubinas pueden ser alimentadas directamente con nauplios de *Artemia* sp. al abrir la boca.

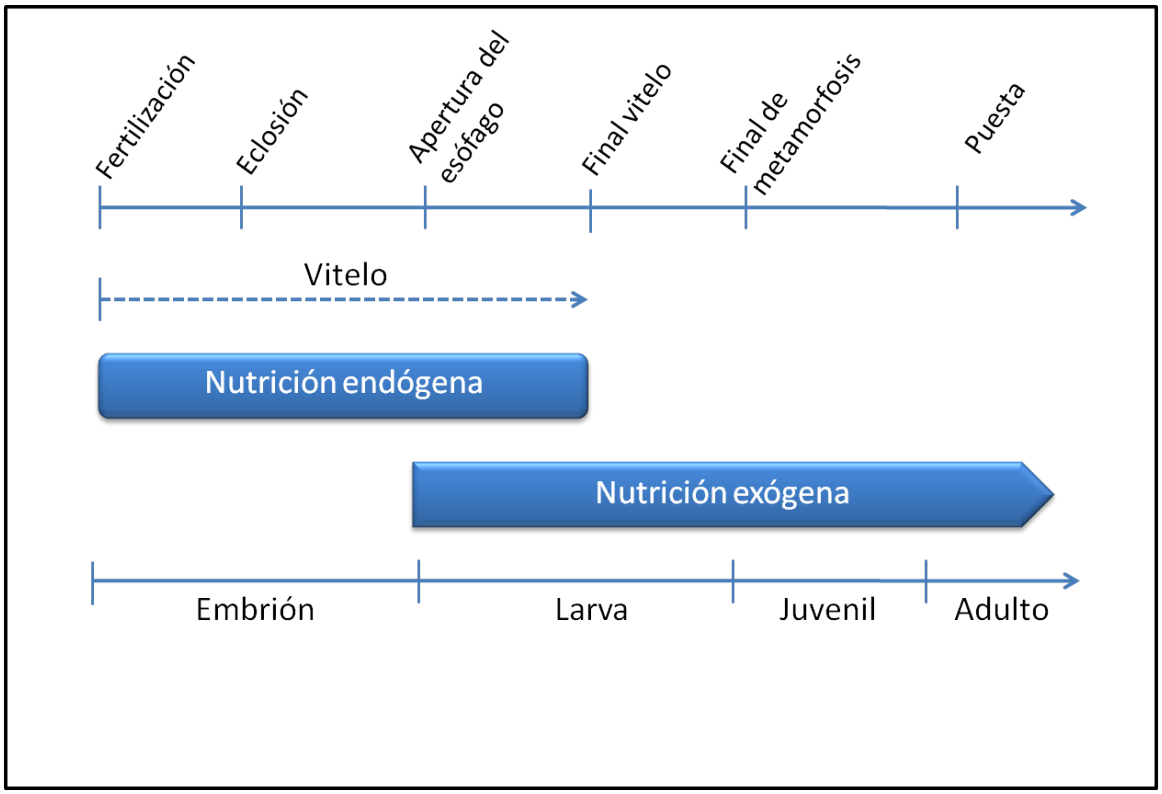

Figura 10.2 Estadios de desarrollo y modos de nutrición larvaria. Adaptado de Gatesoupe *et al.*, 2001.

Hay que tener en cuenta que ni los rotíferos ni la *Artemia* son las presas naturales de las larvas de peces marinos, lo que frecuentemente causa problemas en la producción de larvas provocando alta mortandad. Por tanto, las mejoras del cultivo larvario, y especialmente de la nutrición, siguen siendo uno de los principales objetivos de la acuicultura para garantizar una actividad económica competitiva.

10.1.3 Uso de microdietas inertes para larvas de especies marinas

El esfuerzo extra en términos de trabajo de los operarios, infraestructuras, tiempo y energía para cultivar las distintas especies de rotíferos y *Artemia* representa un coste significativo. En este respecto, Person-Le Ruyet y colaboradores (1993) calcularon el coste necesario para alimentar a larvas de lubina con presas vivas. Las presas vivas supusieron el 79% del coste de producción total de juveniles de 45 días de edad.

Además, en los primeros tres meses de vida, las presas vivas supusieron el 50% del coste de alimentación, aunque sólo representaron el 1,6% del total de comida requerido en peso seco.

Así mismo, el aporte y la calidad nutricional de las presas vivas puede variar, por lo que las larvas podrían encontrarse en condiciones nutricionales sub-óptimas (Jones *et al*., 1993; Kolkovski *et al*., 1993; Barnabé y Guissi, 1994; Roselund *et al*., 1997). Por otro lado, desde un punto de vista experimental, no es posible enriquecer presas vivas con determinados tipos de nutrientes como fosfolípidos o proteínas de calidad, puesto que están determinados genéticamente y no pueden ser alterados con el enriquecimiento (Roselund *et al*., 1997; Koven *et al*., 2001). Teniendo en cuenta que ni los rotíferos ni la *Artemia* son las presas naturales de las larvas de peces marinos, la sustitución de presas vivas por microdietas es crucial para rebajar el precio de la producción y garantizar el suministro continuado de juveniles de alta calidad.

Una disminución de la producción de larvas se ha observado al emplear dietas inertes desde el inicio de la alimentación exógena. Esto podría ser debido a la composición, palatabilidad, características físicas de la microdieta (Person Le Ruyet *et al*., 1993) o a la incapacidad de las larvas para digerir el alimento (Holt, 1993; Kolkovski *et al*., 1993; Walford y Lam, 1993; Zambonino-Infante y Cahu, 1994; Kolkovski, 2001). Además, al suministrar una dieta inerte podemos empeorar la calidad del agua si no controlamos la cantidad de alimento empleado (Leu *et al*., 1991). Sin embargo, los resultados obtenidos utilizando dietas inertes pueden mejorar si se combinan con alimento vivo (co-alimentación) desde la primera toma (Fernández-Díaz y Yúfera, 1997; Kolkovski *et al*., 1997; Rosenlund *et al*., 1997; Sandel *et al*., 2010).

Las dietas microparticuladas pueden ser preparadas de tres formas: microligadas (microbound), microcubiertas (microcoated) o microencapsuladas. En las dietas microligadas, los ingredientes pulverulentos se ligan con una matriz acuosa tal como agar, carragenina o alginato de calcio (López-Alavarado *et al*., 1994). También se puede emplear una matriz de proteína como caseina o zeína (Person-Le Ruyet *et al*., 1993). Este tipo de microdieta no está indicado para especies de agua dulce, dado que su capacidad para romper y digerir la matriz de carragenina es limitada (Gawlicka *et al*., 1996). Las dietas microcubiertas se producen empleando un aglutinante glucídico (carragenina, alginato) o proteico (gelatina, zeína). Las dietas microencapsuladas pueden definirse como dietas microparticuladas hechas mediante la encapsulación enuna solución coloide o suspensión de los ingredientes de la dieta, dentro de una membrana (Yufera *et al*., 1999). La microencapsulación produce micropartículas

solubles en agua y de forma regular, sin embargo, como inconveniente cabe destacar que son difíciles de digerir.

La producción de dietas microparticuladas podría ser una alternativa conveniente y económica a las presas vivas, a pesar de los problemas de pérdidas de nutrientes por lixiviación y su estabilidad en el agua (Baskerville-Bridges y Kling, 2000; Onald y Landgon, 2000; Poussao-Ferreira *et al.,* 2003). Los componentes nutricionales de las microdietas deberían de establecerse en base a los requerimientos de proteínas, aminoácidos, lípidos, carbohidratos, vitaminas y minerales de las larvas. Es más, el desarrollo eficiente de microdietas para larvas lleva emparejado el desarrollo de estudios de los requerimientos nutricionales.

El nivel óptimo de proteína para las larvas de peces parece variar debido a diversos factores, como diferencias en los hábitos de alimentación, edad de la larva, temperatura del agua, fuentes de proteína empleada, así como del nivel energético de la dieta. Dado que los requerimientos de las larvas de peces no están definidos, para la elaboración de las microdietas se emplean fuentes altamente proteicas como harina de krill, de calamar, de vieira o de pescado (Teshima *et al*., 1982). Los carbohidratos son considerados como la fuente de energía más barata para la acuicultura, aunque su utilización por los peces varía y es poco conocida (Kanzawa, 2003). Los conocimientos sobre los requerimientos de vitaminas son limitados. La mayoría de estudios de requerimientos vitamínicos se han llevado a cabo en juveniles, existiendo pocos estudios en larvas, debido principalmente a la falta de una microdieta adecuada. Por otro lado, son numerosos los estudios llevados a cabo para estudiar la composición óptima de lípidos en dietas formuladas para larvas de especies marinas, prestando especial atención a los requerimientos de fosfolípidos y de ácidos grasos poliinsaturados de cadena larga (LC-PUFA) (Takeuchi, 1997). Los lípidos presentes en las microdietas vienen en parte de las harinas incorporadas en la dieta como fuentes de proteína. Otros lípidos, como aceite de hígado de bacalao, de las huevas o de sardina lacha se añaden a la microdieta como fuente de triglicéridos, mientras que los fosfolípidos pueden ser de origen terrestre (lecitina de soja) o marino (fosfolípidos de krill o de pescado). Para tener un mejor control de la fracción lipídica de la dieta, se pueden desengrasar las harinas, por lo que los lípidos de la misma derivarán únicamente de los añadidos.

10.1.4 Importancia de los lípidos para la nutrición de larvas de peces marinos

Los lípidos son junto con las proteínas los principales constituyentes orgánicos en los peces, siendo los carbohidratos menos importantes. Es más, el contenido lipídico puede exceder al de proteínas, denotando la importancia de los lípidos en los peces. Los lípidos son una fuente importante de energía metabólica, constituyen una fuente rica de fosfolípidos, los cuales son componentes de membranas biológicas (Sargent *et al*., 1989). Los lípidos de la dieta también sirven como transportadores para la absorción de algunos nutrientes, incluyendo las vitaminas liposolubles A, D, E y K, así como pigmentos naturales o sintéticos. Por otra parte, los lípidos son precursores de la síntesis de varios metabolitos funcionales, como las prostaglandinas. Además, los lípidos tienen una gran importancia para las larvas de peces teleósteos, ya que estas tienes tasas de crecimiento extremadamente altas, hecho que está unido a altas demandas de energía así como de elementos estructurales (Conceiçao, 1997). Es más, se sabe que existen relaciones entre los lípidos de la dieta y los procesos de formación esquelética en los animales acuáticos (Cahu y Zambonino-Infante, 2003; Villeneuve *et al.*, 2005, 2006; Sandel *et al*., 2010; Izquierdo *et al*., enviado). Como muestra de esta importancia, cabe decir que las dietas para especies de peces marinos como la dorada o la lubina, se han convertido en altamente energéticas (~25% de lípidos) en comparación con las de hace una década (~12% de lípidos) (Izquierdo *et al*., 2003). Por tanto, el papel fundamental de los lípidos en la nutrición de larvas de peces les ha convertido en el foco de numerosos estudios. Por lo tanto, el éxito del cultivo larvario está claramente influenciado por la primera alimentación, así como por la calidad nutricional de las dietas empleadas, estando reconocidos los lípidos como el factor nutricional más importante capaz de afectar al crecimiento y a la supervivencia de las larvas (Izquierdo *et al*., 2000).

Sin embargo, la utilización de los lípidos de la dieta por parte de la larva puede estar directa o indirectamente afectada por diferentes cambios morfológicos y fisiológicos que tienen lugar durante el desarrollo larvario. Por ejemplo, aunque al final de la fase lecitotrófica los enterocitos de la lubina son funcionales, aún están poco desarrollados ya que el tamaño, número y expansión de los orgánulos se incrementará en los días venideros (Deplano *et al*., 1991; Zambonino-Infante *et al*., 1997). Por tanto, a medida que la larva se va desarrollando, el número de vellosidades intestinales también irá incrementando y el estómago se desarrollará, mejorando así su función. Estos cambios en los enterocitos y en el sistema digestivo se traducen en una mejora de la digestión y de la eficiencia de absorción en los juveniles. Otro hecho destacado es que el nivel de lípidos podría afectar a la capacidad de digestión. Hoehne (1999) y Olsen y

colaboradores (2000) señalaron que un nivel alto de lípidos en la dieta puede conducir a una menor capacidad de digestión por parte de la larva. En este sentido, Kjørsvik *et al*., (1991) indicaron que larvas de rodaballo alimentadas con rotíferos enriquecidos con alto contenido de lípidos presentaban una sobrecarga de la capacidad digestiva del intestino posterior. Por el contrario, Zambonino-Infante y Cahu (1999) demostraron una maduración de los enterocitos más rápida, así como un mejor desarrollo larvario en lubinas alimentadas con niveles altos de lípidos.

Los lípidos están constituidos por ácidos grasos (AG), siendo los peces ricos en LC-PUFA, los cuales poseen cadenas de carbono de 20 o más átomos y 3 o más dobles enlaces etilénicos. Los peces requieren de tres LC-PUFA para conseguir un crecimiento y desarrollo normales: el ácido docosahexaenoico (DHA; 22:6*n*-3), el ácido eicosapentaenoico (EPA; 20:5*n*-3) y el ácido araquidónico (ARA; 20:4*n*-6). La esencialidad de estos AG se demuestra por la capacidad de ser retenidos a lo largo del desarrollo embrionario (Rainuzzo *et al*., 1993; Lie, 1993) o a expensas de otros AG durante periodos de ayuno (Tandler *et al*., 1989). Además, estos AG se consideran esenciales en los peces marinos debido a la limitada actividad de las enzimas Δ5- y Δ6-desaturasa y elongasa para sintetizar ARA, EPA y DHA cuando se incluyen sus precursores en la dieta, al contrario de lo que ocurre en especies de agua dulce (Figura 10.3). En este sentido, aunque se ha encontrado cierta expresión de la Δ6-desaturasa en las larvas de dorada, su actividad no fue suficiente para cubrir los requerimientos de este ácido graso (Izquierdo *et al*., 2008).

Las funciones bioquímicas, celulares y fisiológicas de estos tres LC-PUFA son prácticamente las mismas entre peces y vertebrados y se engloban en dos categorías:

(1) Papel en el mantenimiento de la estructura e integridad funcional de las membranas: Los tejidos de los peces tienen en términos generales mayores cantidades de DHA y EPA que de ARA, y estas mayores concentraciones se correlacionan con los requerimientos de estos ácidos grasos.
(2) Precursores de un grupo de moléculas biológicamente muy activas, conocidas como eicosanoides: Los eicosanoides son un conjunto de compuestos C20 biológicamente muy activos producidos en muy pequeñas cantidades por cada tejido del organismo. Están implicados en una gran variedad de funciones fisiológicas y se producen en respuesta a condiciones estresantes. El principal precursor de eicosanoides en los peces es el ARA, produciendo además compuestos biológicamente más activos que los producidos por el EPA.

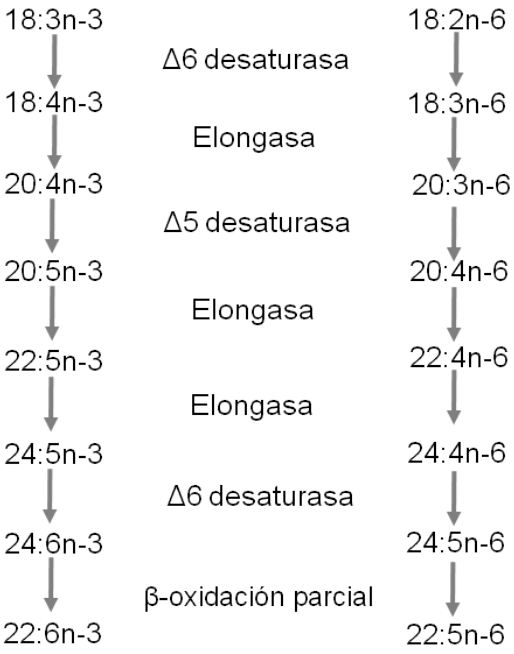

Figura 10.3 Principales rutas de biosíntesis de LC-PUFA a partir de sus precursores C18, ácido α-linoleico (18:2*n*-6) y γ-linolénico (18:3*n*-3). La enzima Δ6 desaturasa actúa en dos pasos, mientras que la Δ5 desaturasa lo hace sólo en uno.

Por tanto, en las dietas de peces marinos se deben incluir cantidades adecuadas de lípidos, especialmente de LC-PUFA. Como ya ha sido señalado previamente, la acuicultura es el sistema de producción animal con el desarrollo más rápido. Para mantener un desarrollo tan elevado, hay un incremento de las demandas de harina y aceite de pescado, sin embargo, el uso de ingredientes de origen vegetal terrestre es necesario para cubrir la demanda de fuentes lipídicas. Este tipo de ingredientes carece de ácidos grasos esenciales y este hecho limita su utilización en dietas para especies marinas. Por tanto, los requerimientos de estos AG deben ser determinados con precisión para determinar sus niveles óptimos de inclusión. Para estudiar los requerimientos de estos AG esenciales (AGE) hay que tener en cuenta algunas premisas:

- Las notorias similitudes químicas entre los tres LC-PUFA hace que existan interacciones competitivas en las reacciones fisiológicas y bioquímicas en las que se ven envueltos estos AG y sus precursores.

- Los peces marinos tienen una capacidad limitada para sintetizar AGEs a partir de sus precursores a través de enzimas elongasas y desaturasas.
- Los requerimientos de estos AGE podrían verse afectados tanto cuantitativa como cualitativamente por factores medioambientales como la temperatura (Farkas *et al*., 1980; Olsen *et al*., 1999), la salinidad (Borlogan y Benítez, 1992) y la luz (Ota y Yamada, 1971).
- Como las larvas crecen más rápido que los juveniles o que los adultos, se espera que el requerimiento de *n*-3 LC-PUFA sea mayor en las primeras etapas de desarrollo que en juveniles (Izquierdo *et al*., 1989a).

La necesidad de estos AG fue señalada por primera vez tras observar el reducido crecimiento en larvas de rodaballo (*Scophtalmus maximus*) (Gatesoupe y Le Milinaire, 1985), dorada del Japón (*Pagrus major*) (Izquierdo *et al*., 1989a, 1989b) y dorada (Rodríguez *et al*., 1993, 1994; Salhi *et al*., 1994) alimentadas con niveles deficientes de estos AG. Así mismo, se observó una disminución de la supervivencia cuando las larvas ingerían bajos niveles de estos AGs en dorada del Japón (Izquierdo *et al*., 1989a, 1989b), dorada (Rodríguez *et al*., 1993, 1994; Salhi *et al.*, 1994) y fletán (*Hippoglossus hippoglossus*) (Holmefjord y Olsen, 1991). Por otro lado, inadecuadas cantidades de estos AG en la dieta dan lugar a la aparición de distintas alteraciones en las larvas como una disminución de las actividades natatorias y de la alimentación (Izquierdo *et al*., 1989; Rodríguez *et al*., 1993, 1994), alteraciones en el comportamiento (Benítez-Santana *et al*., 2007), hidropesía (Yamashita, 1981), inflación defectuosa de la vejiga natatoria (Koven, 1991), pigmentación anormal (Kanazawa, 1993; Rainuzzo *et al*., 1994), desagregación del epitelio branquial (Arnaiz *et al*., 1993) o inmunodeficiencia e incremento de los niveles de cortisol (Izquierdo, 1996).

Existen interacciones competitivas entre el DHA y el EPA y el EPA y el ARA. La competición entre el DHA y el EPA radica en que ambas moléculas usan las mismas enzimas para esterificar ácidos grasos en fosfolípidos (Mourente *et al*., 1991; Sargent *et al.*, 1999). Sin embargo, en todas las larvas de peces marinos el DHA ha demostrado ser superior al EPA en conferir vitalidad a las larvas (Watanabe, 1993). Además, aunque se sabe que ambos AG son esenciales para las larvas de peces marinos, muchas especies han demostrado tener una mayor especificidad de membrana por el DHA que el EPA (Rodríguez *et al*., 1997; Copeman *et al*., 2002) demostrando la esencialidad de este AG respecto a los otros AGE.

10.1.5 Importancia del DHA en larvas de peces

El DHA es un ácido carboxílico con una cadena de 22 carbonos y seis dobles enlaces *cis*. Como ha sido indicado anteriormente, las larvas de especies marinas tienen una capacidad limitada, casi nula, para sintetizar AGEs a partir de sus precursores, los ácidos linoleico y linolénico, por lo que los alimentos para larvas deben contener cantidades adecuadas de los mismos. Así mismo, aportar un correcto nivel de DHA es muy importante para las larvas de peces, dado su rápido crecimiento y desarrollo (Tabla 10.1). La composición de la larva refleja los altos requerimientos por el DHA, el cual se incorpora principalmente en los tejidos visuales y neurales en desarrollo (Mourente, 2003), que suponen un gran porcentaje de tejido en comparación con su pequeña masa corporal.

Tabla 10.1 Requerimientos de DHA para larvas de distintas especies marinas

Especies	Parámetro	Requerimiento (%dieta peso seco)	Referencia
Calotomus japonicus	Tasa de crecimiento	1-2	Kanazawa, 1993
Dentex dentex	Tasa de crecimiento Supervivencia	4	Mourente *et al*., 1999
Latris lineata	Tasa de crecimiento Supervivencia Comportamiento	1,3	Brandsen *et al*., 2004
Pagrus major	Tasa de crecimiento Supervivencia Test salinidad	0,95-1,62	Furuita *et al*., 1996a
Pagrus pagrus	Tasa de crecimiento Supervivencia	3,4	Hernández-Cruz *et al*., 1999
Paralychthis olivaceous	Tasa de crecimiento	1	Kanazawa, 1995
Pseudocaranx dentex	Tasa de crecimiento	1,6-2,2	Takeuchi *et al*., 1996
Pseudopleuronectes herzensteini	Tasa de crecimiento Supervivencia Resistencia ayuno Desarrollo larvario	0,6 Rotíferos 1,4-2,8 *Artemia*	Sato y Takeuchi, 2009
Sparus aurata	Tasa de crecimiento Supervivencia	>3	Izquierdo, 2005
Scophtalmus maximus	Tasa de crecimiento	3,2	Le Milinaire, 1984
Seriola dumerilii	Tasa de crecimiento Supervivencia	4	Izquierdo, 2005

La particular estructura del DHA le confiere muchas funciones importantes en el metabolismo de los peces (Izquierdo, 2005). El DHA se incorpora en las membranas celulares para regular su integridad y función, además de ser un componente importante en los fosfogliceridos en las larvas, formando principalmente parte de la fosfatidiletanolamina y la fosfatidilcolina. Además, la esencialidad de este AG está

corroborada por la retención del mismo en larvas sometidas a inanición o alimentadas con niveles bajos de AGE, lo que puede ser debido a la baja tasa de oxidación del DHA en comparación con otros AG (Koven *et al*., 1989; Madsen *et al*., 1999). Por otro lado hay que tener en cuante que el DHA parece tener mayor potencial que el EPA para promover el crecimiento y la resistencia al estrés en larvas de pargo japonés (Watanabe *et al.,* 1989; Watanabe y Kiron, 1994). Además, sus requerimientos son más limitantes para el crecimiento y la supervivencia que los de los otros *n*-3 LC-PUFA (Izquierdo, 1996).

La importancia del DHA para las larvas de peces marinos comienza antes de la eclosión. Los huevos contienen una adecuada cantidad de DHA (Laurel *et al*., 2010) para asegurar que la larva tenga un adecuado desarrollo a la eclosión, y esto puede modificarse mediante el control de la alimentación de los reproductores (Fernández-Palacios *et al*., 2011). Por tanto, el DHA, vital para la supervivencia y el desarrollo de las larvas recién eclosionadas, está determinado por los lípidos que derivan directamente del aporte de la dieta de los reproductores en el periodo que precede a la gonadogénesis (Kjørsvik *et al*., 1990; Sargent, 1995). Sin embargo, Watanabe (1993) afirmó que el contenido de DHA en larvas de especies marinas disminuía rápidamente durante los primeros 10 días tras la eclosión. Por tanto, para mantener el contenido de DHA en las larvas, se debe aportar altos contenidos de este AG para mantener un adecuado crecimiento.

La deficiencia de este AG causa alteraciones del sistema nervioso del pez, tal como problemas en la visión a bajas intensidades de luz en el arenque (*Clupea harengus*) (Bell *et al*., 1995) o desarrollo tardío del comportamiento en larvas de medregal del Japón (*Seriola quinqueradiata*) (Masuda *et al*., 1988), *Latris lineata* (Brandsen *et al*., 2005) y dorada (Benítez-Santana *et al*., 2007). Además, bajos niveles dietéticos de DHA incrementaron la incidencia de deformidades esqueléticas en larvas de lubina (Cahu *et al*., 2003), dorada (Roo *et al*., 2009) o acedía de Japón (*Pseudopleuronectes herzensteini*) (Satoh y Takeuchi, 2009), redujeron la tolerancia al estrés en larvas de beluga (*Huso huso*) (Jalali *et al*., 2008), causaron malpigmentación y migración irregular del ojo en larvas de peces planos (McEvoy *et al*., 1998; Bell *et al*., 2003) o alteraciones de la estructura de hígado e intestino en *Latris lineata* (Brandsen *et al*., 2005). Pero quizás el principal efecto negativo observado está relacionado con el bajo rendimiento del cultivo y supervivencia en larvas de distintas especies (Watanabe *et al*., 1989; Furuita *et al*., 1996a, b; Copeman *et al*., 2002; Rezek *et al*., 2010).

No sólo el déficit de DHA puede causar alteraciones en los peces, también niveles excesivos de este AG pueden generar efectos adversos en los peces, especialmente si el incremento de *n*-3 LC-PUFA no viene acompañado por cantidades adecuadas de antioxidantes. Por ejemplo, larvas de lubina alimentadas con altos niveles de LC-PUFA y bajo contenido de vitamina A, mostraron una aceleración en la diferenciación de los osteoclastos, así como vértebras supranumerarias (Villeneuve *et al*., 2006). Por tanto, cuando se incorporan niveles elevados de *n*-3 LC-PUFA en la dieta, el suplemento de antioxidantes, como la vitamina E, podría ayudar a mejorar el rendimiento. Por ejemplo, larvas de beluga alimentadas con *Artemia* enriquecida con niveles altos de LC-PUFA, mostraron un mejor rendimiento, en términos de crecimiento y tolerancia al test de salinidad, cuando se incluyó un 20% de α-tocoferol en el medio de enriquecimiento (Jalali *et al*., 2008). Igualmente, Stéphan *et al.* (1995) señalaron que la oxidación *in vivo* e *in vitro* de los lípidos en el músculo de rodaballo disminuía cuando se añadía a la dietaα -tocoferol. La adición de antioxidantes se hace necesaria ya que la susceptibilidad de un AG poliinsaturado en concreto a oxidarse depende del número de enlaces insaturados en la cadena lipídica (Nagaoka *et al.,* 1990). Por tanto, el DHA será más susceptible a la oxidación que cualquier otro PUFA.

Tradicionalmente, los enriquecedores comerciales se han formulado añadiendo grandes cantidades de DHA para enriquecer las presas vivas (Koven *et al*., 2001). La razón por la que se incluyen estas altas cantidades responde a la capacidad limitada de las larvas de peces marinos para convertir el ácido linolénico en DHA. Esta tendencia se ha seguido en la formulación de microdietas para larvas, de acuerdo a la premisa de que contenidos altos de DHA potenciarán el rendimiento del cultivo larvario. Por tanto, el gasto económico es elevado debido a la obligación de incluir aceites concentrados en DHA en las microdietas. Sin embargo, se ha demostrado que niveles demasiado elevados de este AGE podrían tener efectos adversos en las larvas o incluso no tener ningún efecto positivo, por tanto, es necesario hacer una revisión sobre la necesidad de incluir grandes cantidades de DHA en las microdietas.

10.1.6 Metabolitos reactivos del oxígeno: Generación. Detoxificación y estrés oxidativo

La evolución de los procesos metabólicos aeróbicos como la respiración, conlleva inevitablemente a la producción de metabolitos reactivos del oxígeno (ROS), todos con un alto potencial para causar daño oxidativo a las proteínas, ADN y lípidos. Como ya ha sido señalado previamente, los peces y especialmente las larvas, tiene un

alto riesgo de sufrir peroxidación de sus membranas, ya que contienen grandes cantidades de LC-PUFA. Para contrarrestar el efecto negativo de los ROS, existen complejos sistemas enzimáticos y no enzimáticos de detoxificación. Sin embargo, cuando la producción de ROS excede su eliminación por parte de los mecanismos antioxidantes, aparece una situación de estrés oxidativo, pudiendo causar los ROS daños celulares y alteraciones en los diferentes elementos celulares.

10.1.6.1 Generación de ROS

El oxígeno molecular, esencial para los organismos aeróbicos, tiene un papel dominante en los eucariotas, al aceptar el electrón terminal en la respiración mitocondrial, donde es reducido en último término a agua durante el proceso de fosforilación oxidativa, la principal ruta de obtención de ATP en los aerobios. El oxígeno puede ser convertido en ROS mucho más reactivos, formados tanto por transferencia de energía como por transferencia de electrones (Figura 10.4). La primera reacción origina oxígeno singlete, mientras que la última causa la reducción secuencial a superóxido, peróxido de hidrógeno y radical hidroxilo (Klotz, 2002).

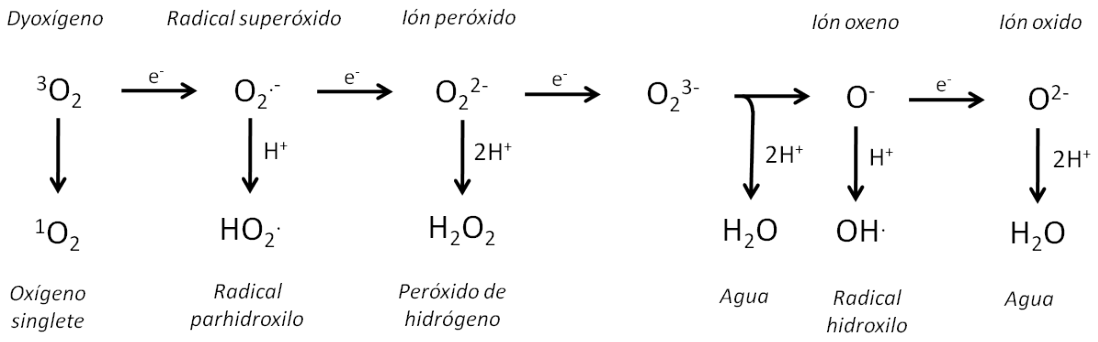

Figura 10.4 Generación de distintos ROS por transferencia de energía o reducción secuencial univalente del oxígeno triplete. Adaptado de Apel y Hirt (2004).

Los efectos patológicos de los ROS dependen del radical libre producido. El ión radical superóxido ($O_2^{\cdot -}$) estimula la activación de leucocitos y de otras células degradantes de enzimas. Puede causar lesiones directas a lípidos, proteínas y ADN y normalmente actúa cerca del sitio donde se produce. El peróxido de hidrógeno (H_2O_2) puede convertirse en el radical hidroxilo ($OH\cdot$) o hipoclorito (OCl^-), ambos con capacidad para destruir los microbios y las células. Este radical libre actúa lejos de su sitio de

producción. El radical hidroxilo representa el radical libre más común derivado del oxígeno y es el principal responsable del ataque a los lípidos, proteínas y ADN.

Además del transporte de electrones mitocondrial, otras fuentes de producción endógena de ROS incluyen la cadena transportadora de electrones de los microsomas (Winston y Cederbaum, 1983), el estallido respiratorio asociado con la fagocitosis por parte de los leucocitos (Chung y Secombes, 1988) y las actividades de enzimas como la xantina oxidasa, triptófano dioxigenasa, diamina oxidasa y prostaglandina sintasa (Fridovich, 1978; Halliwell, 1978).

10.1.6.2 Detoxificación de ROS: mecanismos de defensa antioxidante

La definición de antioxidante es la de cualquier sustancia que cuando está presente a baja concentración, comparada con la de un substrato oxidable, es capaz de interaccionar con los radicales libres para terminar la reacción (Halliwell y Guttridge, 1989). Los organismos han desarrollado un sistema de defensas antioxidantes para detoxificar los ROS y evitar los efectos potencialmente adversos del oxígeno activado (Yu, 1994). Los sistemas antioxidantes pueden dividirse en dos tipos, uno está representado por enzimas y el otro por moléculas de bajo peso molecular. Estos compuestos se encuentran en el plasma celular, las mitocondrias o las membranas celulares (Figura 10.5). Los antioxidantes pueden actuar en diferentes estadios de los procesos oxidativos, pudiendo tener algunos más de un mecanismo de acción. Pueden ejercer su función mediante diferentes mecanismos como romper la cadena de la secuencia iniciada, neutralizar el oxígeno singlete o disminuir las concentraciones locales de O_2.

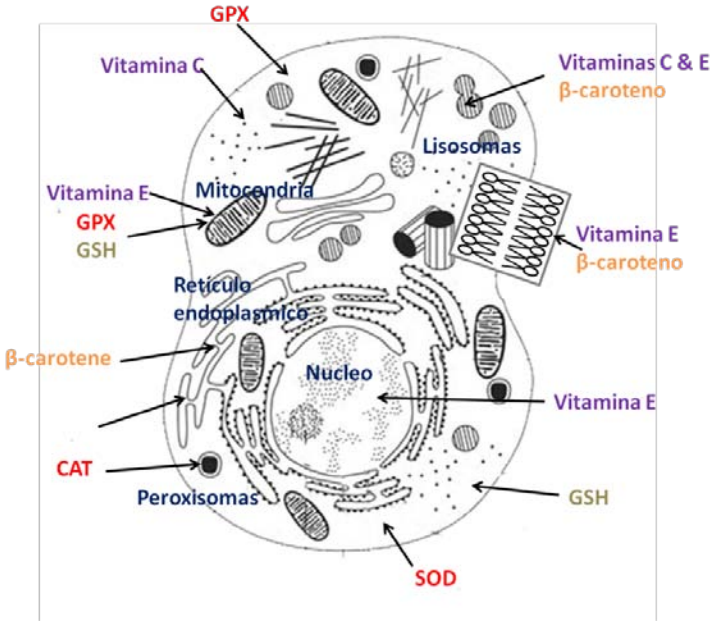

Figura 10.5 Mecanismos antioxidantes del interior de la célula. Adaptado de Machlin y Bendich (1987).

10.1.6.2.1 Sistemas enzimáticos

Distintos sistemas enzimáticos antioxidantes (AOE) son capaces de prevenir la cascada de la oxidación, interceptando e inactivando los metabolitos reactivos intermedios del oxígeno. Se han detectado enzimas especialmente adaptadas, como la superóxido dismutasa (SOD; EC 1.15.1.1), glutatión peroxidasa (GPX; EC 1.11.1.19), glutatión reductasa (GR) o catalasa (CAT; EC 1.11.1.6) en la mayoría de las especies de peces investigadas hasta la fecha (Rudneva, 1997). Todas estas enzimas son muy importantes en las defensas antioxidantes, aunque la SOD juega un papel crucial ya que es la primera enzima que responde a los radicales oxígeno, previniendo el inicio de la cadena de reacción de los radicales que produce el anión superóxido (McCord y Fridovich, 1969; Winston y Di Giulio, 1991). El superóxido es convertido en peróxido de hidrógeno por la SOD (Figura 10.6). Esta enzima no cruza fácilmente las membranas biológicas, por tanto, debe ser detoxificada en el compartimento donde se ha generado (Fridovich *et al*., 1995). Esto explica la presencia de diferentes SOD en los eucariotas, los cuales pueden clasificarse en función de su contenido de metal; SOD con manganeso o con cobre/zinc.

El peróxido de hidrógeno es metabolizado consecuentemente en oxígeno y agua por la enzima GPX, la cual usa glutatión (GSH) como cofactor en la reacción. La GPX neutraliza la mayoría del peróxido de hidrógeno en el citoplasma, aunque también hay

indicios de que juega un papel en la señalización celular al controlar las concentraciones de peróxido de hidrógeno (Brigelius-Flohe, 1999; Figura 10.6). Existen cuatro GPX principales; citosólica (GPX1), gastrointestinal (GPX2), plasmática (GPX3) y la hidroperoxidasa de los fosfolípidos (GPX4). En los sitios activos de las GPXs es necesario la presencia de selenio.

La CAT es una hemoproteína con cuatro átomos de hierro por molécula y su función es transformar el peróxido de hidrógeno en oxígeno molecular y agua (Figura 10.6). En sitios con altas concentraciones de peróxido de hidrógeno, como los peroxisomas, la catalasa juega un papel importante como enzima antioxidante. El radical hidroxilo y los peroxinitritos son de las especies más reactivas presentes en los sistemas biológicos, siendo capaces de oxidar ácidos nucléicos, proteínas, lípidos y carbohidratos en las células.

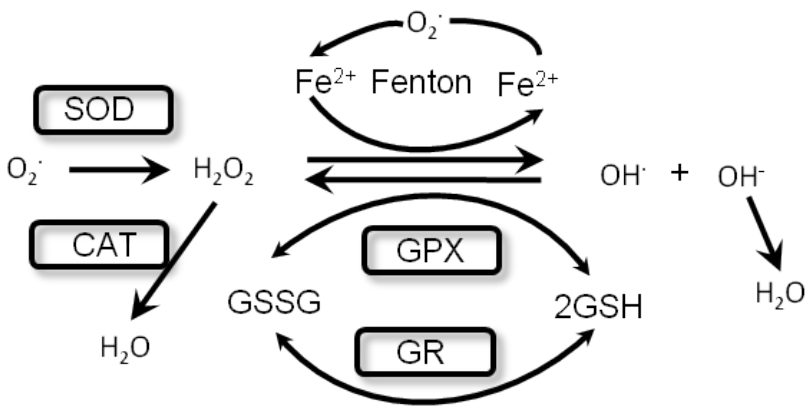

Figura 10.6 Mecanismos de protección de las AOE. El superóxido ($O_2^{\cdot}$) es dismutado en peróxido de hidrógeno (H_2O_2) por la SOD. El H_2O_2 es también reducido a agua por la acción de la GPX o la CAT. Se muestran los ciclos relacionados de oxidación-reducción del glutatión entre las formas oxidadas (GSSG) y reducidas (GSH) en función de las actividades de la GPX y la GR. El H_2O_2 puede regenerar el $OH^{\cdot}$ al reaccionar con iones metálicos como el hierro en la reacción fentón.

Se ha comprobado que en distintas especies de peces el nivel de AOE incrementa con el desarrollo de la larva, mientras que el nivel de antioxidantes de bajo peso molecular disminuye (Aceto *et al*., 1994; Rudneva, 1999; Zhang *et al*., 2009). Este hecho indica que hay una compensación de los bajos niveles de AOE mediante el aumento de las moléculas antioxidantes. De este modo, el incremento en los niveles de AOE podría ser debido al mayor consumo de oxígeno durante los estadios iniciales del desarrollo larvario. Por tanto, se podría pensar que el sistema de enzimas AOE está

inducido como una respuesta a estas condiciones desfavorables. Por ejemplo, las actividades de la CAT y la GPX se vieron incrementadas desde la eclosión hasta los 9 dph en larvas en inanición de dentón *(Dentex* dentex), mientras que el contenido de vitamina E disminuyó con la edad (Mourente *et al*., 1999). La actividad de la CAT también se vio incrementada tras la eclosión en trucha arcoíris (*Oncorhynchus mykiss*; Aceto *et al*., 1994) y desde los embriones hasta los 11 dph en larvas de rodaballo (Peters y Livingstone, 1996), mientras que en larvas de lenguado senegalense (*Solea senegalensis*) las actividades de la CAT y la GPX tendieron a incrementar con la edad (Solé *et al*., 2004).

10.1.6.2.2 Sistemas no enzimáticos

La segunda línea de defensa de los organismos frente a los ROS está compuesta por antioxidantes que sólo pueden ser adquiridos a través de la dieta (Sen, 1995) como son las vitaminas C y E, los carotenoides o el selenio. Debido al alto contenido de PUFA presente en los organismos marinos, es necesario la presencia de antioxidantes hidro y lipsolubles, para promover la resistencia de las membranas celulares en los estadios iniciales de desarrollo.

10.1.6.2.2.1 Vitamina E

El término vitamina E es el término general se emplea para definir a un grupo de compuestos liposolubles, los tocoferoles y los tocotrienoles, encargados de proteger a los lípidos poliinsaturados de la oxidación. Los tocoferoles y tocotrienoles son potentes antioxidantes encontrados en los alimentos y aceites, siendo la actividad del α-tocoferol superior a la de otros homólogos en el salmón (Hamre, 2011).

Los tocoferoles puros son sustancias liposolubles capaces de esterificarse en compuestos cristalinos. En ausencia de oxígeno, los tocoferoles son estables a los ácidos y al calor, aunque pueden oxidarse rápidamente en presencia de peróxidos y otros agentes oxidantes (Dam y Sondergaard, 1964). Así mismo, los tocoferoles son muy sensibles a la luz ultravioleta, siendo excelentes antioxidantes cuando se encuentran de forma libre, mientras que los esteres del tocoferol tienen una baja capacidad oxidante *in vitro*. Sin embargo, los esteres son más estables, por lo que se usan frecuentemente como suplementos alimenticios, facilitando su hidrólisis en el

estómago así como la absorción del alcohol libre, actuando como un antioxidante intra y extracelular.

El α-tocoferol es un componente estructural de las membranas biológicas (Putnam y Comben, 1987), además de tener una potente función antioxidante (Sargent *et al*., 1997). Por otro lado, se cree que tanto en peces como en otros animales, la vitamina E ejerce un cierto efecto sobre la salud y la resistencia a enfermedades a través de la modulación de la respuesta inmune (Waagbø, 1994, 2006; Montero *et al*., 1998; Verlach Trichet, 2010).

Los tocoferoles actúan como antioxidantes inter e intracelulares para mantener el equilibrio de metabolitos lábiles tanto en plasma como en las células. El α-tocoferol es capaz de detener la cascada de la oxidación, interceptando al radical peróxil ($ROO^{\cdot}$) más rápidamente que un PUFA. El α-tocoferol puede donar su átomo de hidrógeno fenólico al radical, quedando así convertido en un producto del hidroperóxido y rompiendo de este modo la cadena de reacciones de la auto-oxidación lipídica. De esta reacción resulta el radical tocoferoxil, que es lo suficientemente estable como para impedir que continúe la cadena de oxidación y que puede ser neutralizado si se une con otro radical peroxil, dando lugar a la formación de un producto no radical. Además, el radical tocoferoxil es muy estable y reacciona lentamente con los PUFA, pudiendo ser reducido por el ascorbato, regenerando de este modo a la vitamina E. Por tanto, a no ser que sea regenerada, para mantener un nivel adecuado de vitamina E en el organismo esta debe ser obtenida de reservas corporales o a través de la dieta (Burton y Traber, 1990).

Ya que el α-tocoferol tiene mayor afinidad por los radicales peroxil que los PUFA, una pequeña cantidad de esta vitamina es suficiente para proteger una gran cantidad de ácidos grasos. En este sentido, Burton y colaboradores (1983) calcularon que una sola molécula de α-tocoferol era capaz de proteger aproximadamente a 1000 moléculas de de PUFA de la oxidación. Además, se ha observado que la suplementación de vitamina E mejora la calidad del filete en distintas especies piscícolas como la trucha arcoíris (Frigg *et al*., 1990; Chaiyapechara *et al*., 2003; Yildiz, 2004), el salmón atlántico (*Salmo salar*) (Hamre *et al*., 1998; Scaife *et al*., 2000), el rodaballo (*Psetta maxima*) (Ruff *et al*., 2003; 2004) y la lubina (Gatta *et al.,* 2000; Pirini *et al.*, 2000). También se ha comprobado que el α-tocoferol es capaz de proteger al filete de la oxidación, mejorar su vida útil, evitar el deterioro del color y prevenir la aparición de la rancidez. De hecho, se ha propuesto que el α-tocoferol es el factor más importante responsable de la estabilidad *post-mortem* de las membranas celulares (Baker, 1997).

El papel que desempeña la vitamina E en la integridad esquelética ha sido estudiado de manera extensa en los mamíferos, al contrario que en los peces. La vitamina E es necesaria para el desarrollo adecuado del sistema esquelético (Xu *et al*., 1995; Jilka *et al*., 1996. El tocoferol se asocia con la membrana bicapa de las células óseas convirtiéndose así en la primera línea de defensa frente a los radicales libres (Arjmandi *et al*., 2002). Los radicales endógenos y exógenos estimulan la diferenciación de los osteoclastos e inhiben la actividad de los osteoblastos (Tintut *et al*., 2002; Parhami, 2003). Esto podría causar resorción del hueso y causar formación inadecuada del mismo, así como anormalidades. No existen muchos estudios sobre el efecto de los ROS en los huesos de los peces. Lewis-McCrea y Lall (2007) describieron las consecuencias de alimentar juveniles de fletán con aceites moderadamente oxidados, observando un incremento de la incidencia de escoliosis en relación con el incremento de la oxidación. Por otro lado, existen algunos estudios sobre los efectos de la contaminación química sobre la columna vertebral (Karen *et al*., 2001; Mochida *et al*., 2008; Danion *et al*., 2011). La intoxicación con estos productos tiene efectos similares a aquellos observados cuando se genera una situación de estrés oxidativo mediante la dieta, ya que los contaminantes suelen ejercer sus efectos negativos alterando también el estado oxidativo.

No se conocen los requerimientos exactos de vitamina E para las larvas de especies marinas, ya que la mayoría de estudios se han enfocado en juveniles o adultos, con valores que oscilan entre 25 y 120 mg kg^{-1}. De forma general, se cree que los requerimientos nutricionales de las larvas son mayores que los de adultos o juveniles, por tanto, si se emplean los niveles estimados para adultos se podría estar infravalorando las necesidades de las larvas (Tabla 10.2).

Tabla 10.2 Requerimientos de vitamina E para larvas de diferentes especies

Especie	Parámetro	Requerimiento	Referencia
Latris lineata	Crecimiento	437 mg kg^{-1}	Brown *et al*., 2005
Salmo salar	Crecimiento	120 mg kg^{-1}	Hamre y Lie, 1995a
Sparus aurata	Crecimiento Supervivencia	136 mg kg^{-1}	González *et al*., 1995
Sparus aurata	Crecimiento Supervivencia	3000 mg kg^{-1}	Atalah *et al*., 2008

Por otro lado, hay que tener en cuenta que los altos requerimientos de LC-PUFA en larvas tienen que estar asociados a niveles elevados de vitamina E, como ya se ha sugerido para algunas especies como la carpa (*Cyprinus carpio*) (Watanabe *et al*.,

1981; Schwarz *et al.,* 1988), el salmón atlántico (Hamre y Lie, 1995b) y la dorada (Atalah *et al*., 2008). En este sentido, Izquierdo y Fernández-Palacios (1997) observaron un incremento del contenido de vitamina E en larvas de dorada desde la eclosión a los 10 días, mientras que este contenido se redujo a partir de este día y hasta llegar a los 20 días *post* eclosión. Curiosamente, esta diminución en los niveles de vitamina E es paralela a la reducción de los contenidos de PUFA durante el desarrollo larvario (Izquierdo, 1988), lo cual sugiere una cercana relación entre ambos nutrientes.

Por otro lado, además de contener altos niveles de PUFA, las microdietas presentan elementos pro-oxidantes, como por ejemplo los minerales. Además, la mayor superficie de las partículas alimenticias respecto a su volumen favorece los procesos oxidativos, ya que los ácidos grasos están más expuestos. Por tanto, es de gran importancia aportar suficiente cantidad de vitamina E en las dietas para larvas de peces marinos.

10.1.6.2.2.2 Vitamina C

El ácido ascórbico (vitamina C) es una vitamina hidrosoluble, considerada un componente esencial en las dietas para teleósteos dado que estos carecen de capacidad para sintetizarla o de sintetizarla con suficiente rapidez como para satisfacer sus requerimientos, debido a la ausencia de la enzima gulonolactona oxidasa (Chatterjee, 1973; Dabrowski, 1990). Es un compuesto blanco, inodoro, cristalino, soluble en agua pero insoluble en solventes lipídicos. Además, el ácido ascórbico es capaz de formar sales y es lábil frente al oxígeno libre. El ácido ascórbico reducido es muy estable en soluciones ácidas debido a la conservación del anillo lactona, mientras que en soluciones alcalinas la vitamina C se hidroliza rápidamente perdiendo así su actividad. La vitamina C es muy lábil a altas temperaturas y susceptible a la oxidación atmosférica, especialmente en presencia de cobre, hierro u otros catalizadores metálicos. La forma reducida es la más activa desde un punto de vista biológico, pero se pueden formar distintas sales o derivados con distinto grado de actividad biológica (Woodruff, 1964; WHO, 1974).

El ácido ascórbico actúa como cofactor en muchos procesos biológicos incluyendo la síntesis de colágeno, así como funciones relacionadas con la neuromodulación, los sistemas hormonales e inmune. Es necesario suplementar las dietas de bagre de canal (*Ictalurus punctatus*) con suficiente cantidad de vitamina C para la adecuada formación de componentes estructurales tales como cartílago,

colágeno o hueso (Wilson y Poe, 1973). Además. el ácido ascórbico actúa como cofactor en la hidroxilación de la prolina y la lisina, paso necesario para la conversión del procolágeno en colágeno maduro (Barnes y Kodicek, 1972; Padh, 1991). Los peces deficientes en ácido ascórbico muestran menores proporciones de hidroxilisina e hidroxiprolina, lo que origina colágeno poco hidroxilado (Sato *et al*., 1982). Cuando los niveles de ácido ascórbico son bajos, se aprecia una disminución de la enzima fosfatasa alcalina, indicando una reducción de la actividad de los osteoblastos. Por tanto, la deficiencia de vitamina C genera una baja osificación y metabolismo óseo (Tietz *et al.,* 1983; Johnston *et al.,* 1994).

Es por esto que los peces deficientes en vitamina C muestran deformidades esqueléticas causada por la biosíntesis irregular de colágeno (Halver, 2002). Una menor hidroxilación de la prolina causa huesos débiles y quebradizos tal y como ya ha sido observado en bagre del canal y en rodaballo (Wilson y Poe, 1973; Coustans *et al.,* 1990), mientras que en labeo roho (*Labeo rohita*) se observó una baja concentración de calcio en hueso, músculo, branquias y piel (Agrawal y Mahajan, 1980). Uno de los primeros síntomas de la deficiencia de vitamina C son las vértebras engrosadas y ligeramente curvadas como ha sido observado en el bagre del canal (Lim y Lovell, 1978). Así mismo, se han detectado algunas alteraciones esqueléticas como la lordosis o la escoliosis en la trucha de arroyo (*Salvelinus fontinalis*) (Poston, 1967), trucha arcoiris (Dabrowski *et al*., 1990) y salmón atlántico (Hardie *et al*., 1991). De acuerdo con esto, se ha visto que restringir los niveles de vitamina C causa reducción y daño del cartílago, generando anomalías en estructuras que experimentan osificación condral (por ejemplo, la mandíbula) así como una sobre estimulación de los receptores de vitamina C (Darias *et al*., 2009).

Dado que el ácido ascórbico es un potente antioxidante, la cantidad de radicales libres puede verse incrementada cuando se emplean piensos de mala calidad, disminuyendo así las reservas tisulares de ascórbico (Sies, 1993) como ya ha sido descrito en trucha arcoíris alimentada con lípidos oxidados (Hung y Slinger, 1980). Otro papel importante de la vitamina C como antioxidante reside en su capacidad para regenerar el radical tocoferoxil, tal y como ha sido demostrado *in vitro* (Tappel, 1962; Packer *et al*., 1979; Niki *et al*., 1985). Así, un incremento en el contenido de vitamina C de 0 a 60 mg kg^{-1} no afectó a la retención de α-tocoferol en salmón del atlántico, ya que los peces no mostraron deficiencia en vitamina C (Hamre *et al.,* 1997). Se han encontrado resultados similares en otras especies piscícolas como la perca amarilla (*Perca flavescens*) o el bagre de canal (Lee y Dabrowski, 2003; Yildirim-Aksoy *et al*., 2008). Además, en salmón del Atlántico, los niveles tisulares de vitamina E fueron

independientes del nivel de vitamina C cuando este se suplementó entre 50 y 2750 mg kg^{-1} (White *et al*., 1993). Por otro lado, se observó una caída marcada en el nivel de vitamina E hepática en salmones del Atlántico deficientes en vitamina C (Hamre *et al*., 1997). Así mismo, también se observó un incremento en los niveles hepáticos de vitamina E cuando se administraba vitamina C en perca amarilla y bagre de canal siendo alimentados con una dieta deficiente en vitamina E (Lee y Dabrowski, 2003; Yildirim-Aksoy *et al*., 2008). Salmones alimentados con un suplemento de vitamina E mostraron un desarrollo paralelo entre la vitamina E hepática, la hidroxiprolina de las vértebras, crecimiento y mortalidad en respuesta a la vitamina C, sugiriendo que son necesarios niveles de vitamina C superiores a los requerimientos para mantener las reservas corporales de vitamina E (Hamre *et al*., 1997). Estos resultados apoyan la hipótesis de que la vitamina C puede regenerar a la vitamina E *in vivo* en adultos y juveniles de peces. Por otro lado, se ha observado un efecto prooxidante de la vitamina E cuando se administran niveles altos a peces deficientes en vitamina C, ya que los radicales tocoferoxil se acumulan en las membranas y pueden causar oxidación irreversible de vitamina C remanente (Hamre, 2011).

Las larvas son especialmente sensibles a la deficiencia de vitamina C (Dabrowski *et al*., 1996), debido probablemente a que tienen un mayor requerimiento en vitaminas por su rápido crecimiento, así como elevados contenidos de PUFA en sus alimentos (Tabla 10.3). Además, se han encontrado altos niveles de vitamina C en huevos de peces (Kossmann, 1988; Dabrowski y Bloom, 1994), lo cual podría ser indicativo de la importancia de este micronutriente durante el desarrollo larvario. En larvas de lubina, rodaballo (Merchie *et al*., 1996) y dorada (Atalah *et al*., 2010) la adición de vitamina C mejoró la supervivencia, el crecimiento, el desarrollo esquelético, la resistencia al estrés y la respuesta inmune. Además, existe la hipótesis de que en los primeros estadios de desarrollo larvario los antioxidantes de bajo peso molecular, como por ejemplo la vitamina C, juegan un papel primordial ante el daño oxidativo, mientras que a medida que va creciendo la larva, se incrementa la actividad de las enzimas antioxidantes (Rudneva, 1999). Por tanto, para que las larvas puedan mantener un equilibrio oxidativo es necesario prestar atención a la vitamina C administrada a los reproductores, así como suministrar la suficiente cantidad de vitamina C a las larvas.

Tabla 10.3 Requerimientos de vitamina C para larvas de distintas especies marinas

Especie	Parámetro	Requerimiento	Referencia
Cirrhina mrigala	Crecimiento Supervivencia Comportamiento Morfología	650-700 mg kg^{-1}	Mahajan y Agrawal, 1980
Clarias gariepinus	Crecimiento	1600 mg kg^{-1}	Merchie *et al*., 1997
Cyprinus carpio	Crecimiento Contenido vitC	45 mg kg^{-1}	Gouillou-Coustans *et al*., 1998
Dicentrarchus labrax	Crecimiento Supervivencia Resistencia estrés	2500 mg kg^{-1}	Merchie *et al*., 1995
Dicentrarchus labrax	Crecimiento Supervivencia Deformidades	30-50 mg kg^{-1}	Darias *et al*., 2011
Scophtalmus maximus	Crecimiento Supervivencia	20-130 mg kg^{-1}	Merchie *et al*., 1997

10.1.6.2.2.3 Selenio

El selenio es un mineral traza y un micronutriente esencial para los vertebrados (Johansson *et al*., 2005), pero también es el elemento con menor rango entre requerimiento y toxicidad (Chassaigne *et al*., 2002; Polatajko *et al*., 2006). Es un componente esencial de distintas rutas metabólicas, incluyendo el metabolismo de las hormonas tiroideas, los sistemas de defensa antioxidante o la función inmune.

El selenio se distribuye a bajas concentraciones en agua dulce (0,2-10 µg $litro^{-1}$) y salada (aproximadamente 0,09 µg $litro^{-1}$) (NRC, 1976). También aparece de forma natural en alimentos en forma de complejos orgánicos, principalmente en forma de selenometionina, selenocistina y selenocisteína. Las harinas de pescado y productos de origen marino representan la mejor fuente natural de selenio de todos los productos empleados normalmente para elaborar piensos de peces. Sin embargo, Bell y Cowey (1989) demostraron que el selenio presente en la harina de pescado presenta una baja digestibilidad, mientras que la selenometionina era muy digestible. La disponibilidad relativa del selenio de los compuestos puros es:

Selenito > selenato > selenometionina > selenido > selenio elemental

En los peces, el selenio también está involucrado en el funcionamiento de hormonas tiroideas y la insulina, la fertilidad y la regulación del crecimiento celular (Lall, 2002; Kohlmeier, 2003). Además, el selenio puede promover tanto la formación del

hueso como la mineralización. Sin embargo, la ingesta excesiva de este mineral tiene efectos negativos sobre el metabolismo del tejido esquelético en los vertebrados (NRC, 2005). Penglase y colaboradores (2010) demostraron que larvas de bacalao alimentadas con rotíferos suplementados con selenio presentaron una mayor incidencia de deformidades vertebrales. Esto podría estar causado por una alteración en la mineralización esquelética causado por el selenio en su forma iónica, o a través de las selenoenzimas como agentes antioxidantes (Lall y Lewis-McCrea, 2007) o por la alteración de las hormonas tiroideas (Power *et al*., 2001).

El selenio se incorpora en forma de selenometionina en el sitio activo de una amplia gama de proteínas. En el pez cebra (*Danio rerio*) se han identificado un total de 18 selenoproteínas, incluyendo tres tipos que no presentan ortólogos en mamíferos (Kryukov y Gladyshev, 2000). Una de las principales funciones del selenio es actuar como componente de las selenoproteínas GPX, una serie de isoenzimas que protegen los componentes lipídicos y las membranas del estrés oxidativo tanto a nivel celular como subcelular (Arteel y Sies, 2001). Otras selenoproteínas pueden presentar también función antioxidante, tal y como puede observarse en la Tabla 10.4. Únicamente la selenometionina puede incorporarse a las proteínas, siendo éste el principal reservorio de selenio en el músculo esquelético. Por el contrario, el selenito de sodio puede incorporarse a selenoproteínas activas como la GPX, pero no a la selenometionina que sirve de reservorio en hígado y músculo (Rider y Sweetman, 2008).

La deficiencia de selenio puede llevar a la aparición de estrés oxidativo en los órganos (Gatlin *et al*., 1986; Bell *et al*., 1986, 1987), reducción del crecimiento (Wang y Lovell, 1997) e incremento de la mortalidad (Gatlin *et al*., 1986; Bell *et al*., 1987) en distintas especies piscícolas. Los principales efectos de la intoxicación por selenio son disminución del crecimiento, baja eficiencia nutricional y alta mortalidad. Por otro lado, niveles de selenio superiores a 13 y 15 mg kg^{-1} fueron suficientes para causar signos de toxicidad en trucha arcoíris y bagre del canal respectivamente (Hilton *et al*., 1980; Gatlin y Wilson, 1984). Se debe tener en cuenta que el requerimiento de selenio de los peces varía junto a la forma de selenio ingerido, el contenido de LC-PUFA y vitamina E de la dieta, así como la concentración de selenio en el agua (Lall, 2002).

Recientemente se ha demostrado que el contenido de selenio de los rotíferos es considerablemente bajo (0,08-0,09 mg kg^{-1} peso seco) en comparación tanto con los requerimientos de los peces (0,5-0,3 mg kg^{-1} peso seco; NRC, 1993) como con el selenio contenido en los copépodos (3-5 mg kg^{-1} peso seco), por lo que podrían no contener suficiente selenio para cubrir los requerimientos de las larvas (Hamre *et al*.,

2008a). Por tanto, el selenio podría ser uno de los elementos traza con mayor potencial de ser deficiente en los rotíferos. Enriquecer rotíferos con selenito de sodio y yoduro de sodio aumenta la supervivencia de larvas de bacalao, aunque no se observaron diferencias en cuanto a crecimiento respecto al grupo control (Hamre *et al*., 2008b). Así mismo, un incremento en el nivel de selenio en los rotíferos incrementó la actividad y expresión del mRNA de la GPX en larvas de bacalao (Penglase *et al*., 2010), sugiriendo que es necesaria una dosis extra de selenio para proteger a las larvas de la oxidación lipídica y a los productos de la oxidación, ya que pueden ser abundantes en las presas vivas enriquecidas con niveles altos de *n*-3 LC-PUFA. Cabe resaltar que la vitamina E puede compensar en situaciones de deficiencia de selenio (Gatlin *et al*., 1986; Awad *et al*., 1994), por tanto, las larvas podrían protegerse de los ROS acumulando otros nutrientes antioxidantes, como ya ha sido observado en las larvas de bacalao (Penglase *et al*., 2010).

Tabla 10.4 Selenoproteínas presentes en el pez cebra y su función

Selenoproteína	Pez cebra	Función conocida (mamíferos)
1. Glutatión peroxidasa 1	GPX1a GPX1b	Catabolismo hidroperóxidos Estructura del esperma
2. Glutatión peroxidasa 2 3. Glutatión peroxidasa 4	GPX2 GPX4a GPX4b	
4. Deionidasa 3	DI3	Activación T4 Inactivación T3
5. Tioredoxina reductasa 2	TR2	Regulación del redox proteico Reciclaje de vitamina C Síntesis de ADN
6. Tioredoxina reductasa 3	TR3	
7. Selenoproteína P	SelPa	Transporte de selenio Antioxidante
8. Selenoproteína Pb	SelPb	
9. Selenoproteína W1	SelW1	Antioxidante
10. Selenoproteína W2	SelW2a SelW2b	
11. Selenoproteína T1	SelT1a SelT1b	Desconocida
12. Selenoproteína T2	SelT2	Desconocida
13. 15 kDa selenoproteína	Sel15	¿¿Etiología cancer??
14. Selenoproteína R	SelR	Desconocida
15. Selenoproteína N	SelN	Desconocida

10.1.6.3 Estrés oxidativo

El estrés oxidativo aparece cuando la tasa de generación de ROS es superior a su eliminación (Sies, 1986; Figure 10.9). Sus efectos adversos incluyen oxidación de proteínas y ADN, así como peroxidación de los lípidos insaturados de las membranas celulares. Todo esto produce hidroperóxidos inestables altamente reactivos que amenazan la integridad de la célula. Además, estos productos pueden convertirse en radicales libres, pudiendo perpetuar el ciclo destructivo de reacciones de peroxidación lipídica. La reparación de las proteínas dañadas por los ROS se limita a la reducción de los derivados oxidados de los residuos de aminoácidos que contienen sulfuro. No se ha demostrado la reparación de otros tipos de proteínas oxidadas. Es más, las proteínas dañadas son degradadas a aminoácidos por la acción de diversas proteasas, incluyendo catepsinas y calpaínas. Por el contrario, el daño oxidativo a los ácidos nucleicos es reparado por mecanismos de excisión/inserción altamente eficaces. Sin embargo, las modificaciones en el ADN tras la exposición a ROS pueden ser el inicio de la inducción de efectos mutagénicos y letales de distintos productos con potencial oxidante (Basu-Modak y Tyrrel, 1993).

En juveniles y adultos de distintas especies de peces, se han relacionado algunas enfermedades con el daño de las radicales libres, como por ejemplo la hemolisis (Kawatsu, 1969), anemia (Cowey *et al*., 1984), ictericia (Sakai *et al.*, 1989), degeneración hepática (Cowey *et al.,* 1984) o alteraciones esqueléticas (Hata y Kaneda, 1980; Watanabe *et al*., 1989; Lewis-McCrea y Lall, 2007). Entre las alteraciones esqueléticas, una de las más frecuentemente descritas en juveniles y adultos es la distrofia muscular (Lovell *et al*., 1984; Gatlin *et al*., 1986; Frischknecht *et al*., 1994; Bowater y Burren, 2007).

10.1.6.3.1 Auto-oxidación de los lípidos

La oxidación de los lípidos puede ser definida como un proceso autocatalítico iniciado por radicales libres que resulta en el deterioro de los PUFA y es una consecuencia importante del estrés oxidativo. Los ROS ejercen su acción quitando electrones de la membrana lipídica de la célula, iniciando así el proceso de oxidación lipídica. La auto-oxidación de los lípidos continúa como una reacción en cadena, en la que una especie radical tiene la capacidad de quitar un átomo de hidrógeno al grupo metileno de los PUFA, convirtiéndolo en un radical lipídico. Como consecuencia, se establece una reacción cíclica en la que el radical peróxido formado por otro radical

reacciona con un nuevo PUFA. En ausencia de antioxidantes, la oxidación de los lípidos continuará hasta que queden PUFA por oxidar (Hamre, 2011). La cadena de peroxidación lipídica puede terminar cuando dos radicales lipídicos reaccionan para formar un producto no radical o cuando es neutralizado por un neutralizador de radicales (Hølmer 1993; Frankel, 1998).

En general, los efectos de la peroxidación lipídica son disminución de la fluidez de membrana, incremento de la permeabilidad de membrana a sustancias normalmente impermeables e inactivación de las enzimas de unión de membrana. Los productos primarios de la oxidación de lípidos son los dienos conjugados que se convertirán en hidroperóxidos, que pueden dar lugar a la formación de distintos productos secundarios de bajo peso molecular como los aldehídos o los hidrocarbonos (Hølmer, 1993; Frankel, 1998). Los hidroperóxidos se pueden oxidar, manteniendo la cadena de carbono intacta y formar isoprostanos, isofuranos y ácidos grasos mono o dihidroxi.

La oxidación del DHA da lugar específicamente al isoprostano de tipo F4, isofurano y mono o dihidroxi DHA. Se pueden formar ocho subfamilias de isoprostanos F4 a partir del DHA, resultado del ataque de los radicales libres a las posiciones C_6, C_9, C_{12}, C_{15} and C_{18}. Además, los hidroperóxidos pueden ocasionar una pérdida completa de la integridad de membrana con una desintegración de la cadena de carbonos, formándose diferentes especies moleculares de aldehídos. El producto más estable resultante de la oxidación del DHA es el 4-hidroxihexenal (Van Kuijk *et al*., 1990). Para reducir la formación de estos productos de la oxidación es necesario una adecuada combinación de antioxidantes hidro y liposolubles. Sin embargo, el efecto de cada una de estas moléculas antioxidantes puede variar en función de los organismos y de tejidos específicos (Dietrich *et al*., 2002). Además, el daño oxidativo a la membrana puede liberar enzimas hidrolíticas que causen más daño a la célula.

Los tejidos de los peces están en riesgo de sufrir oxidación de sus lípidos, ya que contienen cantidades relativamente altas de LC-PUFA. Este riesgo será incluso mayor en las larvas de especies marinas, ya que su mayor requerimiento de PUFA supone una mayor presencia en sus tejidos. Sin embargo, la susceptibilidad de los peces a la oxidación depende en gran parte del perfil de ácidos grasos y de los niveles y tipo de antioxidantes presentes. Por tanto, para evitar la peroxidación *in vivo*, se debe de incluir cantidad suficiente de antioxidantes en las dietas.

10.1.7.2 Efectos patológicos de la oxidación en tejidos de peces

Distintos estudios han demostrado los efectos adversos de la oxidación en distintas especies de agua dulce y salada. Además de una alteración de los parámetros de producción, tales como reducción del crecimiento o incremento de la mortalidad (Watanabe *et al*., 1970; Blazer, 1982; Wang *et al*., 2006), se han asociado distintas patologías al estrés oxidativo tanto en adultos como en juveniles de distintas especies (Tabla 10.5). Como puede observarse en esta Tabla, uno de los signos más notorios es la aparición de distrofia muscular.

Tabla 10.5 Algunos efectos patológicos de los radicales libres en los tejidos de peces

Patología	Especies	Fuente de oxidación	Autor
Ictericia	*Seriola quinqueradiata*	Desconocida	Sakai *et al*., 1989. 1998
Distrofia muscular nutricional	*Cyprinus carpio*	VitE DEF	Watanabe *et al*., 1970
	Cyprinus carpio	AO+VitE DEF	Miyazaki, 1986
	Cyprinus carpio	AO+VitE DEF	Miyazaki, 1986
	Ictalurus punctatus	Se+VitE DEF	Gatlin *et al*., 1986
	Ictalurus punctatus	↑ FA	Lewis *et al.,* 1985
	Ictalurus punctatus	AO+VitE DEF	Murai y Andrews, 1974
	Lates calcarifer	VitE DEF	Bowater, 2007
	Salmo gairdneri	APO	Cowey *et al*., 1984
	Salmo gairdneri	VitE+Se DEF	Bell *et al*., 1985
	Oncorhynchus mykiss	VitE, VitC, VitE+C DEF	Frischknecht *et al.,* 1994
Hemolisis	*Salvelinus fontanalis*		Kawatsu, 1969
	Salmo gairdneri	AO+VitE/C DEF	Smith, 1979
	Salmo gairdneri	APO+VitE/Ethoxyquin	Moccia *et al.,* 1984
	Salmo salar	VitE+Se DEF	Poston *et al.,* 1976
Anormalidades esqueléticas	*Hippoglossus hippoglossus*	APO	Lewis McCrea y Lall, 2007
Pigmento ceroide	*Seriola quinqueradiata*	Desconocida	Sakai *et al*., 1989
	Salmo salar	AO	Roald *et al*., 1981

DEF: Deficiencia; AO: Aceite oxidado; APO: Aceite de pescado oxidado

10.1.6.3.2 Los efectos adversos del estrés oxidativo en el sistema músculo esquelético

10.1.6.3.2.1 Tejido muscular

En los peces teleósteos, la musculatura axial está organizada en series de miotomos segmentados con una compleja morfología tridimensional. Los miotomos están separados entre sí por láminas de colágeno llamadas miosepto y tienen una

compleja geometría para conceder estabilidad mecánica al músculo durante el curvado del cuerpo (Figura 10.7; Van Leeuwen, 1999). El número y forma de los miotomos varía significativamente según el estado de desarrollo, posición en el tronco, morfología del cuerpo, filogenia y estilo de locomoción (Johnston *et al*., 2011). Cada miotomo contiene una región superficial, con forma de cuña, que reposa directamente bajo la línea lateral, donde las fibras musculares están dispuestas de manera helicoidal, formando ángulos de hasta 40º. Esta orientación típica de las fibras musculares se asocia con la necesidad de acortar los sarcómeros cuando el cuerpo se flexiona (Rome y Sosnicki, 1990). En la lubina, como en muchas otras especies de peces, existe un miómero epaxial e hipoaxial para cada vértebra, separado por el septo horizontal y la línea lateral (Figura 10.7).

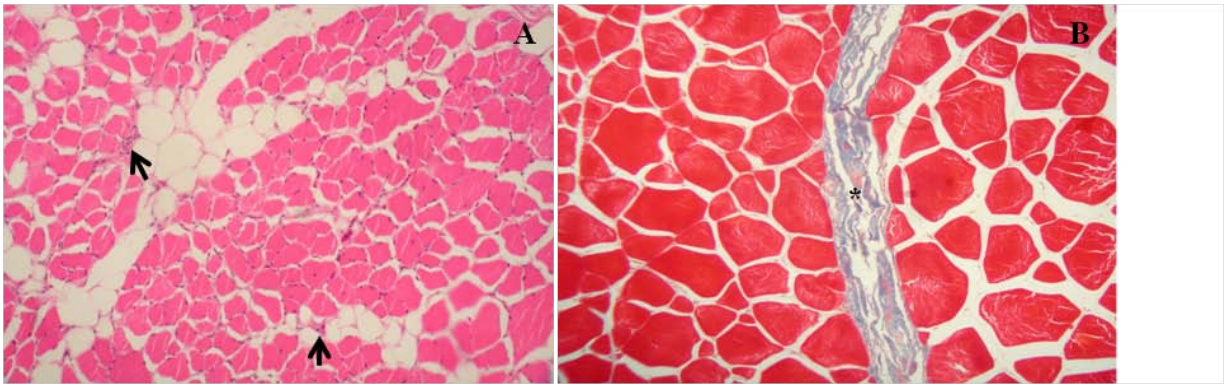

Figura 10.7 Sección transversal del músculo blanco de dorada, tinción de H&E (A) y tricrómico de Masson (B). (**A**) Presencia de tejido adiposo entre las fibras musculares así como vasos sanguíneos (flecha) (x200). (**B**) Se puede apreciar el miosepto de colágeno teñido de azul (asterisco) (x400).

Los músculos locomotores de todos los grupos de peces están muy especializados para enfrentarse a la amplia gama de fuerzas que requiere el sistema esquelético tanto para desplazarse a una velocidad constante como a una repentina alta velocidad. La solución adoptada universalmente a estos problemas ha sido dividir el sistema locomotor en distintas partes con distintos tipos de fibras musculares. Por tanto, los músculos locomotores se organizan en musculatura axial, formada principalmente por fibras blancas rápidas, cubiertas por una fina capa de fibras rojas lentas, y una capa de fibras rosas o intermedias entre ambas. El color del músculo es indicativo del grado de vascularización de cada tipo muscular. Así, el músculo rojo superficial, o músculo lento, aparece de color oscuro debido a su gran cantidad de mioglobina, teniendo también abundantes mitocondrias en su centro y periferia, así como un alto contenido en grasas y glucógeno (Figure 1.12; Johsnton, 1980; Shindo *et al*., 1986; Ayala *et al*., 1999). Las fibras rojas son aeróbicas y tienen una alta actividad respiratoria. Estas fibras

son pequeñas en diámetro (25 – 45 µm) y normalmente representan menos del 10% y nunca más del 30% de la musculatura del miotomo (Boddeke *et al*., 1959). Las principales características de este tipo de músculo son la gran cantidad de mitocondrias de tipo lamelar en el sarcolema y entre las miofibrillas, así como la presencia de gotas lipídicas. En corte transversal, estas fibras muestran una morfología rectangular. Generalmente este tipo de músculo se usa para la natación a altas velocidades.

El músculo blanco, también conocido como músculo rápido, nunca representa menos del 70% y muestra el mayor diámetro de fibras, entre 50 y 100 µm y a veces mayor (Figura 10.7). Ultraestructuralmente, muestra una marcada orientación radial y mantiene un ancho relativamente uniforme. Estas fibras tienen una morfología poligonal, abundantes miofilamentos y escasas mitocondrias. Las fibras musculares blancas son de contracción rápida y se fatigan velozmente.

En concordancia con su nombre, las fibras intermedias o rosas se hayan entre las fibras rojas y blancas. La cantidad relativa de fibras intermedias varía tanto entre las especies de peces como en la fase de desarrollo, de hecho, en las larvas de lubina se puede observar este tipo de fibra al final de la etapa larvaria (Scapolo *et al*., 1988; Veggetti *et al*., 1990; Ramírez-Zarzosa *et al*., 1995; López-Albors *et al*., 2005).

El crecimiento muscular en los peces difiere del de los mamíferos ya que en peces continúa a lo largo de casi todo el ciclo vital (Gree-Walker, 1970; Stickland, 1983). Además, los procesos de hiperplasia como los de hipertrofia tienen lugar tanto en músculo de larvas como de adultos, llegando a alcanzar una gran talla de adulto (Weatherley *et al*., 1988). Sin embargo, el número de miotomos se fija antes o poco después de la eclosión (Blaxter, 1988). Pueden distinguirse tres fases en la formación del músculo de los peces:

(1) Miogénesis embrionaria: Tiene lugar la formación de la fibra embrionaria junto con una población de mioblastos indiferenciados.
(2) Hiperplasia estratificada: Esta fase puede observarse en las larvas con saco vitelino. Se aprecian zonas germinales de proliferación de mioblastos en los ápices dorsales y ventrales de los miotomos (Veggetti *et al.,* 1990; Brooks y Johnston, 1993; Rowlerson *et al*., 1995).
(3) Hiperplasia de mosaico: Se produce la activación de los mioblastos en la superficie de las fibras musculares embrionales. Este proceso puede continuar a lo largo de la vida adulta (Koumans *et al*., 1994; Johnston *et al*., 1995; Rowlerson *et al*., 1995).

Se sabe que existen distintos factores abióticos que pueden afectar a la miogénesis (Figura 10.8). La alimentación es un factor clave que afecta al desarrollo y crecimiento de los músculos de peces, como sugieren las diferencias en talla, morfología y tasa de proliferación de las células progenitoras miogénicas aisladas de peces alimentados o en inanición (Fauconneau y Paboeuf, 2000). Además, en los estadios larvarios, el aporte de alimento adecuado que cubra los requerimientos de órganos con un rápido desarrollo (incluyendo el músculo) es crucial (Koumans y Akster, 1995). Sin embargo, la incidencia que pueden tener la falta o el exceso de determinados nutrientes sobre el músculo de larvas marinas es desconocida. Por ejemplo, a pesar de que algunos informes han señalado el efecto que la deficiencia de selenio y/o vitamina E puede tener en músculo de adultos o juveniles de salmón del atlántico, bagre del canal o perca gigante (*Lates calcarifer*) (Poston *et al*., 1976; Lovell *et al*., 1984; Bowater y Burren, 2007), ninguno de ellos se refiere a los estadios larvarios. Además, el nivel de lípidos o proteínas podría tener un efecto directo sobre la oxidación en el músculo como ya ha sido descrito en juveniles de lubina y trucha arcoíris (Álvarez *et al*., 1998).

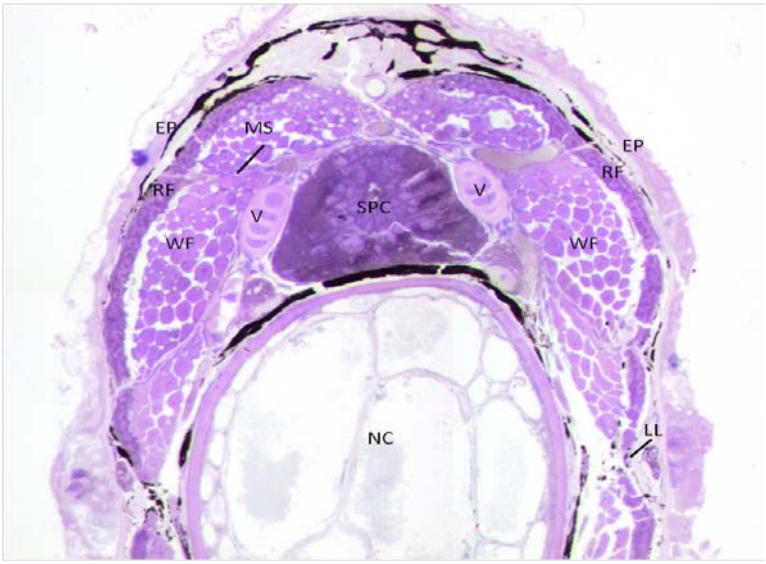

Figura 10.8 Tipo de fibras y estructura de las fibras musculares en una sección transversal en una preparación de larva de lubina teñida con azul de toluidina. El tronco está dividido en dos mitades laterales, sirviendo la columna vertebral y los procesos esqueléticos como soporte estructural. Puede observarse que la monocapa superficial de fibras rojas (RF) está en contacto con la epidermis (EP). Las fibras blancas (WF) son de mayor tamaño que las rojas y se encuentran dispuestas en distintas capas. NC notocorda; LL línea lateral; V vertebra, SPC médula espinal; MS miosepto.

Aunque la biología celular de la miogénesis en teleósteos es distinta a la descrita en mamíferos, los genes implicados en la regulación del crecimiento están aparentemente muy conservados (Watabe, 2001). Sin embargo, existen mecanismos de regulación del desarrollo y crecimiento del músculo ligeramente diferentes en peces. En las larvas de teleósteos, células madres pluripotentes se convierten en mioblastos, que darán lugar a la población de células miogénicas progenitoras (MPC), mediante la expresión de factores reguladores de la miogénesis (MRFs). Después de ello, se cree que las MPC experimentan una división asimétrica y forman células hijas destinadas a una diferenciación terminal. La progenia de las MPC puede experimentar una fase proliferativa controlada por rutas de señalización positivas y negativas, pudiendo migrar a través del músculo llegando a diferentes destinos. Los mioblastos de la musculatura rápida pueden fusionarse para formar miotubos mediante la fusión mioblasto-mioblasto, en la que posiblemente esté implicada la calpaína. Las calpaínas (EC 3.4.22.17) son proteasas citosólicas neutras dependientes de calcio que existen en dos formas principales ubicuas, dependiendo de la concentración de Ca^{2+} necesaria para su activación: μ-calpaína y m-calpaína. Aunque se ha hipotetizado que las calpaínas pueden tener un papel regulador en el crecimiento muscular, la mayoría de estudios en peces se han enfocado en estudiar la actividad proteolítica *post mortem* de estas enzimas, así como su efecto en la calidad del filete (Geesink *et al*., 2000; Chéreta *et al*., 2009; Caballero *et al*., 2009; Terova *et al.,* 2011).

Una vez formados, los miotubos comienzan el programa de formación de miofibrillas y maduran en fibras musculares. Cuando se observan en una sección longitudinal, las fibras musculares maduras muestran estriaciones transversas de bandas alternas claras (I) y oscuras (A), con un marcado grado de regularidad (Figura 10.9). La banda A está compuesta por miofilamentos gruesos (miosina) y finos (actina), mientras que la banda I está formada exclusivamente por filamentos finos. Cada banda I está atravesada por una línea transversa oscura, la línea Z. La estructura que queda entre dos líneas Z se llama sarcómero y es la unidad de contracción muscular. En el centro de la banda A hay una banda más pequeña, exclusivamente formada por miofilamentos gruesos y llamada banda H. En el medio de esta banda H existe otra línea llamada línea M, donde los miofilamentos gruesos están incrustados en distintos tipos de proteína. La formación de miofibrillas se inicia en las fibras de estrés de actina formadas sobre los cuerpos Z (precursores de las líneas Z) antes de la integración y alineación de los filamentos gruesos y proteínas asociadas en un proceso que parece conservado en los vertebrados (Sanger *et al*., 2009). Por otro lado, la miosina es un hexámero constituido por dos cadenas pesadas (MyHC) y cuatro cadenas ligeras,

incluyendo isoformas embrionarias. Por tanto, las MyHC son los principales marcadores cuando se estudia la determinación fenotípica de las fibras y del músculo completo durante el crecimiento y la adaptación. Tanto la actina como la miosina pueden usarse como marcadores tardíos de la miogénesis ya que se expresan al final de dicho proceso.

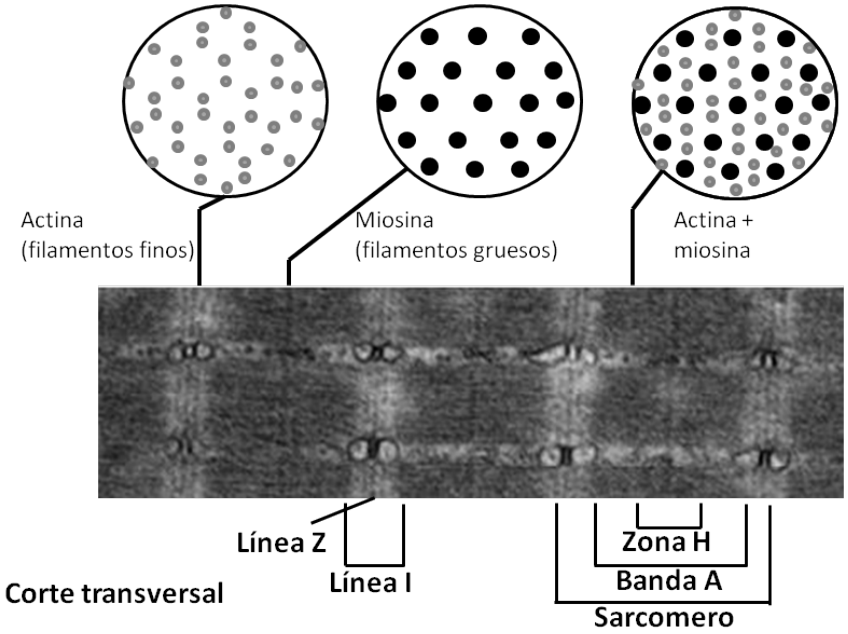

Figura 10.9 Representación esquemática de la disposición de los filamentos de actina y miosina en el músculo de larvas de lubina (35 dph).

Las MPC también se llaman células satélite, aunque algunos autores afirman que no deberían de llamarse así, ya que las MPC no siempre se encuentran en la lámina basal de las fibras musculares (Veggetti *et al*., 1990; Johnston *et al*., 2003). Además, una vez que se activan las células satélite son las responsables del crecimiento postembriónico en teleósteos. Por otro lado, algunos trabajos han analizado el papel de las células satélites en la regeneración muscular de los teleósteos (Rowlerson *et al*., 1997), encontrando rasgos similares con la regeneración muscular en mamíferos. Las células satélite se activan en respuesta al daño muscular para proliferar y fusionarse para así formar nuevas fibras musculares (Goldspink *et al*., 2001). Estas nuevas fibras expresan, transitoriamente, MyHC, similar a la que se expresa durante los estadios embrionarios (Whalen *et al*., 1990).

Se cree que la regulación de la masa muscular está controlada por rutas de señalización en las que se encuentran los factores de crecimiento insulínico tipo I y II

(IGF-I y II). Las IGF son polipéptidos de cadena simple, homólogos estructuralmente a la proinsulina y capaces de promover el crecimiento. Dependiendo del contexto biológico, la IGF puede estimular el crecimiento celular, promover la diferenciación celular e inhibir la apoptosis (Jones y Clemmons, 1995). La acción de las IGF puede verse influenciada tanto positiva como negativamente por una familia de proteínas vinculadas a las IGF (IGFBP) y la mayoría, si no todas sus acciones, están mediadas por los receptores de IGF (Jones y Clemmons, 1995). Las IGFs conforman una de las rutas centrales de regulación de síntesis de proteínas en el músculo esquelético. Por ejemplo, en embriones de pez cebra, dos parálogos de la IGF-II regulan el desarrollo de la línea media (White *et al*., 2009). Así mismo, el músculo de los peces tiene una mayor abundancia de receptores de la IGF-I que de receptores de insulina (Parrizas *et al*., 1995). Esto indica que la IGF-I contribuye más a la regulación de la función muscular que la insulina en los peces, al contrario de lo que ocurre en los mamíferos.

Se ha comprobado que algunas de las propiedades biológicas del músculo de los mamíferos lo vuelven particularmente susceptible al daño de los radicales libres, incluso en situaciones de estrés oxidativo sistémico. Por tanto, parece ser que el músculo se ve afectado de manera primaria o incluso selectivamente. Esto podría ser debido a la capacidad del músculo para asumir cambios rápidos y coordinados en el aporte de energía y el flujo de oxígeno durante la contracción. Esto podría hacer al músculo muy susceptible de padecer estrés oxidativo como resultado de un mayor flujo de electrones (Haycock *et al*., 1996). Por otro lado, hay una alta concentración de hemoglobina en el músculo, y se sabe que las proteínas que contienen el grupo hemo son muy sensibles al ataque de los radicales libres (Ostdal *et al*., 1997). Finalmente, los fosfolípidos de las membranas musculares las hacen particularmente susceptibles a padecer oxidación (Murphy y Kehrer, 1989).

Por tanto, parece ser que el músculo tiene una mayor probabilidad de sufrir estrés oxidativo que cualquier otro tejido. Es lógico pensar que los mecanismos de protección antioxidante deberían estar mejorados en el tejido muscular. Sin embargo, en salmón del Atlántico se ha demostrado que la retención de -tocoferol es particularmente baja en el músculo blanco, especialmente si se compara con otros órganos como por ejemplo el hígado, que muestra una retención exponencial de vitamina E (Hardie *et al.,* 1990; Hamre y Lie, 1997). El modelo lineal de retención de vitamina E en el músculo y el exponencial para el hígado están confirmados en otras especies como la tilapia (*Oreochromis aureus*; Satoh *et al*., 1987) y la trucha arcoíris Hung *et al*., 1980; Frigg *et al.,* 1990; Puangkaew *et al*., 2005). Se han encontrado resultados similares en otros nutrientes antioxidantes como el selenio. Monteiro y

colaboradores (2009) encontraron una baja retención de selenio en el músculo de *Brycon cephalus* alimentados con una dieta rica en selenio, en comparación con las branquias o el hígado. Además, las actividades de la SOD, CAT y GPX fueron más bajas en el músculo de larvas de *Brachymystax lenok* en comparación con las vísceras, cerebro y branquias (Zhang *et al*., 2009). Por tanto, todas estas condiciones tendrían que considerarse como causas adicionales a la aparición de lesiones musculares en peces.

10.1.6.3.2.2 Sistema esquelético

El sistema esquelético tiene muchas funciones fisiológicas, incluyendo la de dar integridad estructural durante el desarrollo y el movimiento. Además, el esqueleto permite la inserción de los músculos, protege a los órganos vitales y sirve como reservorio de minerales (Lall y Lewis-McCrea, 2007). Los teleósteos muestran una amplia variedad de tejidos esqueléticos. Es más, más que hueso o cartílago, el tejido esquelético de los peces se puede describir como un espectro continuo, variando desde el tejido conectivo al cartílago y al hueso (Hall y Witten, 2007). Por tanto, se han identificado en peces, el cartílago y otros muchos tejidos con características histológicas entre hueso y cartílago y se sabe que juegan un papel fundamental en el desarrollo esquelético (Benjamin, 1990; Beresford, 1993; Huysseune, 2000). El hueso es un tejido conectivo vascularizado especializado constituido por células y matriz extracelular mineralizada. Antes de la mineralización, la matriz extracelular está compuesta principalmente de colágeno tipo I que se va mineralizando subsecuentemente debido a la deposición de hidroxiapatita mediada por los osteoblastos (Hall y Witten, 2007; Nordvick, 2007). El cartílago es un tejido avascular compuesto por condrocitos que están incluidos en una matriz extracelular, compuesta principalmente por colágeno tipo II y proteoglicanos (Witten *et al*., 2010). El hueso condroide es un tejido intermedio que se halla, por ejemplo, en los tejidos mandibular y maxilar de los teleósteos. Tiene características intermedia tanto de hueso como de cartílago, pudiendo estar mineralizado.

En la formación y remodelado del esqueleto óseo axial están implicados distintos tipos celulares. Los osteoblastos son células formadoras de hueso y su función es la de secretar matriz ósea no mineralizada así como controlar la mineralización de la matriz. Los osteoclastos intervienen en la resorción del tejido óseo y pueden ser visualizados como macrófagos multinucleados. Los osteocitos son células atrapadas en el interior de

la matriz ósea y están implicados en el mantenimiento de las sustancias del hueso y el intercambio de iones desde los fluidos orgánicos.

Distintos estudios relacionados con el impacto de la primera alimentación en el desarrollo de los peces han demostrado que algunos nutrientes juegan un papel fundamental en la aparición de deformidades esqueléticas cuando no se administran durante la fase larvaria (Cahu, 2003). Además, el desarrollo de alteraciones esqueléticas en larvas y juveniles puede estar relacionado al poco conocimiento que se tiene de las interrelaciones entre nutrición, ambiente y factores genéticos. Por tanto, para intentar evitar la aparición de deformidades esqueléticas en larvas cultivadas, hay que prestar una especial atención al aporte adecuado de nutrientes como las vitaminas, minerales o lípidos.

No existe mucha información sobre el papel de los lípidos oxidados y los radicales libres en el desarrollo de anomalías esqueléticas en teleósteos. En humanos, se sabe que los ROS contribuyen principalmente a la remodelación del hueso al promover la resorción ósea (Bai *et al*., 2005). Esto probablemente se deba a una inhibición de los osteoblastos y una estimulación de los osteoclastos, causando una pérdida neta del hueso (Parhami *et al*., 1997; Parhami, 2003). La reducción en la formación del hueso, acompañada de una estimulación de la resorción del mismo, podría causar anormalidades esqueléticas, como ya ha sido observado en fletán alimentado con lípidos oxidados (Lewis-McCrea y Lall, 2007). Estudios recientes en larvas de dorada demostraron que altos niveles de DHA en la dieta causan un mayor porcentaje de deformidades esqueléticas, encontrando además en estas larvas el mayor contenido de TBARS, lo cual es indicativo de procesos peroxidativos (Izquierdo *et al*., enviado). El aporte de vitamina E no redujo la frecuencia de las anomalías observada en fletán juvenil alimentado con dietas oxidadas (Lewis-McCrea y Lall, 2007), ni tampoco en larvas de lubina alimentadas con rotíferos enriquecidos con altos niveles de DHA (Izquierdo *et al*., enviado). Sin embargo, el aporte de vitamina E mejoró la calidad del hueso en ratones adultos expuestos a estrés oxidativo (Wang *et al*., 2000). Por tanto, dietas oxidadas pueden causar deficiencias de nutrientes antioxidantes, lo cual puede llevar a la aparición de deformidades. Sería importante llevar a cabo más estudios sobre el efecto de los lípidos oxidados sobre el desarrollo del sistema esquelético para poder entender su efecto patológico sobre el hueso de los peces.

10.2 Objetivos

Hoy en día, la producción de juveniles sigue representando el cuello de botella en la acuicultura de especies marinas. El uso de una dieta compuesta seca para mantener la alta producción de juveniles de calidad es crucial. Sin embargo, se desconocen los requerimientos exactos de las larvas de peces. Se sabe que altos requerimientos de PUFA, y particularmente de DHA, incrementan el riesgo de padecer estrés oxidativo. Sin embargo, poco se sabe sobre los daños causados por los radicales libres en las larvas de especies marinas y del efecto de los distintos nutrientes antioxidantes. Por tanto, los objetivos de la presente tesis fueron los siguientes:

1.- Determinar el daño potencial de niveles altos de DHA en larvas de lubina. Tradicionalmente en las dietas para larvas se han empleado gran cantidad de PUFA, especialmente DHA para promover su crecimiento y supervivencia. Sin embargo, debido a su alta insaturación, el DHA es muy susceptible de padecer peroxidación lipídica. Este hecho junto con otras características, puede exponer a las larvas a sufrir estrés oxidativo en sus propios tejidos.

2.- Evaluar el efecto protector antioxidante de la vitamina E cuando se administran distintos niveles de DHA. Se deben de incluir en las dietas cantidades suficientes de nutrientes antioxidantes para contrarrestar los efectos adversos de los ROS. Se probaron distintos niveles de α-tocoferil acetato para intentar evitar los efectos negativos del estrés oxidativo.

3.- Investigar el efecto protector de la combinación de vitamina E y selenio cuando se emplean niveles altos de DHA en la dieta. Se probó una dieta con niveles elevados de DHA y vitamina E y suplementada con Se, otro nutriente con potencial antioxidante.

4.- Investigar el efecto protector de la combinación de vitaminas C y E cuando se emplean niveles altos de DHA en la dieta. Para alcanzar este objetivo se añadió niveles altos de vitamina C a una dieta con alto potencial de causar estrés oxidativo.

10.3 Material y Métodos Generales

10.3.1 Condiciones y animales experimentales

Se obtuvieron larvas de lubina de puestas naturales de la *Ecloserie Marine de Gravelines* (Gravelines, Francia, Capítulo 3) y del *Instituto de Acuicultura de Torre la Sal* (Castellón, España, Capítulos 4, 5 y 6). Durante los primeros días de aclimatación se controló la temperatura por medio de enfriadores (16ºC), incrementándose la tasa de renovación paulatinamente hasta llegar a la temperatura ambiente (19.5-20ºC). Las larvas se cultivaron en tanques madre hasta alcanzar los 12 (Capítulos 4, 5, 6 y 7) o 32 (Capítulo 3) dph cuando se distribuyeron al azar para, tras un periodo de aclimatación de dos días, dar comienzo a los distintos experimentos. Todos los experimentos se realizaron en las instalaciones del Instituto Canario de Ciencias Marinas (ICCM, Telde, Islas Canarias, España).

10.3.1.2 Cultivo en agua verde

Las larvas de lubina recién eclosionadas se cultivaron en tanques de 2000 L de capacidad, en sistema abierto (7 renovaciones al día) durante los primeros cinco días. Al sexto día, cuando las larvas ya habían abierto la boca, se cerró el circuito abierto y las larvas se cultivaron en agua verde. Para ello, cada día se añadían 20 L de *Nanochloropsis gaditana* (205 x 10^3 células ml^{-1}) y se mantuvo la densidad de rotíferos a 10 ind ml^{-1}. Para conseguir este propósito, se añadían rotíferos dos veces al día (8:00; 15:00). Los rotíferos restantes en el tanque se eliminaron por filtración, evitando así la presencia de rotíferos con bajo valor nutricional en el tanque. Se siguió el fotoperiodo natural (alrededor de 10 h de luz) durante 12 (Capítulos 4, 5, 6 y 7) o 32 días (Capítulo 3), hasta que las larvas se transfirieron a los tanques experimentales.

10.3.1.3 Cultivo en tanques experimentales

Cada tanque (tanques cilíndricos de fibra de vidrio y color gris de 170 L) fue abastecido por agua de mar filtrada (salinidad de alrededor de 34 g L^{-1}) almacenada previamente en una tanque de 500 L para facilitar la eliminación del exceso de gases disueltos. El agua se filtró a través de una malla de 50μm y entró en los tanques a una tasa creciente de 1.0 - 1.5 L min^{-1} en un sistema abierto. El agua era continuamente aireada (125 ml min^{-1}), alcanzando niveles de oxígeno de 5-8 g L^{-1} y entre un 60 y 80% de saturación. La temperatura y el oxígeno se midieron diariamente utilizando el oxímetro Guard-handy beta (Zeigler Bros, Gardners, EEUU). La intensidad de la luz se mantuvo a 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia,

Australia). Se utilizó un fotoperiodo artificial de 12 h luz: 12 h oscuridad mediante luces fluorescentes. Los tanques se limpiaron diariamente de manera manual entre las 18:00 y las 20:00 mediante un sifón.

10.3.2 Dietas y alimentación

10.3.2.1 Rotíferos

Los rotíferos (*Brachionus plicatilis*) se cultivaron en tanques cilíndricos de 1700 L de capacidad. Estos tanques poseían un sistema central de aireación a través de una piedra porosa separada del fondo 20 cm. Los cultivos se hicieron con una mezcla de agua salada (80%) y dulce (20%), siendo inoculados los rotíferos (línea S-1; 150-250 µm de longitud) a una densidad inicial de 100 ind ml^{-1}. La alimentación de los rotíferos se basó en levadura de panificación (1,2 g 10^6 ind^{-1} d^{-1}), repartida en dos tomas diarias.

Los rotíferos se emplearon para alimentar a las larvas de lubina durante el cultivo en agua verde y los primeros cinco días de experimentación. Los rotíferos usados en el agua verde estaban enriquecidos (24 h) con DHA Selco® (INVE, Bélgica; 0,125 g Selco l^{-1} en dos dosis). Durante los cinco primeros días de ensayo con microdietas, se administraron rotíferos alimentados con levadura en un régimen de co-alimentación, por lo que no existió aporte de LC-PUFA diferente del aportado por la dieta.

10.3.2.2 Microdietas

Para la realización de esta Tesis se formularon distintas dietas isolípidicas e isoprotéicas, pero con niveles variables de DHA, vitaminas C y E o selenio. El contenido proteico y lipídico osciló alrededor del 65 y 15% respectivamente.

10.3.2.2.1 Formulación de las microdietas

Como fuente de EPA y DHA se empleó EPA 50 y DHA 50 en forma de triglicéridos (Croda, East Yorkshire, Reino Unido). La vitamina E, en forma de tocoferil acetato, se obtuvo de Sigma-Aldrich (Madrid, España), mientras que el ROVIMIX Stay-C-35 (ascorbil monofosfato; Roche, Paris, Francia) se usó como fuente de vitamina C. Además, se usó selenio orgánico extraído de levaduras (Sel-Plex, Alltech Inc, Lexington, KY, EEUU). Para ecualizar el contenido lipídico, se empleó un aceite que no es fuente de ácidos grasos esenciales, como es el ácido oléico (Merck, Darmstadt, Alemania). La lecitina de soja (Acrofarma, Barcelona, España), con un contenido de alrededor del 50% de lípidos polares, fue la fuente elegida de fosfolípidos. La mezcla de atractantes se basó en la estimada por Kanazawa *et al.* (1989), mientras que las vitaminas se basaron

en la de Teshima *et al.* (1982) con algunas modificaciones. Como fuente de proteína, se empleó la harina de calamar, la cual fue desengrasada tres veces consecutivas para poder controlar el perfil de ácidos grasos de la dieta. Para ello, se mezcló un volumen de harina con 3 volúmenes de cloroformo, se agitó unos minutos y se filtró a través de silica con la ayuda de una bomba de vacío. Tras cada extracción se dejó evaporar el cloroformo introduciendo la harina en una estufa a 38º durante al menos 12 horas.

10.3.2.2.2 Preparación de las microdietas

Las microdietas se prepararon siguiendo el protocolo establecido por Liu *et al*. (1992). Primero, se mezcló la harina con los componentes hidrosolubles en un mortero hasta conseguir una mezcla lo más fina posible. Por separado se preparó la mezcla de vitaminas liposolubles y aceites, que se añadió a los componentes hidrosolubles. Se preparó gelatina, disolviéndola en agua caliente y se añadió a la mezcla. La pasta resultante fue comprimida, moldeada en trozos pequeños (Severin, Suderm, Alemania) y secada en una estufa a 38ºC durante 24 horas. A continuación se molió (Braun, Kronberg, Alemania) y tamizó (Filtra, Barcelona, España) la dieta hasta obtener el tamaño de partícula deseado (alrededor de 125-500 µm). Las dietas fueron analizadas para conocer su contenido proximal y de ácidos grasos, testando cada dieta en triplicado (Capítulos 3, 5 y 6) o cuadriplicado (Capítulos 4 y 7).

10.3.2.3 Alimentación

Las dietas se administraron cada 45 min desde las 8:00 hasta 19:00. Se alimentó a las larvas al menos durante los cinco primeros días de vida con rotíferos alimentados con levadura, para así evitar cualquier aporte de LC-PUFA. La densidad inicial fue de 2 rotíferos ml^{-1} y fue luego reducida a 1 rotífero ml^{-1}. Para garantizar la disponibilidad de alimento, la administración de las dietas experimentales fue al inicio 2.0 g y se fue incrementando 0.5 g cada semana.

10.3.3 Muestreos

10.3.3.1 Evaluación del crecimiento

Se determinó el crecimiento de las larvas valorando su peso corporal y longitud total. El peso total se calculó en tres réplicas de 10 larvas en inanición, lavadas con agua destilada y secadas en un portaobjetos en un horno a 110ºC hasta alcanzar un peso constante, aproximadamente tras 24 h. Para los estudios de longitud total, se midieron 30 larvas por tanque en el proyector de perfiles (V-12A Nikon, Nikon Co., Tokio, Japón).

10.3.3.2 Análisis proximal

Para analizar la composición bioquímica se tomaron todas las larvas que restaron en los tanques experimentales, tras un periodo de inanición de 12 h, se lavaron con agua destilada y se mantuvieron a -80ºC hasta su posterior análisis. Antes del inicio de las pruebas experimentales se tomaron muestras de los tanques madre para conocer la composición bioquímica inicial.

10.3.3.3 Histología

En cada punto de muestreo se fijaron 30 larvas por tanque en formol tamponado al 10%. Otras 30 larvas se fijaron durante 24 h a 4ºC en glutaraldehído al 2,5% en tampón fosfato 0,2M (pH 7,2) para el estudio de microscopía electrónica de transmisión (TEM).

10.3.3.4 Biología molecular

Se tomaron aproximadamente 200 mg de larvas no alimentadas durante 12 h en cada punto de muestreo. Estas larvas fueron lavadas con agua DEPC (dietil pirocarbonato) y conservadas en 1000 µl de RNA later (Sigma-Aldrich, Madrid, España) durante toda una noche a 4ºC. A continuación se retiró el RNAlater y las larvas se conservaron a -80ºC hasta la extracción del ARN.

10.3.3.5 Test de actividad y supervivencia final

Antes del final de algunos de los experimentos (Capítulos 3 y 4) se llevó a cabo un test de actividad, manteniendo 20 larvas en una red fuera del agua durante 1 min, para después ponerlas de nuevo en el agua y observar la supervivencia a las 24 h. La supervivencia final se calculó tras contar todas las larvas remanentes en los tanques experimentales.

10.3.4 Análisis bioquímicos

10.3.4.1 Análisis proximales

10.3.4.1.1 Humedad

Se determinó el contenido de humedad secando en la estufa las muestras a 110ºC hasta alcanzar peso constante. Se anotó el peso de la muestra (aproximadamente 100 mg) antes y después de secarlas, después de dejarlas enfriar en un desecador. La humedad se expresó como un porcentaje del peso siguiendo el

Método Oficial de la Asociación Química Analítica de los EEUU (A.O.A.C., 1995), empleando la siguiente ecuación:

$$Humedad(\%) = \frac{100 - (B - A) - (C - A)}{B - A}$$

Siendo A el peso del tubo vacío, B el peso de la muestra húmeda y el tubo y C el peso de muestra seca y tubo.

10.3.4.1.2 Cenizas

El contenido de cenizas se midió tras secar las muestras (aproximadamente 200 mg) en una estufa a 450º hasta que se alcanzó un peso constante (A.O.A.C., 1995).

10.3.4.1.3 Proteínas

Las proteínas se estimaron a partir del nitrógeno total contenido en la muestra (~250 mg) usando el método Kjeldhal (A.O.A.C., 1995) tras la digestión de la misma con ácido sulfúrico a una temperatura de 420ºC. El nitrógeno total fue convertido en proteínas crudas totales tras multiplicar por el factor empírico 6,25.

10.3.4.1.4 Lípidos totales

Los lípidos se extrajeron siguiendo el método de Folch *et al*. (1957). La extracción comienza con el homogeneizado de la muestra (50-200 mg) en un Ultra Turrax (IKA-Werke, T25 BASIC, Staufen, Alemania) durante 5 min en una solución de 5 ml de cloroformo:metanol (2:1) con BHT al 0,01%. La solución resultante fue filtrada y a continuación se añadió KCl al 0,88% para incrementar la polaridad de la fase acuosa. Esto fue seguido de decantación y centrifugación a 2000 rpm durante 5 min para así separar la fase acuosa de la orgánica. Una vez descartada la fase acuosa, se evaporó el solvente bajo atmósfera de nitrógeno y se pesaron los lípidos.

10.3.4.2 Preparación y cuantificación de ésteres metílicos de ácidos grasos

Los ésteres metílicos de ácidos grasos (FAMEs) se obtuvieron por transmetilación ácida de los lípidos totales con 1% de ácido sulfúrico en metanol según el método de Christie (1982). La reacción se llevó a cabo en la oscuridad durante 16 h a 50ºC en atmósfera de nitrógeno. Después, los ésteres metílicos de los ácidos grasos se extrajeron con hexano:éter dietílico (1:1; v:v) y fueron purificados por cromatografía de adsorción en cartuchos NH_2 Sep-pack (Waters S.A., Massachussets, EEUU) tal y como describió Christie (1982). Los FAME fueron separados por GLC (GC-14A, Shimadzu, Tokyo, Japan) en una columna capilar de sílica (Supercolvax-10-fused; longitu: 30 mm,

diámetro interno: 0.32 mm; Supelco, Bellefonte, EEUU) utilizando helio como gas transportador. La temperatura de la columna fue de 180ºC durante los primeros 10 min, aumentando a 215ºC durante 10 min, según las condiciones descritas por Izquierdo *et al*., (1992). Los FAME se cuantificaron por FID y se identificaron por comparación con estándares externos y aceites de pescado bien caracterizados (EPA 28, Nippai, Ltd. Tokio, Japón).

10.3.5 Medida de las sustancias reactivas al ácido tiobarbitúrico (TBARS)

La medida de TBARS en triplicado se determinó según el método de de Burk *et al*. (1980) con algunas modificaciones. Se homogeneizaron aproximadamente 20-30 mg de larvas en 1,5 ml de ácido tricoloroacético al 20% con 0,05 ml de BHT (Ultra Turrax; IKA-Werke, T25 BASIC, Staufen, Alemania). A continuación se añadieron 2,95 ml de ácido tiobarbitúrico 50mM, antes de mezclarlo y calentarlo 10 min a 100ºC. Tras dejarlo enfriar y retirar los precipitados de proteína por centrifugación (Sigma 4K15, Osterode y Harmz, Alemania) a 2000 X g. El sobrenadante se leyó en un espectrofotómetro (Evolution 300, Thermo Scientific, Cheshire, Reino Unido) a 532 nm. La concentración de malonaldehído se expresó como nmol de MDA por g de tejido usando el coeficiente de extinción 0.156 μM^{-1} cm^{-1} aplicando la siguiente fórmula:

$$\text{nmol MDA/g tejido} = \frac{A}{0.156} \times \frac{50}{Pesomuestra}$$

10.3.6 Determinación del contenido de vitamina E

La concentración de vitamina Eα(-tocoferol) se estimó en muestras de dietas y larvas usando cromatografía líquida de alta presión (HPLC) con detección UV tras la saponificación de las muestras. Este análisis se realizó durante una estancia en el Instituto de Acuicultura de la Universidad de Stirling (Escocia, Reino Unido).

10.236.1 Preparación de los estándares y curva de calibrado

La cuantificación de la vitamina E se llevó a cabo tras la comparación con (+)-α-tocoferol (Sigma-Aldrich, Madrid, España) como estándar externo. Para preparar los estándares, se preparó una solución madre de 1 mg ml^{-1} en 25 ml de metanol, añadiendo 37 mg de aceite en 25 ml de metanol. Se tomaron 200 µl de la solución madre y se añadieron a 10 ml de metanol para así obtener una concentración de trabajo de 20 µg ml^{-1}. Su absorbancia se leyó a una longitud de 293 nm en una cubeta de plástico usando como blanco el metanol. Se empleó la siguiente ecuación para calcular la concentración de vitamina E:

$$\frac{A\ 293\ nm}{71} = \text{g vit E/100 ml}$$

Donde A es la absorbancia y 71 es el coeficiente de extinción molar de la vitamina E a 293 nm. Por tanto;

$$\frac{\frac{g\ vit\ E}{100\ ml \times 10}}{20} = \text{µg vit E/50 µl de inyección}$$

La curva de calibrado se preparó usando cinco concentraciones entre 1 y 100 µg ml^{-1} como se indica en la Tabla 10.6.

Tabla 10.6 Diluciones empleadas para la preparación de la curva estándar del α-tocoferol

Concentración (µg ml^{-1})	Solución madre (µl)	100 µg ml^{-1} vial (µl)	Metanol (ml)
100	400	-	3.6
50	200	-	3.8
25	100	-	3.9
10	-	400	3.6
1	-	40	3.96

Se midió la concentración de cada vial en un espectrofotómetro (CECIL CE 2021 series, CECIL instruments, Cambridge, Reino Unido) a 293 nm. Los estándares se inyectaron en triplicados en el HPLC y se almacenaron bajo atmósfera de nitrógeno en el -20ºC. Cada vez que se analizaban nuevas muestras, se inyectaba también el estándar.

10.3.6.2 Extracción de vitamina E de las dietas

Se hizo en triplicados según el método de McMurray *et al*. (1980). Se pesó aproximadamente 1 g de pienso, al que se le añadieron 25 ml de pirogalol etanólico al 6% (w/v), incubando la mezcla a 70ºC durante aproximadamente 5 min. Tras esto, se añadieron 5 ml de KOH al 60% a los tubos que, tras ser agitados vigorosamente, se devolvieron al baño maría donde estuvieron unos 10 min. A continuación, se enfriaron los tubos en hielo antes de añadir 40 ml de agua filtrada fría y 30 ml de isohexano + BHT diluido. Se agitó cada tubo exactamente durante un min para permitir la separación

de las dos fases. Para facilitar este proceso, los tubos se introdujeron en el -20ºC durante unos 10 min. Una vez que las dos fases estaban separadas, se extrajeron unos 20 ml de la fase superior y se dispusieron en nuevos tubos, se evaporó el solvente a sequedad con nitrógeno y se resuspendió en 5 ml de metanol. Este se transfirió a viales de cristal y se almacenó en el congelador en atmósfera de nitrógeno y oscuridad hasta su posterior lectura en el HPLC.

10.3.6.3 Extracción de vitamina E de larvas

Para llevar a cabo este procedimiento en triplicado se siguió el método de Cowey *et al*. (1981). Se homogeneizaron aproximadamente 50 mg de larvas en 5 ml de pirogalol etanólico al 2% (w/v) usando un Ultra turrax (IKA-Werke, T25 BASIC, Staufen, Alemania). A continuación se incubó en un baño maría a 70ºC durante 5 min. Una vez enfriados los tubos, se les añadió 1 ml de KOH al 60% y se volvieron a colocar en el baño durante otros 20 min. Después, se enfriaron en hielo y se les añadieron 4 ml de agua destilada y 6 ml de isohexano + BHT. Los tubos se agitaron durante exactamente 1 min para extraer la vitamina E. Para facilitar la separación de las dos fases, los tubos se colocaron en el -20ºC durante unos 10 min. Una vez que las dos fases se habían separado, se extrajeron 4 ml de la fase superior, se evaporaron a sequedad y se redisolvió en 1 ml de etanol, para posteriormente ser transferido a una vial de cristal de 2 ml para ejecutar el análisis.

10.3.7 Determinación del contenido de vitamina C

Se extrajo el ascorbil-2-monofosfato de las dietas usando un tampón fosfato y cuantificando con HPLC siguiendo el método desarrollado por Roche Vitamins Ltd. El análisis de la vitamina C se realizó durante una estancia en el Instituto de Acuicultura de la Universidad de Stirling (Escocia, Reino Unido).

10.3.7.1 Extracción de vitamina C de las dietas

Se molieron las muestras y se pesaron 5 g que fueron dispuestos dentro de un matraz. Se añadieron 50 ml de tampón fosfato 0,4 M (pH 3,0) y se agitó durante 15 minutos a temperatura ambiente. Se centrifugaron (1610 x *g*) dos alícuotas durante 5 min a temperatura ambiente y se transfirió el sobrenadante a un nuevo tubo. Se tomó 1 ml con una aguja desechable y se filtró a través de un filtro de 0,45 µm a un vial de 2 ml. Se mantuvo en refrigeración (4ºC) hasta el análisis con HPLC.

10.3.8 Determinación de selenio

Se midió la concentración total de selenio en larvas y dietas. La muestras fueron acidificadas en un digestor microondas (MarsXpress, CEM, Kamp-Lintfort, Alemania) con 5 ml de ácido nítrico puro al 69%. La solución resultante se vertió tras la digestión en un tubo de plástico y se llevó a volumen de 10 ml con agua destilada. Se transfirieron un total de 0,4 ml de esta solución a un tubo de 10 ml y se añadió 10 µl del estándar interno (Ga y Sc, 10 ppm) y 0,3 ml de metanol. Los tubos se llevaron a un volumen de 10 ml con agua destilada y se midió el selenio total con un ICP-MS de colisión/reacción de células (Thermo Scientific, Cheshire, Reino Unido) empleando argón e hidrógeno como gases transportadores. La determinación de selenio se realizó durante una estancia en el Instituto de Acuicultura de la Universidad de Stirling (Escocia, Reino Unido).

10.3.9 Estudios histológicos

10.3.9.2 Inclusión en parafina

Las larvas de lubina fijadas en formol tamponado al 10% hasta se incluyeron en parafina, pasando previamente por una escala de alcoholes (70-96º) con el uso de un procesador (Histokinette 2000, Leica, Nussloch, Alemania). Finalmente se incluyeron en bloques de parafina (Jung Histoembedder, Leica, Nussloch, Alemania) que se cortaron con un grosor de 3 µm en un micrótomo (Leica, RM2135, Leica Instruments, Nussloch, Alemania). Las secciones se tiñeron con Hematoxilina y Eosina (H&E) para proceder a su evaluación histopatológica (Martoja y Martoja-Pearson, 1970). Así mismo, se emplearon distintas tinciones especiales como el ácido periódico de Schiff (PAS), el azul Prusia de Perl (PB), el Ziehl-Neelsen modificado (ZN) o el negro sudán (SB) (Martoja and Martoja-Pearson, 1970) para comprobar la presencia de distintos componentes (Tabla 10.7). Las preparaciones se examinaron en un microscopio óptico Olympus CX41 (Olympus, Hamburgo, Alemania) conectado a una cámara Olympus XC30 (Olympus, Hamburgo, Alemania), la cual estaba unida a un ordenador con el programa de captura de imágenes (CellB®, Olympus, Hamburgo, Alemania).

10.3.9.2 Inclusión en resina

Se fijaron 10 larvas por tanque en glutaraldehído al 2,5% en tampón fosfato durante 24 h a 4ºC y se dejaron en tampón cacodilato hasta su posterior procesado según el método de Millonig (Bancroft y Stevens, 1996). A continuación las muestras se enjuagaron en buffer fosfato, se post-fijaron durante una hora en tetróxido de osmio al 2% en ferrocianuro potásico y se deshidrataron en acetona. Después cada larva fue

incluida en un bloque de resina de Araldita (Durcupan, Fluka, Buchs, Suiza) y se mantuvo en estufa a 60ºC durante la noche. Se hicieron cortes transversales y longitudinales a 1 µm en un ultramicrótomo (Leica LKB Ultratome Nova, Nussloch, Alemania). Las secciones se tiñeron con azul de toluidina al 1% en tetraborato de sodio al 1% y se examinaron con un microscopio óptico convencional.

Tabla 10.7 Técnicas de tinción empleadas en la presente Tesis y su significado

Técnica	Uso	Resultados
H&E	Evaluación histopatológica	Colágeno – Rosa pálido Músculo – Rosa fuerte Citoplasma acidófilo – Rojo Citoplasma basófilo – Púrpura Núcleos – Azul Eritrocitos – Rojo cereza
PB	Demuestra la presencia de hierro, derivado de la lisis de los eritrocitos	Hierro – Azul Núcleos – Rojo
PAS	Detecta sustancias resultantes de la peroxidación lipídica como la lipofucsina o el pigmento ceroide	Ceroide – Magenta Núcleos – Azul
ZN	Detecta sustancias resultantes de la oxidación de lípidos y lipoproteínas como la lipofucsina o el pigmento ceroide	Ceroide– Magenta Núcleos – Azul Fondo – De magenta claro a azul claro
SB	Detecta sustancias resultantes de la oxidación de lípidos y lipoproteínas como la lipofucsina o el pigmento ceroide	Ceroid and red blood cells – Black Background – Pale grey

Los cortes ultrafinos se realizaron con una cuchilla de diamante con un grosor de 50 nm, se colocaron en una rejilla de cobre y se contrastaron con citrato de plomo antes de su observación en el microscopio electrónico de transmisión ZEISS EM 910 (Carl ZEISS, Oberkochen, Alemania) del Servicio de Microscopía Electrónica de la Universidad de Las Palmas de Gran Canaria. Las fotografías se tomaron empleando una cámara CCD (Froscan Elektronische Systeme, Alemania) la cual estaba conectada

a un ordenador que usaba el programa de captura de imágenes (Soft Imaging System, Alemania).

10.3.9.3 Tinción de hueso y cartílago

Se utilizó un protocolo de tinción para determinar el grado de mineralización y la incidencia de deformidades de las larvas en los Capítulos 5 y 6. A los 35 dph (final del periodo experimental) se almacenaron 100 larvas por tanque en formol tamponado al 10% para examinar las deformidades esqueléticas y 50 para determinar el grado de osificación. Las larvas se tiñeron con rojo Alizarina S para detectar el hueso según el protocolo de Vandewalle *et al*. (1998). Las larvas de los distintos tratamientos se tiñeron a la vez para evitar cualquier variabilidad técnica.

Tras la tinción las larvas se midieron en un proyector de perfiles (V-12A Nikon, Nikon Co., Tokio, Japón) bajo un aumento de 50X y fueron clasificadas en tres clases de tamaño (<10 mm; 10-12 mm and >12 mm). Para el estudio de deformidades se seleccionaron las larvas pertenecientes a la segunda clase de tamaño y se observaron bajo la lupa (Leica DM2500, Nussloch, Alemania). Las deformidades se clasificaron en tres grupos según su localización:

- **Deformidades craneales**: Incluye deformidades como alteraciones operculares o de los radios braquioestegales.
- **Lordosis**: Curvatura hacia dentro de la columna vertebral.
- **Cifosis**: Curvatura de la parte superior de la columna.
- **Otras**: Incluye deformidades como la escoliosis, compresión vertebral o vertebras aplastadas.

La superficie correspondiente con el tejido óseo en las larvas se visualizó utilizando un programa de análisis de imagen (Image-Pro Plus®, Media Cybernetics, Maryland, EEUU) tras la tinción con el rojo Alizarina S. Se usaron una serie de comandos para seleccionar la gama de color de los pixeles y cuantificarlos. Seleccionado gamas de píxeles en imágenes en color se pudieron distinguir los pixeles asociados con el rojo. Se aplicó una máscara para convertir todos los pixeles seleccionados en objetos brillantes y a continuación se cuantificó el número de pixeles seleccionados usando un comando de análisis de partículas, contando así los objetos brillantes (en pixeles). El tamaño de la larva fue estimado calculando el área (en pixeles) ocupado por la superficie de las larvas teñidas.

10.3.10 Biología molecular

Todos los procedimientos indicados en la presente sección se realizaron durante una estancia en el Departamento de Biotecnología y Ciencias Moleculares de la Universidad de Insubria (Varese, Italia). Los genes estudiados se muestran en la Tabla 10.8. Las secuencias de los genes catalasa (CAT), superóxido dismutasa (SOD) y glutatión peroxidasa (GPX) fueron reconfirmadas antes de su uso para la PCR cuantitativa a tiempo real (RT-PCR). Los cebadores de estos genes se encargaron a la empresa Eurofins (Ebersberg, Alemania).

Tabla 10.8 Genes estudiados en la presente Tesis

Gen	Número de acceso
Superóxido dismutasa (SOD)	FJ860004
Catalasa (CAT)	FJ860003
Glutatión peroxidasa (GPX)	FM013606
Factor de crecimiento insulínico I (IGF-I)	AY800248
Factor de crecimiento insulínico II (IGF-II)	AY839105
α-Actina (α-actin)	FJ716131
Cadena pesada de la miosina (MyHC)	DQ317302
Calpaína 1 (Calpn)	FJ821591

10.3.10.1 Extracción total del ARN

El ARN total se extrajo de las larvas de lubina (≈200 mg) usando el kit PureYield RNA Midiprep System (Promega, Milán, Italia). Las larvas se homogeneizaron en hielo con 2 ml del tampón de lisis y 40 µl of β-Mercaptoetanol utilizando un Ultra turrax (IKA-Werke, T25 BASIC, Staufen, Alemania). El brazo del Ultra turrax se lavó entre muestras con NaOH 1M, etanol 100º y agua DEPC. Posteriormente se incubaron los lisados en hielo durante 10 min para así completar la lisis y se transfirieron 2 ml a un tubo desechable. Para limpiar los lisados, se añadieron 4 ml del tampón de dilución de RNA y se mezclaron mediante agitación. Después, se añadió 1 ml del agente aclarante y se volvió a agitar la mezcla. A continuación, los tubos se colocaron en un baño maría a 70ºC donde se incubaron durante 5 min para desnaturalizar las muestras y posteriormente se dejaron enfriar hasta alcanzar la temperatura ambiente durante al menos 5 min. Las muestras se colocaron en el interior de una columna Pureyield™

Clearing Column dentro de un tubo de 50 ml, para ser centrifugadas a 12000 x *g* durante 10 min a una temperatura de 20-25ºC. Se traspasó el lisado y se le añadió 4 ml de isopropanol. La purificación del ARN se llevó a cabo usando una bomba de vacío (Vacuum Manifold, Promega, Milán, Italia), por lo que no fue necesario centrifugar las muestras. Se vertieron las muestras lisadas dentro de una columna PureYield^{TM} Binding Column y a continuación se le aplicó el vació, para que así la muestra pasase a través de la columna. Sucesivamente, se añadieron 20 y 10 ml de la solución de lavado del ARN a la columna y se volvió a aplicar el vacío. Después de 3 min de vacío en seco, se añadió 1 ml de agua libre de nucleasas a la columna, se incubó a temperatura ambiente durante 2 min y se aplicó nuevamente el vacio. El ARN purificado se almacenó en el -80ºC en eppendorf libres de ARNasas.

10.3.10.2 Control de calidad del ARN

La medida de la concentración del ARN así como de su calidad se hizo usando un espectrofotómetro Bio-Rad SmartSpec Plus. La pureza se estimó a un radio de absorbancia 260:280, estimando que un ratio mayor de 1,8 indicaba que la muestra poseía un alto nivel de pureza y que no estaba contaminada por proteínas (McKenna *et al*., 2000). Además, se estimó la degradación del ARN al analizar 2µg de ARN total por electroforesis en un gel de agarosa al 1%. Las muestras se sometieron a una corriente eléctrica de 90 V durante 45 min y a continuación el gel fue visualizado con un transluminador de UV (Bio-Rad UV Transluminator 2000).

10.3.10.3 Síntesis de cADN

El ADN complementario (cADN) se genera típicamente del ARN mensajero (mARN) por la acción de la transcriptasa retroviral reversa que retrotranscribe una única hebra de ARN en un único cADN. Tras el tratamiento con ADNasa (Invitrogen, Milán, Italia), se retrotranscribieron 3 µg de ARN total en cADN en un volumen de 12 µl, incluyendo 1 µl de cebadores dT16 (50pmol) y 1 µl de trifosfatos deoxynucleotido 10 mM (dNTPS). Esta mezcla se calentó a 65ºC durante 5 min, después se enfrió en hielo y se añadieron 4 µl de tampón de transcripción 5x, 2 µl de ditiotreitol 0,1 M, 1 µl de ARNase out y 1 µl de virus de leucemia murina de Moloney (M-MLVRT). Tras la incubación a 37ºC durante 50 min, la reacción se paró al calentarla a 75ºC durante 15 min.

10.3.10.4 Clonado y secuenciación

Para amplificar las secuencias seleccionadas de los cebadores, se llevó a cabo una PCR “touch up”. La selección de este tipo específico de PCR se debió a la amplia variedad de temperaturas de “melting” de los primers. En este sentido, con una PCR “touch up”, la temperatura inicial de 54ºC incrementa 0,5ºC en cada ciclo, llegando a una temperatura final de 72ºC. Se llevaron a cabo un total de 30 ciclos para todos los cebadores, usando un termociclador (Mycycler, Bio-Rad, Italia).

Se amplificó una alícuota de 4 µl de cADN usando 25 µl de GoTaq Green Master Mix (Promega, Milán, Italia) en 50 µl de volumen final, conteniendo 5 µl de tampón de alta fidelidad, 10 mM dNTPS y 50 pmol de cada cebador diseñado.

Se realizaron un total de 31 ciclos de amplificación de PCR para todos los cebadores utilizando un termociclador automatizado (MyCycler, BioRad, Italia). Una alícuota de cada muestra se sometió a electroforesis en gel de agarosa al 1% en tampón 1X tris-acetato-EDTA (TAE) (BioRad, Italia) y se detectaron las bandas con bromuro de etidio. Junto a las muestras se aplicó un marcador de peso molecular (100 bp+1,5 Kb) para controlar el peso molecular del ADN, así como con un control negativo que constaba de la mezcla de reacción sin contener el cADN, conformando de este modo la ausencia de contaminación genómica.

Antes de clonar, se purificó el producto de la PCR cortando un fragmento de ADN del peso molecular previsto del gel y procesándolo con el kit Wizard Clean-up System (Promega, Milán, Italia) de acuerdo a las instrucciones de los fabricantes. Tras disolver el fragmento de gel se colocó en una minicolumna SV y se incubó durante 1 min a temperatura ambiente. Las columnas se centrifugaron a 14000 x g durante 1 min, se desechó la fase acuosa y se lavó con 700 µl de la solución de lavado de membrana diluída en etanol al 95%. Tras la centrifugación, la minicolumna se dispuso en un nuevo tubo de microcentrifugación y se disolvió con 50 µl de agua libre de ARNasas. La concentración del producto de PCR purificado se midió utilizando el espectrofotómetro. La cantidad de producto de PCR a incluir en la reacción de clonación se calculó con la siguiente fórmula:

$$ng\ Insert = \frac{ng\ Vector \times Kb\ Insert}{Kb\ Vector} \times Insert : Vector\ molar\ ratio$$

Siendo:

ng vector = 50 ng

Kb vector = 3,0 Kb

Radio molar Inserción:Vector = 3:1

Kb inserción = En este caso los genes tenían una longitud de 200 pares de bases, por tanto 0.2 Kb.

El vector de clonaje empleado en este estudio fue pGEM®-T Easy cloning vector system (Promega, Milán, Italia). La unión del producto de PCR se realizó gracias a la actividad intrínseca de la topoisomerasa asociada con preparación comercial del vector de clonación. La reacción de clonación se realizó a 4ºC durante una noche y al día siguiente se añadió 50 µl de bacteria *Escherichia coli* (Promega, Milán, Italia) a la mezcla y se incubó en hielo durante 30 min. A continuación se realizó un choque térmico en un baño maría (40ºC) sin agitar durante 45 seg para posteriormente ponerlo en hielo durante 2 min. A continuación se añadieron 940 µl de medio SOC (Sigma) y la mezcla se incubó a 37ºC durante 2 h en un agitador. La mezcla conteniendo las células transformadas se extendió en tres placas de petri por gen, con el medio selectivo LB Agar y fueron incubadas durante la noche a 37ºC. Las placas de petri contenían además ampicilina (100µg ml^{-1}), X-gal e IPTG. Como resultado, sólo las células que contenían un fragmento de PCR ligado, que contiene un gen resistente a la ampicilina podían crecer. Además, la selección de células con un fragmento de PCR ligado se basó en la reacción metabólica colorimétrica del X-gal por el gen Laczα, ya que la ligación del producto de PCR resultaría en una interrupción del gen, lo que causa colonias blancas. Se eligieron cinco colonias por gen, que fueron disueltas en 20 µl de agua libre de ARNasas, utilizando 4 µl de esta solución para hacer una nueva PCR.

De acuerdo a la calidad de las bandas, se eligió una colonia y se cultivó toda la noche a 37ºC en agar LB con ampicilina. El vector del plásmido fue purificado usando el kit Pureyield Midiprep System (Promega, Milán, Italia), siguiendo las indicaciones del manual. La columna se purificó con 30 µl de agua libre de ARNasas, se calculó su concentración con un espectrofotómetro y se conservó a -20ºC. Los extractos se secaron y se enviaron a BMR Genetics (Padova, Italia) para su secuenciación en ambas direcciones (T7 y SP6).

10.3.10.5 Cuantificación por "one-step" RT-PCR con sondas TaqMan

Cien nanogramos del ARN total extraído de las muestras experimentales se sometieron a PCR a tiempo real en las mismas condiciones que se emplearon para

crear curvas estándares, necesarias para determinar la concentración absoluta del ARNm. Los cebadores de tiempo real Assays-by-Design™ y las sondas fluorogénicas específicas de cada gen se diseñaron por Applied Biosystems (ABI). Las sondas TaqMan® usadas se muestran en la Tabla 10.9.

Table 10.9 Sondas TaqMan® de los genes empleados en la presente Tesis

Genes	Tipo de cebador	Secuencia (5´→3´)
CAT	Cebador Forward	ATGGTGTGGGACTTCTGGAG
	Cebador Reverse	GCTGAACAAGAAAGACACCTGATG
	Sonda TaqMan®	CAGACACTCAGGCCTCA
SOD	Cebador Forward	TGGAGACCTGGGAGATGTAACTG
	Cebador Reverse	TCTTGTCCGTGATGTCGATCTTG
	Sonda TaqMan®	CAGGAGGAGATAACATTG
GPX	Cebador Forward	AGTTAATCCGGAATTCGTGAG
	Cebador Reverse	AGCTTAGCTGTCAGGTCGTAAAAC
	Sonda TaqMan®	AATGGCTGGAAACGTG
IGF-I	Cebador Forward	GCAGTTTGTGTGTGGAGAGAGA
	Cebador Reverse	GACCGCCGTGCATTGG
	Sonda TaqMan®	CTGTAGGTTTACTGAAATAAAA
IGF-II	Cebador Forward	TGCAGAGACGCTGTGTGG
	Cebador Reverse	GCCTA CTGAAATAGAAGCCTCTGT
	Sonda TaqMan®	CAAACTGCAGCGCATCC
Calpn	Cebador Forward	ACTTTACAGGCGGCGTGA
	Cebador Reverse	GGCTCTGCTGATGATGTTGTAGA
	Sonda TaqMan®	TCAGATCGTACATTTCCG
α-actin	Cebador Forward	CCTCTTCCAGCCTTCCTTCA
	Cebador Reverse	TGTTGTAGGCGGTCTCATGGATA
	Sonda TaqMan®	CCAGCAGACTCCATACCGA
MyHC	Cebador Forward	TGGAGAAGATGTGCCGTACTCT
	Cebador Reverse	CGTGTCATTGATTTGACGGACATTT
	Sonda TaqMan®	AACTGAGTGAACTGAAGACC

La PCR se llevó a cabo en un sistema de PCR a tiempo real StepOne (Applied Biosystems). Para reducir errores de pipeteo, se prepararon cantidad suficiente de mezcla para hacer reacciones en duplicado (2 x 30 µl) de cada muestra.

10.3.10.5.4. Cuantificación de las muestras

Los datos de las PCR se recogieron con el programa StepOne™ v 2.0. Los valores de Ct corresponden con el número de ciclos en los que la emisión fluorescente superaba el límite umbral. Los valores Ct se usaron para crear curvas estándares que sirviesen como base para el cálculo de las cantidades absolutas de ARNm en el ARN total (nº de moléculas/ng de ARN total).

10.4.11 Análisis estadístico

Los análisis estadísticos realizados en la presente Tesis se llevaron a cabo empleando en programa SPSS (SPSS para Windows 14.0; SPSS Inc., Chicago, IL, EEUU, 2005). Se consideró un nivel de significancia del 5% (P<0.05) en todas las pruebas estadísticas. Para cada parámetro medido se calculó la media aritmética como una estima de la media poblacional junto con la desviación estándar (SD) para representar así la distribución de las muestras.

10.4.11.1 Pruebas paramétricas

Las pruebas paramétricas se realizaron una vez que se comprobó que las observaciones se hicieron al azar y que las varianzas eran independientes. Además, las varianzas debían de ser homogéneas y los datos estar distribuidos normalmente. Cuando los datos no cumplían estos requisitos, se realizaba una prueba no paramétrica (Sección 10.2.11.2).

Se usó el test de Levene para determinar la normalidad y homogeneidad de las varianzas y una vez que se cumplieron los supuestos, se analizaron usando un análisis de varianza de una vía (ANOVA) y cuando existieron diferencias entre los datos (P<0.05), se aplicó la comparación *post-hoc* de Duncan usando el SPSS. Para la aplicación del ANOVA de una vía se usó el siguiente modelo linear general:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}$$

donde Y_{ij} es la media del valor del tanque, μ es la media de la población, α_i es el efecto fijo de la dieta y ε_{ij} es el error residual.

Además, se usó un modelo lineal general de dos variables para analizar los datos de biología molecular. Así, el factor “tiempo de muestreo” se incluyó dentro del modelo, permitiendo observar diferencias entre las variables dieta, efecto del tiempo y sus interacciones. El modelo empleado fue:

$$Y_{ijk} = \mu + \alpha_i + \delta_j + (\alpha\delta)_{ij} + \varepsilon_{ijk}$$

Donde Y_{ij} es el valor medio del tanque, μ es la media de la población, α_i es el valor fijo de la dieta, δ_j es el factor fijo del tiempo, $(\alpha\delta)_{ij}$ es la interacción entre dieta y tiempo y e_{ij} es el error residual.

10.4.11.2 Pruebas no paramétricas

Los datos que no cumplieron los supuestos de las pruebas paramétricas o los categóricos se analizaron usando pruebas no paramétricas. Se utilizó el test de Kruskall-Wallis y las medias con diferencias significativas se identificaron con el *post-hoc* chi cuadrado.

10.5 Resúmenes de los experimentos

10.5.1 Capítulo 3: La inclusión de α-tocoferol en microdietas para larvas de lubina (*Dicentrarchus labrax*) mejora la supervivencia y reduce la incidencia de lesiones musculares causadas por un exceso de DHA

El objetivo del presente trabajo fue investigar el efecto de distintos niveles de vitamina E y ácidos grasos poliinsaturados (LC-PUFA), principalmente DHA, sobre el crecimiento, supervivencia, composición bioquímica y morfología muscular de la lubina durante los primeros estadios de su desarrollo larvario. Para ello, se llevó a cabo un experimento empleando dietas con la misma composición proximal y distintos niveles de aceite concentrado en DHA (1, 3 y 5 % peso seco) y vitamina E, α-tocoferil acetato; (150 y 300 mg 100 g^{-1} peso seco). El DHA se depositó rápidamente en los tejidos y se asoció a alta mortandad larvaria, probablemente debido a efectos de la peroxidación. Además, el incremento del DHA en dietas hasta un 5% aumentó plausiblemente la incidencia de lesiones musculares y la presencia de pigmento ceroide en los hepatocitos. Sin embargo, el aumento de vitamina E redujo marcadamente la incidencia de estos signos, incrementando el contenido de diversos PUFA en los tejidos larvarios así como mejorando el crecimiento y la resistencia al estrés. Además, cuando las larvas ingirieron altos niveles de vitamina E, mostraron una mejora en su crecimiento cuando el DHA se incrementó de un 1 a un 3%. Por tanto, un nivel de 3 % de DHA y 300 mg 100 g^{-1} de vitamina E parecen ser adecuados para conseguir un buen rendimiento en larvas de lubina y evitar la aparición de lesiones musculares.

10.5.2 Capítulo 4: Estado oxidativo y cambios estructurales en el músculo de larvas de lubina alimentadas con dietas con un alto contenido de DHA

En el estudio anterior se observó distrofia muscular en larvas de lubina de 48 días de edad alimentadas con dietas desequilibradas en DHA y vitamina E. Para comprender los efectos asociados al estrés oxidativo, se llevó a cabo un estudio estructural tras alimentar a larvas de 14 días de edad con microdietas con distintos ratios de DHA/vitamina E (1/150, 5/150 y 5/300). No se apreciaron lesiones en las larvas alimentadas con la dieta 1/150, en contraposición con las larvas alimentadas con un 5% de DHA, en las que se observaron lesiones musculares y valores altos de TBARS. En las secciones semifinas se apreciaron lesiones focales constituidas por miofilamentos hipercontraidos, así como extensa vacuolización citoplasmática afectando tanto a músculo blanco como rojo. En las secciones ultrafinas se observó la dilatación difusa del retículo sarcoplásmico, desorganización de miofilamentos y vacuolas autofágicas conteniendo cuerpos densos y figuras de mielina. Además se observaron algunos monocitos-macrófagos alrededor de las fibras afectadas, así como numerosas células satélite. Los resultados del presente trabajo refuerzan las conclusiones observadas en el Capítulo 3 donde se muestra el potencial patológico de los radicales libres en la musculatura de las larvas de lubina, sin que este efecto haya podido ser atenuado mediante la inclusión de vitamina E en las dietas.

10.5.3 Capítulo 5: La inclusión de selenio disminuye los indicadores de estrés oxidativo en larvas de lubina alimentadas con microdietas altas en DHA

En los estudios anteriores se demostró que el músculo de las larvas de lubina es muy sensible al daño de los radicales libres generados por un exceso de ácido docosahexaenóico (DHA) en sus dietas. Sin embargo, el incremento de vitamina E combinado con un alto nivel de DHA no consigue reducir la incidencia de estas lesiones. Este hecho sugiere que la inclusión de otros nutrientes con función antioxidante, como el selenio (Se), podría ayudar a prevenir estas lesiones. Por tanto, el objetivo del presente trabajo fue estudiar el efecto de la inclusión de Se en microdietas con un alto contenido de DHA y vitamina E (5/300 y 5/300+Se) sobre el crecimiento, supervivencia, composición bioquímica, morfología muscular, así como la expresión de miosina, IGFs y enzimas antioxidantes. La inclusión de Se favoreció el crecimiento de las larvas y redujo la incidencia de lesiones musculares, contenido de malonaldehído (MDA) y la expresión de las enzimas antioxidantes. Por el contrario, la expresión de IGFs se vio incrementada en las larvas alimentadas con la dieta 5/300 sugiriendo un aumento de la mitogénesis a nivel muscular, hecho que fue corroborado por el incremento en la expresión de miosina en las mismas. Los resultados del presente estudio confirman el efecto beneficioso del Se en la prevención del estrés oxidativo, no solo como una cofactor de la GPX, si no probablemente debido a otras funciones fisiológicas no tan conocidas.

10.5.4 Capítulo 6: La adición de vitaminas C y E mejora la disponibilidad de la vitamina E y reduce los marcadores del estrés oxidativo en larvas de lubina (*Dicentrarchus labrax)* alimentadas con contenidos altos de DHA.

El ácido docosahexaenóico (DHA) es un ácido graso esencial necesario para muchas funciones bioquímicas, celulares y fisiológicas. Sin embargo, el músculo de las larvas de lubina es muy sensible a altos niveles de DHA en la dieta. A pesar de que niveles altos de vitamina E (α-tocoferol, α-TOH) pueden reducir la incidencia de las lesiones, estas aún aparecen. Por tanto, la inclusión de otros nutrientes con funciones antioxidantes complementarias, como la vitamina C (ácido ascórbico, AA), podrían contribuir a prevenir estas lesiones. El objetivo de este estudio fue determinar el efecto de la inclusión de AA en microdietas para larvas de lubina con niveles elevados de DHA y α-TOH (5/300 and 5/300+AA) en comparación con una dieta control (1/150). Para ello se estudió el crecimiento, la supervivencia, la composición bioquímica, el contenido en malonaldehído, la morfología del músculo, las deformidades esqueléticas y la expresión de las enzimas antioxidantes, IGFs y miosina (MyHC). El AA demostró ser efectivo para controlar el daño oxidativo en el músculo y reducir el contenido de MDA, aunque no se observó ningún efecto en la prevención de las deformidades. La expresión de las IGFs fue elevada en las larvas 5/300 sugiriendo un incremento en la mitogénesis muscular, hecho que fue corroborado por el incremento en las copias de ARNm de la MyHC. Los resultados del presente estudio demuestran el sinergismo entre las vitaminas E y C cuando las dietas para larvas de lubina contienen altos niveles de DHA.

10.5.5 Capítulo 7: Factores moleculares implicados en la aparición y regeneración de la distrofia muscular nutricional en larvas de lubina

Estudios previos han señalado el efecto de la inclusión de niveles altos de ácido docosahexaenóico (DHA) en el músculo de larvas de lubina bajo la influencia de distintos niveles de vitamina E, selenio o vitamina C. Sin embargo, no se ha estudiado el potencial de regeneración de estas lesiones, ni las rutas moleculares implicadas en la misma. Por tanto, para poder entender mejor este proceso, se llevó a cabo un experimento de tres semanas de duración en el que se alimentaron larvas de 14 dph con microdietas con distintos niveles de DHA (1 o 5%) y 150 mg 100 g^{-1} de vitamina E. Tras este periodo, parte de las larvas alimentadas con la dieta 5/150 comenzaron a comer la dieta 1/150 (dieta control) continuando el experimento durante otras dos semanas. Las larvas alimentadas con la dieta 5/150 mostraron un estado oxidativo alterado, indicado por un incremento del MDA, expresión de las AOE así como mayor incidencia de lesiones musculares. A los 49 dph se observó una disminución en la expresión de las AOE, indicando que a esta edad un 5% de DHA podría ser adecuado para las larvas. Así mismo, las larvas realimentadas con la dieta 1/150 mostraron un menor peso seco y contenido de vitamina E. La expresión de la IGF-I fue alta en las larvas alimentadas con la dieta 5/150 a los 35 dph, sugiriendo una mayor miogénesis, corroborada por el incremento de la MyHC. Sin embargo, ni la IGF-II ni la α-actina mostraron esta tendencia. Con estos resultados se puede concluir que altos contenidos de DHA alteran el estado oxidativo y causan lesiones musculares en larvas de lubina, con aspectos morfológicos y moleculares similares a los encontrados en las enfermedades degenerativas musculares de los mamíferos.

10.6 Discusión general

10.6.1 Objetivos logrados

1.- En la presente tesis, se probó el efecto de la inclusión de altos niveles de DHA (1, 3 y 5%) en larvas de lubina por primera vez. Se corroboró que niveles demasiado altos de este ácido graso podrían no ser beneficiosos para el cultivo de las larvas de lubina.

2.- También se evaluó el efecto protector antioxidante del α-tocoferol asociado a altos niveles de DHA (1, 3 y 5%). La vitamina E parecía no ser capaz de proteger los tejidos de las larvas de lubina cuando se empleaba un nivel de DHA del 5%. Esto sugiere que para conseguir una protección antioxidante adecuada, se requerirían otros nutrientes.

3.- Atendiendo a los resultados obtenidos previamente, se planteó un nuevo experimento utilizando tanto vitamina E como selenio, para investigar el papel de este mineral en las defensas antioxidantes de las larvas de lubina, comprobando así que ambos antioxidantes fueron efectivos a la hora de contrarrestar los efectos adversos de los ROS.

4.- Así mismo, también se emplearon niveles altos de vitamina C junto con vitamina E y DHA para estudiar su papel específico en la defensa antioxidante cuando existe ya un estado oxidativo alterado.

¿Los niveles altos de DHA pueden mejorar el rendimiento del cultivo larvario?

Parece ser que las larvas de especies marinas tienen mayores requerimientos específicos de DHA que los juveniles o los pre-adultos, por tanto la formulación de alimentos para larvas de especies marinas tradicionalmente han incluido grandes cantidades de este ácido graso. Muchos estudios han señalado los efectos negativos asociados a la deficiencia de DHA en larvas de peces (Watanabe *et al*., 1989; Masuda *et al.*, 1998; Brandsen *et al.*, 2005; Benítez-Santana *et al.,* 2007) o como el incremento de este ácido graso puede mejorar el rendimiento del cultivo (McEvoy *et al*., 1998; Cahu *et al*., 2003; Brandsen *et al*., 2005; Jalali *et al.,* 2008; Roo *et al.,* 2009; Satoh y Takeuchi, 2009). Sin embargo, en la presente tesis se ha comprobado que niveles demasiado altos de DHA pueden conducir a la alteración del estado oxidativo, con efectos negativos para el cultivo larvario de la lubina. El crecimiento y la supervivencia,

indicadores del rendimiento larvario, no se vieron siempre afectados de manera negativa por niveles de DHA del 5%. En el Capítulo 3, el crecimiento y la supervivencia más baja se observó en las larvas alimentadas con la dieta 5/150 y en los Capítulos 5 y 6, las que mostraron menor longitud total fueron aquellas alimentadas con la dieta 5/300, mientras que en los Capítulos 4 y 7, estos parámetros no se vieron afectados por el contenido de DHA en las dietas (5%). Estas diferencias podrían atribuirse a las diferentes edades de las larvas, a la calidad de la puesta o a la nutrición de los reproductores, ya que los requerimientos de ciertos nutrientes en los primeros estadios larvarios depende del aporte materno previo. En este sentido, las larvas del Capítulo 3 se obtuvieron de la granja Ecloserie Marine de Gravelines (Francia), mientras que las de los otros capítulos provinieron del Instituto de Acuicultura de Torre la Sal (Castellón, España), por tanto los reproductores siguieron regímenes alimenticios diferentes, que podrían haber afectado a sus puestas. Además, las larvas utilizadas en el Capítulo 3 comieron previamente una dieta comercial, lo que las podría hacer más resistentes a los ROS que aquellas empleadas en los otros Capítulos, que sólo comieron rotíferos. Además, como ya se ha indicado previamente, según van creciendo las larvas, sus defensas antioxidantes se basan principalmente en la actividad de las AOE más que en los antioxidantes de bajo peso molecular, por tanto, se espera que la respuesta de larvas de distinta edad sea diferente ante el estrés oxidativo.

Un hallazgo común en todos los experimentos fue la elevada incidencia de lesiones en la musculatura axial de las larvas de lubina cuando se alimentaron con un 5% de DHA. Estas larvas desarrollaron un tipo de lesión conocida como distrofia muscular, que se caracteriza por necrosis segmentaria tanto de fibras blancas como rojas. Estas lesiones aparecen debido al incremento de la producción de ROS, que atacan a los LC-PUFA de la membrana muscular, incrementando de este modo la permeabilidad celular y llevando a la fibra muscular a la necrosis. El desequilibrio entre producción y eliminación de los ROS se ha podido comprobar gracias al contenido de TBARS (Capítulos 4, 5, 6 y 7), así como con la expresión de los genes antioxidantes (Capítulos 5, 6 y 7). La importancia de estas lesiones musculares no solo radica en el bajo rendimiento del cultivo obtenido, si no en las consecuencias que puede tener a largo plazo, como un incremento en la tasa de deformidades debido a las tensiones generadas durante la cicatrización de las heridas (Cotran *et al*., 2004). La fibrosis de la herida, se debe al acumulo excesivo de colágeno y se sabe que también ocurre en peces tras las infecciones de piel y músculo causadas por bacteria o daños mecánicos (Lunder *et al*., 1995; Ingerslev *et al*., 2010), afectando a la calidad del filete. Por

ejemplo, salmones Atlánticos infectados por *Moritella viscosus* muestran una peor calidad debido a la cicatrización de los filetes (Lunder *et al*., 1995).

Otra alteración morfológica observada tras usar dietas altas en DHA fue la presencia de pigmento ceroide en los hepatocitos (Capítulo 3). Esta alteración ha sido relacionada anteriormente con la ingesta de dietas deficientes en vitamina E o con lípidos oxidados en distintas especies de peces (Moccia *et al*., 1984; Miyazaki, 1995; Sakai *et al*., 1998), utilizándose como indicador de peroxidación lipídica. Sin embargo, en el Capítulo 3, se observó una leve ceroidosis, sugiriendo que el músculo es el primer y principal tejido atacado por los radicales libres en las larvas de lubina. Esta hipótesis se ve reforzada por la ausencia de depósito de pigmento ceroide observada en los otros experimentos.

Los niveles de DHA tuvieron también un efecto en la incidencia de deformidades y en la osificación, observando un mayor número de deformidades en las larvas alimentadas con un 5% de DHA, así como menor tasa de mineralización (Capítulos 5 y 6). Por tanto, los radicales libres podrían ejercer su efecto adverso sobre el hueso mediante mecanismos directos e indirectos; alterando directamente el proceso de mineralización e indirectamente causando lesiones musculares que podrían causar deformidades en un futuro, tras su reparación en los juveniles.

¿La vitamina E es un antoxidante efectivo cuando las larvas de lubina consumen niveles altos de DHA?

Ha sido probado que la vitamina E es un potente antioxidante capaz de proteger la musculatura de la larva del daño de los radicales libres. En el Capítulo 3, para un nivel determinado de DHA, el incremento de vitamina E de 150 a 300 mg 100 g^{-1} redujo la incidencia de lesiones musculares, incrementó el crecimiento y la supervivencia tras un estrés. Sin embargo, cuando el nivel de DHA era del 5%, el incremento de α-tocoferol no fue suficiente para contrarrestar el efecto de los radicales libres, observando una mayor incidencia de las lesiones musculares, así como de los TBARS y expresión de las enzimas antioxidantes (Capítulos 4, 6, 7 y 8). El radical tocoferoxil, que surge como resultado de la donación de un electrón al radical peroxil, reacciona lentamente con los LC-PUFA, sin embargo, si no se regenera, los radicales tocoferoxil pueden sustraer átomos de hidrógeno a los LC-PUFA, iniciando así la peroxidación lipídica (Ingold *et al.,* 1993). Por lo tanto, parece ser que cuando se incluye un 5% de DHA en las microdietas para lubina, se producen gran cantidad de ROS, no siendo capaz la vitamina E de

neutralizarlos. Además, si la vitamina E no es regenerada, los radicales tocoferoxil continúan la reacción en cadena de peroxidación lipídica *in vivo*, actuando así la vitamina E como un prooxidante. Resumiendo, la vitamina E es un antioxidante eficaz, sin embargo, se necesitan otros antioxidantes para contrarrestar los ROS cuando se emplean niveles de DHA del 5%.

¿Qué es más efectivo para prevenir la distrofia muscular causada por el estrés oxidativo? ¿Incrementar el selenio o la vitamina C de la dieta?

El incremento tanto de vitamina C como de selenio mejoró el crecimiento larvario y redujo las lesiones musculares y el contenido de MDA en comparación con las larvas que comieron las dietas sin suplementar. Sin embargo, se apreciaron diferencias en la expresión de las AOE, sugiriendo que ambos nutrientes antioxidantes actúan a diferentes niveles. El selenio redujo el número de copias de ARNm de la SOD y de la GPX a niveles comparables con el grupo control (1/150), mientras que la vitamina C no tuvo ningún efecto en la expresión de estas enzimas.

El selenio fue efectivo al ejercer una determinada acción sobre la expresión de las AOE. Además, se sabe que el selenio se incorpora en forma de selenocisteína en el sitio activo de una amplia gama de proteínas, dando lugar a distintas selenoproteínas, algunas de ellas con una marcada capacidad antioxidante. Hoy en día se han purificado o clonado 18 selenoproteínas en peces (Tubajeva *et al*., 2000), incluyendo cuatro GPX, con diferentes localizaciones orgánicas y celulares, lo cual representa una clase primordial de selenoproteínas funcionalmente importantes.

Una de las principales funciones de la vitamina C como antioxidante es la de regenerar radicales tocoferoxil, por tanto sería lógico pensar que la protección antioxidante observada en las larvas alimentadas con la dieta suplementada con vitamina C podría ser debido en parte a la vitamina E. En este sentido, se encontraron mayores contenidos de vitamina E en las larvas alimentadas con la dieta suplementada en vitamina C, sugiriendo que la vitamina E estaba siendo reciclada por la vitamina C o que ésta estaba siendo usada preferentemente como antioxidante, ahorrando así α-tocoferol. Además, se considera que la vitamina C es el antioxidante más importante en los fluidos extracelulares (Stocker y Frei, 1991) y que es capaz de inhibir la peroxidación lipídica iniciada por un radical peroxil en los lípidos del plasma (Frei *et al*., 1991). Además, la vitamina C es donadora de electrones para ocho enzimas diferentes. Algunas de estas enzimas participan en la hidroxilación del colágeno, añadiendo grupos

hidroxilo a los aminoácidos prolina o lisina en la molécula de colágeno. Finalmente, aunque los niveles de vitamina C empleados pueden parecer bastante elevados, se ha demostrado que son necesarios niveles de ácido ascórbico superiores a los requeridos para satisfacer las demandas de otros nutrientes como la vitamina E en casos de deficiencia (Hamre *et al.,* 1997).

Por tanto, como se ha observado en juveniles y adultos de diversas especies de peces, existe un sinergismo entre la vitamina E y el selenio (Poston *et al*., 1976; Bell *et al*, 1985) y un mecanismo conservador entre vitamina E y C (Lovell *et al*.,1984; Sealey y Gatlin, 2002; Shiau y Hsu, 2002; Yildirim-Aksoy *et al*., 2008). Cabe señalar que no se sabe qué tipo de radical se genera de manera endógena en cada enfermedad en concreto, por tanto, estos nutrientes podrían ser adecuados cuando niveles altos de DHA se incluyen en las dietas, pero podrían no ser capaces de contrarrestar radicales libres en presencia de otros prooxidantes.

¿Cuál es el nivel adecuado de inclusión de DHA?

Antes que nada, hay que tener en cuenta que el contenido de DHA siempre tiene que estar relacionado con el contenido antioxidante. Por tanto, niveles incrementados de DHA en las dietas y en los tejidos deben estar acompañados de niveles adecuados de antioxidantes para prevenir el daño oxidativo.

De acuerdo a los resultados obtenidos en la presente tesis, en los primeros estadios (14 a 35 dph) un contenido del 1% de DHA parece ser adecuado, puesto que con este nivel se obtuvieron los mejores resultados de crecimiento, supervivencia, incidencia de lesiones musculares y TBARS (Capítulos 4, 5, 6 y 7). Este contenido parece ser bajo si lo comparamos con los requerimientos de larvas de otras especies como dorada (3%; Izquierdo, 2005), rodaballo (3,2%; LeMilinaire, 1984) o el dentón (*Dentex dentex*) (4%; Mourente *et al*., 1999). La dieta suplementada tanto con selenio como con vitamina C redujo la incidencia de lesiones musculares y mejoró el rendimiento del cultivo, sin embargo hacen falta más estudios para conocer la combinación exacta de cada nutriente así como la forma de suplementación idónea, para que así las larvas puedan emplear mejor los niveles altos de DHA y conseguir mejores tasas de crecimiento.

En estadios más tardíos (a partir de los 35 dph), parece ser que un 5% de DHA es adecuado para alcanzar un mayor crecimiento, tal y como se observó en el Capítulo 7, aunque la incidencia de lesiones musculares aún era mayor que en las larvas alimentadas con la dieta 1/150. Quizás en estos estadios de desarrollo la inclusión de 300 mg 100 g^{-1} de vitamina E es adecuada para evitar la incidencia de lesiones musculares, no obstante, no se probó esta dieta en larvas mayores de 35 dph y además estas larvas comieron una dieta con el 5% de DHA a partir de los 14 dph. Atendiendo a los resultados obtenidos en el Capítulo 3, la dieta con un 3% de DHA y 300 mg 100 g^{-1} de vitamina E fue la más adecuada para conseguir mejor rendimiento del cultivo y más baja incidencia de lesiones musculares. A falta de otros parámetros, a partir de los 35 dph, la dieta 3/300 parece ser la idónea. Por tanto, es necesario un incremento de DHA cuando las larvas están creciendo. Este hallazgo indica que aunque las larvas tienen mayor tasa metabólica que los juveniles, la cantidad de cada nutriente en las dietas para larvas no tiene que ser necesariamente mayor que en el caso de los juveniles para satisfacer las demandas fisiológicas de las larvas.

¿Cuáles son los cambios fisiológicos implicados en la distrofia muscular en larvas de lubina?

Las lesiones microscópicas observadas en el músculo esquelético muestran las características típicas de la distrofia muscular nutricional, caracterizada por una necrosis segmentaria que afecta a ambos tipos de fibra. Ya se había descrito la distrofia muscular en relación con el déficit de vitamina E en adultos y juveniles de distintas especies de peces (Lovell *et al.,* 1984; Gatlin *et al*., 1986; Frischknecht *et al*., 1994; Bowater y Burren, 2007). Sin embargo no hay información disponible en larvas o sobre las alteraciones implicadas en la aparición de estas lesiones. Los cortes semifinos aportaron más información sobre la cronología de la distrofia muscular nutricional (Capítulos 4, 5, 6 y 7). Uno de los cambios más evidentes fue la presencia de vacuolas grandes y claras dentro del citoplasma, que desplazaban otros elementos del sarcoplasma. Además, se apreciaron variaciones en el diámetro de las fibras en los cortes transversales, así como un marcado edema entre las fibras musculares, con pérdida de la arquitectura normal del miotomo. Además se observaron fibras oscuras y encogidas rodeadas por una membrana celular intacta, lo cual se correspondía con una condensación del sarcoplasma.

Las secciones de TEM revelaron la dilatación de los orgánulos, especialmente del retículo sarcoplásmico y de las mitocondrias. Una consecuencia conocida del ataque de los ROS es la modificación oxidativa de los lípidos de unión de membrana, lo que causa daño en la membrana (Farooqui y Horrocks, 1998) junto a la inhibición de enzimas responsables de la regulación del metabolismo celular como la Na/K-ATPasa. La alteración de estas enzimas puede causar entrada masiva de Ca^{2+}, Na^{+} y agua en el retículo endoplásmico, lo que causa la alteración de esta estructura y la consiguiente formación de vacuolas. Además, el incremento del Ca^{2+} sarcoplásmico puede ocasionar la apertura del poro de permeabilidad transitoria de la mitocondria, lo que causa una pérdida de componentes de la matriz, alteración en la funcionalidad mitocondrial e hinchazón de los orgánulos con la subsecuente ruptura de la membrana externa y liberación del citocromo *c* (Green y Reed, 1998; Kolkatowski y Vercesi, 1999). El incremento en Ca^{2+} ha sido corroborado por la mayor expresión de la μ-calpaína (Capítulo 7), ya que las concentraciones de Ca^{2+} intracelular estimulan la actividad de las calpaínas (Mongini *et al*., 1988; Hopf *et al*., 1996).

El citocromo *c* es un factor inductor de la apoptosis y su liberación al sarcoplasma puede disparar la apoptosis. Sin embargo, en el presente estudio no se observaron signos morfológicos de apoptosis. El análisis morfológico de la apoptosis es difícil, debido a la rapidez de la destrucción celular y a que normalmente solo implica la muerte de una célula aislada (Fidzianska, 2002). Por tanto, el único tipo de muerte celular observado en este estudio fue la necrosis, sin embargo, la apoptosis no puede ser descartada como muerte celular en el músculo de lubina.

En términos generales, los hallazgos de TEM fueron similares a los encontrados en mamíferos con trastornos neuromusculares, como la distrofia muscular de Duchene, la isquemia o la atrofia por denervación. Por ejemplo, las figuras de mielina representan material endógeno producido por peroxidación lipídica en mamíferos (DeGritz *et al*., 1994) o las vacuolas autofágicas son lisosomas secundarios frecuentes en muchas alteraciones neuromusculares de los mamíferos (Nishino, 2006). Finalmente, los halos sarcoplásmicos son un hallazgo común en las alteraciones musculares genéticas de los humanos (Farkas *et al*., 1974). Para resumir, un exceso de producción de ROS debido al mayor contenido de PUFA en los tejidos es el causante de la distrofia muscular, que posee rasgos similares a las distrofias musculares genéticas de los mamíferos.

¿Hay un proceso de regeneración muscular en peces?

Se sabe poco sobre la regeneración tisular en peces, pero parece lógico pensar que podría haber cierta regeneración en las larvas, dado que son organismos de crecimiento rápido, con una gran capacidad para formar células nuevas. Uno de los estudios que versan sobre regeneración muscular en peces (Rowlerson *et al*., 1997) demostró una regeneración marcada en juveniles de dorada tras un daño mecánico, siendo la expresión de miosina en las fibras regeneradas similar a la de las nuevas fibras producidas en el músculo blanco post-larval. Igualmente, en la presente tesis se observó una mayor expresión de la MyHC en las larvas alimentadas con un 5% de DHA independientemente del nivel de vitamina E (Capítulos 5, 6 y 7). En este sentido, se encontró una correlación positiva entre la incidencia de lesiones musculares y el número de copias de ARNm de la MyHC (Capítulos 5 y 6). Sin embargo, la expresión de la -actina no indicó ningún proceso regenerativo, al contrario de lo que ha sido establecido en mamíferos (Takano *et al*., 2010). Otro indicador de la regeneración muscular en mamíferos es el incremento en la expresión de la IGF-I (Chargé y Rudnicki, 2004). En los Capítulos 5, 6 y 7 se relacionó el incremento de la IGF-I con las larvas con mayor incidencia de lesiones musculares, confirmando que estas larvas estaban experimentando regeneración muscular. Además la IGF-I parece ser capaz de regular la población de células satélite, que son las responsables de la capacidad de regeneración del músculo. Sin embargo en la presente tesis no se cuantificó ni el número ni el diámetro de estas células, aunque parecía que el mayor número de las mismas estaba relacionado con la mayor incidencia de lesiones musculares (observación personal, Capítulo 4). Además, se ha comprobado que las IGFs pueden activar la proliferación celular y la síntesis de ADN en las células embrionarias del pez cebra, sugiriendo que el incremento del número de copias del mARN del IGF-I observado en la presente tesis en las larvas con mayor número de lesiones puede ser debido al proceso regenerativo llevado a cabo por las células satélite.

¿Niveles altos de DHA pueden afectar a la mineralización en larvas de lubina?

A la vista de los resultados obtenidos en la presente tesis, la respuesta a esta pregunta es rotundamente sí. En contraste con trabajos previos en los que el DHA parecía tener un efecto positivo en la mineralización y la incidencia de deformidades en el chano (*Chanos chanos*; Gapasin y Duray, 2001) y en el bocinegro (*Pagrus pagrus*, Roo *et al*., 2009), en esta tesis el incremento de la peroxidación debido a niveles

elevados de DHA reducen la mineralización e incrementan las deformidades, como ha sido señalado en larvas de dorada (Izquierdo *et al*., enviado). Por tanto, el ajuste de los niveles de DHA es necesario para reducir las deformidades y favorecer la mineralización ósea.

Por otro lado, la inclusión de selenio orgánico en dietas con un 5% de DHA no parece reducir la incidencia de deformidades (Capítulo 5), en contraste a la adición de vitamina C. Estos resultados no son sorprendentes ya que indican que la vitamina C juega un papel más activo que el selenio en el desarrollo óseo. En este sentido, un trabajo previo en el que se estudió el efecto del selenio en el desarrollo óseo de larvas de bacalao demostró que este mineral no tenía efectos sobre la tasa de deformidades (Penglase *et al*., 2010).

10.7 Conclusiones

1. Las dietas con niveles altos de DHA causan bajo crecimiento y supervivencia, aumento de deformidades esqueléticas y favorecen la aparición de lesiones musculares en larvas de lubina.

2. La distrofia muscular aparece como consecuencia de la peroxidación lipídica *in vivo* causada por niveles excesivos de DHA, tal y como indican los contenidos elevados de TBARS y la sobreexpresión de las enzimas antioxidantes.

3. La evaluación microscópica de las lesiones reveló que una de las primeras alteraciones tiene lugar en la membrana muscular, probablemente debido al ataque directo de los radicales libres a la membrana de fosfolípidos, causando alteración de la permeabilidad y consecuente hinchazón de los orgánulos. El calcio entra de forma masiva en la célula activando la μ-calpaína, induciendo la lisis de las proteínas.

4. Las secciones ultrafinas mostraron dilatación difusa del retículo sarcoplásmico, desorganización de los miofilamentos y vacuolas autofágicas. Así mismo, se observaron figuras de mielina y cuerpos densos, así como halos sarcoplásmicos. No se encontraron signos de apoptosis, sugiriendo que la muerte celular que acontece en la distrofia nutricional muscular en las larvas de lubina es preferentemente la necrosis.

5. Cuando se incluye un 1 o un 3% de DHA en las dietas para larvas de lubina, el incremento de vitamina E de 150 a 300 mg 100 g^{-1} mejora el crecimiento y la supervivencia. Sin embargo cuando el nivel de DHA sube hasta un 5%, aparece distrofia muscular en las larvas, sin que el incremento de vitamina E hasta 300 mg 100 g^{-1} pueda evitarlo.

6. Niveles de DHA del 5% en combinación con 300 mg 100 g^{-1} de vitamina E indujeron de manera contundente la expresión de las enzimas antioxidantes, como un intento para dismutar las especies reactivas al oxígeno y defender a las células de su ataque.

7. Con la inclusión de selenio orgánico (5 mg kg^{-1}) en dietas con un 5% de DHA y 300 mg 100 g^{-1} de vitamina E se controló la peroxidación lipídica *in vivo*, se disminuyó la incidencia de lesiones musculares y se incrementó el crecimiento en comparación con la dieta sin este mineral, pero no tuvo ningún efecto en las deformidades. No se

observó ningún efecto directo entre la suplementación con selenio y la expresión de la enzima glutatión peroxidasa.

8. La adición de 180 mg 100 g^{-1} de vitamina C a dietas con alto riesgo de causar oxidación ayudó a reducir el estrés oxidativo en las larvas de lubina, aumentando el crecimiento y reduciendo la incidencia de lesiones musculares y deformidades craneales. La falta de efectos en la expresión de las enzimas antioxidantes indicó que la vitamina C ejerce su función antioxidante mediante otro mecanismo.

9. Se encontró un crecimiento muscular compensatorio en las larvas alimentadas con contenidos altos de DHA, tal y como indica el incremento de expresión de los factores de crecimiento insulínico y de la cadena pesada de la miosina, así como por las abundantes células satélites observadas alrededor de las fibras musculares dañadas.

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