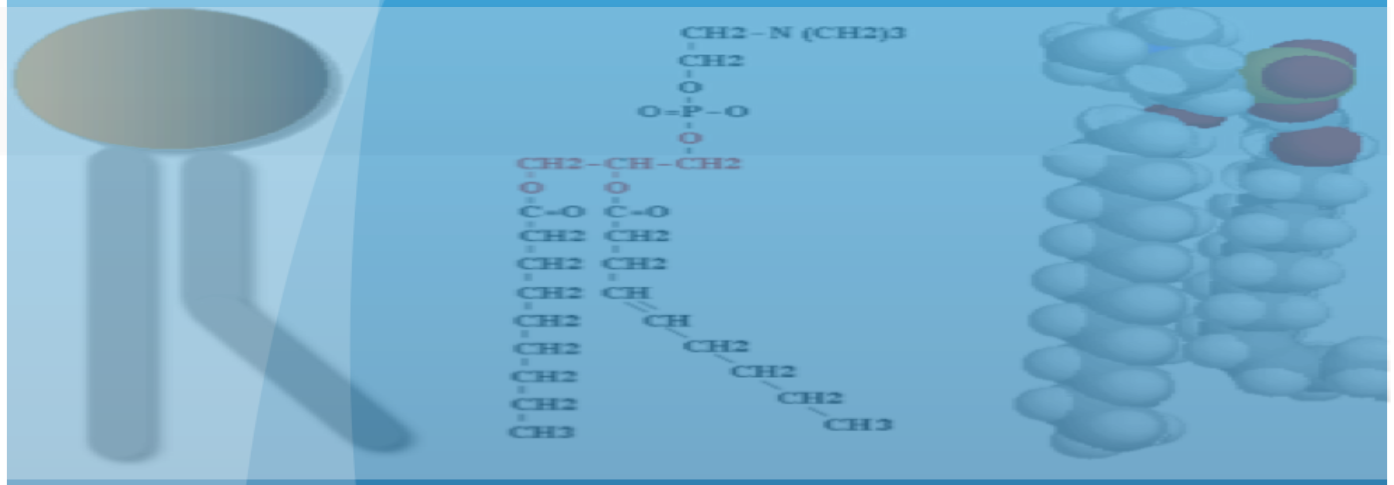
PhD Thesis

Optimum phospholipids and antioxidant levels to develop novel microdiets for gilthead seabream larvae



Reda Saleh Mohamed Ibrahim

Las Palmas of Gran Canaria, Spain 2013

University of Las Palmas of Gran Canaria

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Anexo I

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

CERTIFICA

Que el Consejo de Doctores del Departamento en su sesión de fecha 12 de diciembre de 2012 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral titulada: **"NIVELES ÓPTIMOS DE FOSFOLÍPIDOS Y ANTIOXIDANTES EN EL DESARROLLO DE MICRODIETAS PARA LARVAS DE DORADA"** presentada por el doctorando D. Reda Saleh y dirigida por los Dres. María Soledad Izquierdo López y Francisco Javier Moyano López.

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

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Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Programa de Doctorado: Acuicultura: Producción Controlada de Animales Acuáticos

Título de la Tesis

Optimum phospholipids and antioxidant levels to develop novel microdiets for gilthead seabream larvae

Tesis Doctoral presentada por **D. Reda Saleh Mohamed Ibrahim**

Dirigida por **La Doctora María Soledad Izquierdo López y**
El Doctor Francisco Javier Moyano López

La Directora	El Director	El Doctorando
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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

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Doctorado en Acuicultura: Producción Controlada de Animales Acuáticos

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Prof. María Soledad Izquierdo and Prof. Francisco Javier Moyano

بسم الله الرحمن الرحيم

قُلْ إِنَّ صَلَاتِي وَنُسُكِي
وَمَحْيَايَ وَمَمَاتِي لِلَّهِ رَبِّ الْعَالَمِينَ (١٦٢) لَا شَرِيكَ
لَهُ وَبِذَلِكَ أُمِرْتُ وَأَنَا أَوَّلُ الْمُسْلِمِينَ (١٦٣)

الأنعام

صدق الله العظيم

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Summary

Phospholipids (PL) are particularly important in fish larvae production for their essential function as necessary components for cellular bio-membranes and organelles formation, as well as for being an endogenous energy source during early development. Besides, during early development, PL seems to induce digestive system maturation, may promote digestive enzymes activities, and consequently play an important role on larval digestive physiology and the metabolic pathways of the assimilated nutrients. However, despite the many studies available denoting the importance of dietary PL, few of them have intended to determine quantitative PL requirements testing diets with at least five different levels of this nutrient. Thus, the aim of the present thesis was to determine the optimum requirements of krill PL (KPL) and soybean lecithin (SBL) for gilthead seabream (*Sparus aurata*) larvae, and its influence on larval production performance and digestive enzymes activity (Chapter 3 & 4). Another aim was to compare the effectiveness of dietary KPL and soybean lecithin on the seabream larval rearing performance, oxidative status, vertebral mineralization, skeletal anomalies and bone formation related genes expression (Chapter 5). However, dietary PL have high levels of polyunsaturated fatty acids which are molecules with a great susceptibility to peroxidation resulting in production of harmful peroxides that affect their biological and physiological functions, so it was important that investigate the combined effect of graded levels of antioxidant nutrients such as α-tocopherol with dietary KPL and SBL, and the effect of graded levels of Se derived yeast, on the seabream larval rearing performance, oxidative status, vertebral mineralization, skeletal anomalies and bone formation related genes expression (Chapter 6 & 7). In the light of these experiments, the results have shown that dietary KPL are an excellent source of lipids and the optimum inclusion levels of this ingredient in microdiets to completely substitute live preys at larval age of 16 dph were found to be 12% KPL, providing about 10% total PL, and 8% SBL, providing about 8.8% total PL. These levels significantly improved digestive enzymes activities, utilization and deposition of dietary essential fatty acids and larval growth, as a consequence of a better digestion, absorption, transport and deposition of dietary nutrients. However, despite increased on dietary SBL up to 9% total PL improved larval survival, stress resistance, growth and skeletal development,

dietary KPL was more effective in promoting all these parameters where their higher content in phosphatidylcholine and n-3 HUFA, particularly, DHA, not only promotes digestion, transport and deposition of dietary lipids, but also contributes to reduce skeleton anomalies by up-regulating of bone formation related genes, inducing early mineralization and resistance of vertebral bodies to reduce anomalies such as lordosis and kyphosis. Also, inclusion of SBL markedly increased the proxidation risk as denoted by the high larval malondialdehyde (MDA) content, as well as a high expression of catalase (CAT), superoxide dismutase, (SOD) and glutathione peroxidase (GPX) genes.

The inclusion of 3000 mg **α**-tocopherol kg^{-1} diet promoted the expression of genes related to bone formation and mineralization and improved larval survival and growth, what could be related to the better utilization of dietary lipids and to the reduction of toxic free radicals as indicated by the low larval MDA content and the low antioxidant enzyme genes expression (AOE). These results denoted the high efficiency of -tocopherol as an antioxidant factor, and its positive effect on genes expression of bone formation and mineralization.

Finally, the inclusion of Se derived yeast at 11.65 µg Se/mg diet improved larval survival and stress resistance what could be related in one hand to the reduction of toxic free radicals as indicated by the low levels of MDA and AOE content, which demonstrate an adaptive response in attempting to neutralize the generated reactive oxygen species (ROS) suggesting better oxidative status, and in another hand to the improved utilization of dietary lipids. Moreover, the Bone Morphogenetic Protein-4, alkaline phosphatase, osteocalcin, osteonectin, osteopontin, and matrix gla protein genes expression in larval tissues were positively correlated to the dietary selenium increased or larval Se tissue content increased, in relation to an adequate skeletal development.

List of Abbreviations

AA	Arachidonic acid
ANOVA	Analysis of variance
AOE	Antioxidant enzymes
APROMAR	Asociación Empresarial de Productores de Cultivos Marinos
BHT	Butylated hydroxytoluene
CAT	Catalase
cDNA	Complementary desoxiribonucleic acid
DEPC	Diethyl pyrocarbonate
DHA	Docosahexaenoic acid (22:6n-3)
dph	Days post hatching
DW	Dry weight
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid (20:5n-3)
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
HPLC	High performance liquid chromatography
ICCM	Instituto Canario de Ciencias Marinas
IGF-I	Insulin-like growth factor I
IGF-II	Insulin-like growth factor II
IGFBP	Insulin-like growth factor binding protein
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSSG	Oxidized glutathione

GSH	Reduced glutathione
HUFA	Highly unsaturated fatty acid
KPL	Krill phospholipids
LC-PUFA	Long chain polyunsaturated fatty acid
LA	Linoleic acid
MDA	Malonaldehyde
mRNA	Messenger ribonucleic acid
n-3 LC-PUFA	Long chain polyunsaturated fatty acids of *n-3* series (20 or more carbon atoms)
NL	Neutral lipids
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PL	Phospholipids
PLA2	Phospholipase A2
PS	Phosphatidylserine
RNA	Ribonucleic acid
ROO^-	Peroxyl radical
ROS	Reactive oxygen species
SBL	Soybean lecithin
SD	Standard deviation
SE	Selenium
SOD	Superoxide dismutase
TBARS	Thiobarbituric reactive substances

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First of all I am greatly thankful to Allah who grant me the opportunity to achieve my dream.

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Chapter 1: Introduction

1.1 Aquaculture

Aquaculture has been the fastest growing food production system and the most rapid developing animal production sector worldwide. In the last decade it has been growing at a rate higher than 5% per year (Izquierdo, 2005), with a global production that increased from 0.6 millions mT in 1950 to 7.3 millions mT in 1980 and 55.1 millions mT in 2009 (APROMAR, 2009; FAO, 2011).

This rapid increase in production was promoted by the improved control of fish reproduction, rearing conditions, diet formulations and other technological innovations that allowed the fast development of land and sea based aquaculture facilities (UNEP/MAP/MED POL, 2004). The future success of the aquaculture sector is closely related to the increasing demand of food from marine origin that make this activity very attractive for investors, as well as an alternative to other labor activities such as wild captures. However, the production success of marine fish species is limited by the quantity and quality of mass production of fry (Izquierdo and Fernandez-Palacios, 1997; Watanabe *et al*., 1983; Yúfera and Pascual, 1984; Sargent *et al*., 1997; Izquierdo *et al*., 2000).

While the Mediterranean production of marine fish species has been increasing in several species, gilthead seabream (*Sparus aurata)* and European sea bass (*Dicentrarchus labrax*) are still the most cultivated marine fish species, characterizing the development of European marine aquaculture over the last three decades (FAO, 1999; Izquierdo, 2005). Demand for good quality fry has been increasing at a rate of 10% annually and the European annual fry production of seabream and of sea bass respectively reached 497.7 and 457.7 millions in 2008 (FEAP, 2008). The improved development of fry production is essentially affected by the effectiveness of first feeding regimes and the high nutritional quality of early weaning diets (Kolkovski *et al*., 1993; Sargent *et al*., 1997; Izquierdo *et al*., 2000). Rearing of marine fish larvae implies the administration of external nutrients by feeding firstly alive preys for some days followed by early weaning diets. Nevertheless, both live preys and microdiets should meet the nutritional requirements of each species to ensure the best growth and survival rates (Kolkovski *et al*., 1993; Sargent *et al*., 1997).

Several strains of the rotifer *Brachionus plicatilis* constitute the first prey fed to

gilthead seabream due to its adequate size (90-130 µm). They are followed by *Artemia* and co-feeding with early weaning diets to fit larval mouth size. However, the extra effort in terms of manpower, infrastructure, time and energy to produce rotifers and *Artemia* species represents a significant cost (Kolkovski, 2009). Moreover, the nutritional value of live preys varies notably according to its feed and environmental conditions such as light intensity and regimes, temperature, salinity, etc., risking the optimal nutrition of the larvae (Jones *et al*., 1993; Kolkovski *et al*., 1993; Barnabé and Guissi, 1994; Rosenlund *et al*., 1997). Besides, from an experimental point of view, effective live prey enrichment is limited to few nutrients such as phospholipids (PL) or proteins (Rosenlund *et al*., 1997; Koven *et al*., 2001). For all these reasons, substitution of live prey by compound microdiets is crucial for sustaining cost effective production of high and constant quality juveniles.

During the past three decades, extensive efforts have been made to develop microdiets as a complete or partial substitution for both rotifers and *Artemia*. The majority of the studies have focused on the larvae's nutritional requirements. Despite substantial achievements, complete replacement of live feeds at first feeding is not yet feasible for most marine species. 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). In addition to diet nutritional quality, important physical characteristics of diet particles within the water column such as sinking rates, nutritional stability, leaching rates and leachate profiles all contribute to the microdiets attractiveness that influence ingestion and digestion rates and can greatly affect larvae growth. Surprisingly, these factors have received very little attention. The production of microdiets 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; Önald and Landgon, 2000; Poussao-Ferreira *et al.*, 2003). Therefore, it is important taking into account the physiology and ontogeny of the larval digestive system, feed regime and technology as well as each species nutritional requirements in the process of microdiets preparation for marine fish larvae.

In the last years, various studies have been conducted to determine the optimum lipid composition in early weaning diets for marine fish larvae, where more attention has been paid to study the long chain polyunsaturated fatty acids (LCPUFA) and

phospholipids requirements and its role in marine fish larvae development (Watanabe 1982; Takeuchi, 1997; Sargent *et al.,* 1999a; Izquierdo *et al*., 2003).

1.2 Marine fish larvae and microdiets utilization

Nowadays, the dependence on the availability of seeds is one of the critical factors for the commercial success of the industrial fish marine production (FAO, 1998). Certain phases are recognized as critical points during the first stages of larval development:

a) The change from the endogenous to the exogenous feeding which starts with supplying of rotifers between day 3 and 4 post hatching.
b) The moment of weaning or the transition from the ingestion of the live food to the early weaning inert food.

The larval culture development of marine fish has been possible thanks to the use of live preys, mainly rotifers and nauplii and metanauplii of *Artemia*. Since the rotifer cultivation was settled down (Ito, 1960), this organism has been widely used for the cultivation of larvae of several species of marine fish. According to Verreth (1994), the bottlenecks in the development of techniques for larval rearing are:

I. The lack of knowledge about the optimal environmental conditions and feeding requirements during the early life stages.

II. The unknown of the optimal nutritional requirements, which are difficult to assess in the absence of a suitable rearing technology.

III. The changes in specific husbandry and feeding practices from one species to another due to ontogenic changes and growth.

IV. The small size of the larvae and the need for small particle sizes, which imposes specific problems on feed technology.

Marine fish larvae are very vulnerable during the first stages of development and have strict requirements for biotic and abiotic conditions to survive, develop and grow. Most research on larval nutrition have contributed to the knowledge necessary to estimate the nutrient requirements of larval fish. However, it is not easy to quantify the nutritional requirements of larval fish. Obviously, a good knowledge of the larval nutritional requirements throughout the larval development would contribute to optimize diets and feeding protocols.

As marine fish species undergo dramatic morphological and physiological changes, including metamorphosis, during ontogenesis, their nutritional requirements may

differ qualitatively and quantitatively from larvae to juvenile or adult stages. Moreover, fish larvae grow extremely rapidly, feed continuously and therefore, total ingestion of nutrients must be high. The larval nutritional requirement for a particular nutrient can be defined from a physiological point of view as the nutrient intake needed to fulfil a physiological role, but is frequently defined as the "requirement for maximal growth and/or survival" where the relation fish-diet-feeding has an important effect in the determination of the quantitative needs (Izquierdo and Lall, 2004). Enormous efforts have been made to improve microdiets efficiency to replace live feed, both rotifers and *Artemia*, as complete or partial replacements for marine fish larvae (Koven *et al*., 2001; Kolkovski, 2004; Kolkovski *et al.*, 2009). There have been substantial achievements in the earlier weaning of larvae onto microdiets and partial replacement of live feeds, but microdiets still cannot completely replace live feeds for most species. The partial substitution of live food has proven to be successful. The formulated microdiets supply with the live food (co-feeding) to enable early weaning, for instance, up to 90% substitution of rotifers with artificial diets has been achieved for red seabream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*) (Kanazawa *et al*., 1989), and gilthead seabream larvae (Liu *et al.,* 2002). Although weaning the larvae from *Artemia* onto a microdiet can be achieved at metamorphosis in many species (Foscarini, 1988; Hardy, 1989; Koven *et al*., 2001; Curnow *et al*., 2006a, b), the early introduction of formulated diets for completely replacement of live food has limited success (Kanazawa *et al*., 1989; Walford *et al*., 1991; Fernández-Díaz and Yúfera, 1997; Rosenlund *et al*., 1997; Kolkovski *et al*., 2009). The addition of *Artemia* nauplii combined with a microdiet, enhances the peristaltic movement of the larval digestive tract enhancing microdiet ingestion and production of digestive enzymes (Kolkovski *et al*., 1995). The design and formulation of these early weaning diets requires meeting the optimal nutritional requirements to fulfil the larval physiological needs (Kolkovski *et al.*, 2009). The efficiency of utilization of feed particles by marine fish larvae is affected by many external and internal factors (Figure 1.1) (Kolkovski, 2001, 2004; Koven *et al*., 2001). However, the studies of nutritional requirement measurements that use direct methods are scarce due to the difficulty of designing experimental diets with full control of nutritional composition and environmental factors in all the experimental tanks. Formulated diets have variable leakage and stability problems resulting in an unknown difference in nutrient composition between the formulated and ingested diet.

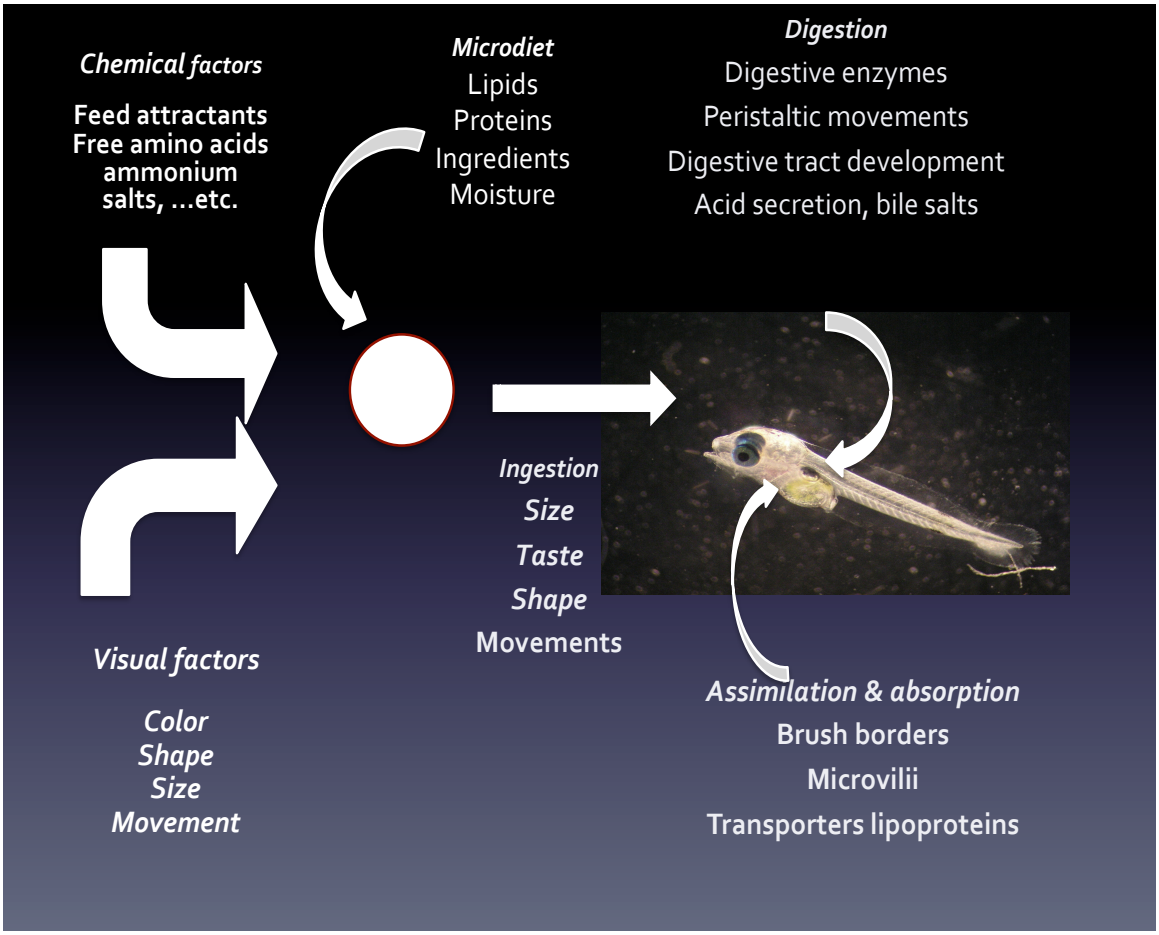

Figure 1.1 Factors affecting food particle utilization (Adapted from Kolkovski *et al.*, 2009).

According to (Kolkovski *et al.*,2009), microparticulated diets can be classified in three general types:

I. Microbound type is the simplest microdiet, where the powdered ingredients are microbound with a water stable matrix such as agar or carrageenan (López-Alvarado *et al.*, 1994).

II. Microcoated type, its manufacturing process relied on coating the powdered ingredients with a glucidic (carraggeenan, alginate), proteic (gelatin, zein) or lipid binder to reduce leaching (Önal and Langdon, 2004).

III. Microencapsulated diets are made by encapsulating a solution, colloid or suspension of diet ingredients within a membrane, and it is produced with a cross linking agent (Yúfera *et al.*, 1999; Kolkovski, 2009).

1.3 Importance of phospholipids in marine fish larvae nutrition

In fish, as in other vertebrates, phosphoglycerides are particularly important in fish larvae production for their essential function as necessary components for cellular bio-membranes and organelles formation (Izquierdo and Koven, 2010). Besides, phosphoglycerides can also serve as an energy source during egg development and endogenous feeding in the pre-larval stages of marine fish (Izquierdo and Koven, 2010). Moreover, phosphoglycerides can act as emulsifiers in the formation of mixed micelles in the digestive tract (Olsen and Ringø, 1997). Indeed, young fish contain abundant phospholipids received during embryo and larval development either from endogenous yolk sac or exogenous live preys lipids (Rainuzzo *et al*., 1997; van der Meeren *et al*., 2008). Studies on dietary phospholipids were initiated by Kanazawa *et al*., (1981, 1983a, b), who showed that despite their significant roles in fish metabolism, developing fish possess a limited ability for phospholipids synthesis (Kanazawa *et al*., 1985) and, consequently, they must be included in young fish diets. Moreover, lipid digestion, absorption and transport are very limited in the first feeding larvae with a poorly developed digestive system, and phospholipids playing different important roles in all these processes. However, fish larvae may be unable to efficiently synthesize phospholipids in an enough quantity to cover this high demand and therefore need to be included in the diet (Izquierdo and Koven, 2010). Indeed, in the first feeding larvae, enterocytes are poorly developed and phospholipid synthesis is scarce (Deplano *et al.*, 1991). In gilthead seabream, isolating microsomes from enterocytes demonstrated that phospholipid synthesis occurs mainly through the glycerol-3-phosphate pathway, whose activity is modulated by dietary lipids (Caballero *et al.,* 2003). Moreover, restrictions in the rate of phospholipid synthesis may constrain lipoprotein synthesis (Liu *et al.*, 2002) that is crucial for lipid transport (Salhi *et al.*, 1999; Caballero *et al*., 2006a). Thus, addition of 20 g kg^{-1} dry weight diet of soybean lecithin in microdiets for 15 dph gilthead seabream containing 220 g kg^{-1} dw lipids, significantly increased the occurrence of lipoprotein particles in the *lamina propria*, promoting transport and utilization of dietary lipids as well as growth (Liu *et al.*, 2002). Dietary PL not only increases synthesis and release of lipoproteins in the *lamina propria* by enterocytes, but also significantly reduces lipoprotein size by promoting very low density lipoprotein (VLDL; higher in PL) synthesis, rather than

chylomicron production (higher in NL) (Liu *et al*., 2002; Caballero *et al*., 2003, 2006b). Thus, phospholipids inclusion in young fish diets has been found to improve culture performance of various fresh water and marine fish species (Izquierdo and Koven 2011), enhancing growth and survival (Kanazawa 1993; Salhi *et al.,* 1999; Izquierdo *et al.,* 2001; Kjørsvik. 2009). Besides their importance for growth and dietary lipid utilization, dietary phospholipids have been also found to be necessary to improve stress resistance, skeleton development and flatfish metamorphosis and pigmentation (Kanazawa *et al*., 1981; Kanazawa, 1993; Fontagné *et al*., 2000; Koven, 2003; Zambonino- Infante and Cahu, 2007; Hamza *et al.*, 2008).

1.3.1 Phospholipids as source of essential fatty acids

Phospholipids can be an important source of energy (fatty acids) in fish, particularly during embryonic and early larval development in species that produce phospholipid-rich eggs (Tocher, 1995). Larval fish at first feeding may be predisposed to digestion and metabolism of phospholipids and the use of fatty acids from phospholipids for energy (Sargent *et al.*, 1997). The main roles of fatty acids in fish larvae are consistent with those in juveniles and adults. Thus, they function as a source of metabolic energy, structural components in the phospholipids of cellular membranes and precursors of bioactive molecules (Sargent *et al*., 1999; Izquierdo *et al*., 2000; Tocher, 2003). Dietary phospholipids tend to be a richer source of essential fatty acid (EFA) than neutral lipids such as triacylglycerols (Tocher, 1995). In addition, phospholipids may be superior to neutral lipids as a source of EFA in larval fish due to improved digestibility (Sargent *et al.*, 1997, 1999). Recently it was shown that phospholipids were the more efficient mode of supply dietary EPA and DHA to sea bass larvae than triglycerides (Gisbert *et al*., 2005; Tocher *et al*., 2008). In any case, most of the studies to determine phospholipid requirements have been performed with soybean or egg lecithin that are rich in 18:2n-6 but also deficient in n-3 HUFA, EPA and DHA, essential fatty acids for marine fish (Watanabe *et al*., 1984; Coutteau *et al*., 1997; Sargent *et al*., 2002; Tocher *et al*., 2008).

1.3.2 Phospholipid requirements

Studies conducted on a large number of species, marine as well as fresh water, confirmed an essential dietary requirement for PL. Optimal level of dietary PL supplementation was found to depend on the species, the PL source and classes, and the criteria used to evaluate this effect: growth, survival, stress resistance, malformations in larvae. Tocher *et al.,* (2008) reported the dietary requirements of different species both at larval and juvenile stages (Table 1.1).

Table 1.1 Quantitative and qualitative phospholipid requirements of teleost fish (Adapted from Tocher *et al.*, 2008)

Species	Developmental stage	Phospholipid supplement[a] and levels studied[b]	Optimal requirement and criteria used[c]	Feeding period	Reference
Ayu (*Plecoglossus altivelis*)	Larvae	0 and 3% SBL or EL	3% (G,S,M)	20 days	Kanazawa *et al.,* (1981)
	Larvae	0, 1, 3 and 5% SL	3% (M), 5% (G,S)	50 days	Kanazawa *et al.*, (1983b)
	Larvae	0 and 3% EL or BPL	3% (G,S,M)	50 days	Kanazawa *et al.,* (1983b)
	Juvenile	0 and 3% SBL or BPL	3% (G)	33 days	Kanazawa *et al.,* (1981)
	Juvenile	0, 1, 3 and 5% EL	3% (G)	33 days	Kanazawa *et al.,* (1981)
Japanese flounder (*Paralichthys olivaceus*)	Larvae	0, 3, 5 and 7% SBL	7% (G,S)	30 days	Kanazawa (1993)
	Juvenile	0, 3, 5 and 7% SBL	7% (G)	30 days	Kanazawa (1993)
Knife jaw (*Oplegnathus fasciatus*)	Larvae	0, 2.5, 5 and 7.4% SBL	7.4% (G,S)	22 days	Kanazawa *et al.,* (1983a)
	Larvae	0, 3, 5 and 7% SBL	5% (G,S,R)	28 days	Kanazawa (1993)
	Juvenile	0, 3, 5 and 7% SBL	3% (G)	60 days	Kanazawa (1993)
European sea bass (*Dicentrarchus labrax*)	Larvae	3, 6, 9 and 12% SBL	12% (G,S,M)	40 days	Cahu *et al.,* (2003a)
	Juvenile	0 and 3% SBL	3% (G)	40 days	Geurden *et al.,* (1995b)
	Juvenile	0 and 2% EPC or SPC	2% (G)	40 days	Geurden *et al.,* (1995a)
Red seabream (*Pagrus major*)	Larvae	0 and 5% SBL	5% (G,S)	20 days	Kanazawa *et al.,* (1983a)
Common carp (*Cyprinus carpio*)	Larvae	0 and 2% EL	2% (G,S)	25 days	Geurden et al. (1995b)
	Larvae	0 and 2% PL	2% (G,S)	21 days	Geurden *et al.,* (1995a)
	Larvae	0 and 2% SPC, SPI or EL	2% (G,S, M except El)	25 days	Geurden *et al.,* (1997a)

Pikeperch (*Sander lucioperca*)	Larvae	1, 5 and 9% SBL	9% (G)	24 days	Hamza *et al*., (2008)
Gilthead seabream (*Sparus aurata*)	Larvae	9, 11 and 15% SL	>9% (G,S)	23 days	Seiliez *et al.,* (2006)
	Larvae	6, 7, 9, 10 and 11 KPL	10% (G,S,R)	30 days	Saleh *et al.,* (2012a)
	Larvae	5, 6, 7, 9 and 10 SBL	9% (G,S,R)	30 days	Saleh *et al.,* (2012b)
Striped Jack (*Pseudocaranx dentex*)	Juvenile	0, 0.5, 1, 1.5 and 2% SPC	1.5% (G,S,R)	6 weeks	Takeuchi *et al.,* (1992)
	Juvenile	0 and 1.5% SPE	1.5% (G)	6 weeks	Takeuchi *et al.,* (1992)
Rainbow trout (*Oncorhynchus mykiss*)	Juvenile	0, 2, 4 and 8% SL	4% (G)	20 weeks	Poston (1990a)
	Juvenile	0 and 14%	14% (G)	8 weeks	Rinchard *et al.,* (2007)
Turbot (*Psetta maximus*)	Juvenile	0 and 2% EL	2% (G)		Geurden *et al.,* (1997b)
Atlantic salmon (*Salmo salar*)	Juvenile (180 mg)	0, 2, 4, 6 and 8% SBL/CPL	6% (G)	14 weeks	Poston (1991)
	Juvenile (180 mg)	0 and 4% SBL	4% (G)	16 weeks	Poston (1990b)
	Juvenile (1.0 g)	0 and 4% SBL	4% (G)	12 weeks	Poston (1990b)
	Juvenile (1.7 g)	0 and 4% SBL	4% (G)	12 weeks	Poston (1990b)
	Juvenile (7.5 g)	0 and 4% SBL	0% (no requirement)	12 weeks	Poston (1990b)
White sturgeon (*Acipenser transmontanus*)	Juvenile (5-10 g)	0 and 8% SBL	0% (no requirement)	6 weeks	Hung and Lutes (1988)

[a]BPL, bonito egg polar lipid; CPL, corn polar lipid; EL, chicken egg lecithin; EPC, purified egg phosphatidylcholine; KPL, Krill phospholipid; PL, various phospholipid sources supplemented to supply 2% dietary phospholipids including EL, SL, sunflower, rapeseed and marine phospholipids: SBL, soybean lecithin; SPC, purified soybean phosphatidylcholine; SPE, purified soybean phosphatidylethanolamine; SPI, purified soybean phosphatidylinositol; [b]Percentage of diet weight; [c]G, growth; S, survival; M, malformations; R, stress resistance.

These authors evidenced a trend suggesting that quantitative PL requirements decrease from larval stages to small juveniles. No requirements were observed in fish greater than 5 g. Phospholipids requirements have been found to be related to the larval age and the degree of digestive system development (Kanazawa, 1993; Izquierdo and Koven, 2010). However, both optimal PL level and composition, as well as the role of dietary PL remain unclear for most species. Sargent *et al.,* (1999) considered that the egg lipid composition give a valuable approximation of the optimal lipid composition of a larval diet. The quantitative requirements of phospholipids for larval fish range from 2 to 12% of diet, being lower in the larvae of common carp (*Cyprinus carpio*) (2% chicken egg) (Geurden *et al.,* 1995a), followed

by red seabream (*Pagrus major*) (5% SBL) and knife jaw (*Oplegnathus fasciatus*) (7.4 % SBL) (Kanazawa *et al.,* 1983a) and Japanese flounder (*Paralichthys olivaceus*) larvae (7 % SBL) (Kanazawa, 1993), with the highest reported value being for gilthead seabream (*Sparus aurata*) (15 % SBL) (Seiliez *et al.,* 2006). However, for the later species, our recent studies (Chapter 3) have found an optimum inclusion of about 10% using krill oil as phospholipids source (Saleh *et al*., 2012a).

1.3.3 Phospholipid digestion, absorption and transport

1.3.3.1 Phospholipid digestion

The digestion and assimilation efficiency in marine fish larvae may be lower than in adult fish because the marine fish larvae have digestive features different from that of juveniles (Cahu and Zambonino-Infante, 1995a, b). It has been hypothesized that marine fish larvae have insufficient digestive capacity to thrive on a compound diet (Lauff and Hofer, 1984). The change from a primary to an adult mode of digestion characterizes developing animals and has been extensively described in upper vertebrates (Henning, 1987). The activities of digestive enzymes in fish larvae are low at first feeding and increase along with development (Govoni *et al.,* 1986). The digestion of ingested food occurs in the larval intestine, where the pH remains alkaline and trypsin-like enzyme activity accounts for the proteolytic activity (Walford and Lam 1993). At first feeding, the pancreatic and intestinal enzyme activities are generally low (Cousin *et al.,* 1987). Digestive enzymes activity increase during the first 10 dph in *Solea senegalensis* (Ribeiro *et al*., 1999), whereas an increase in alkaline phosphatase activity has been found to reflect the development of the brush border membranes of enterocytes in Atlantic cod (*Gadus morhua*) (Wold *et al*., 2007). Moreover, the addition of dietary PL enhanced gut maturation index in this species, based on the relation between brush border alkaline phosphatase and cytosolic leucine-alanine aminopeptidase. Enhancement of gut maturation by dietary PL could be related with a higher intracellular availability of PL for cell membrane and cell organelles formation, since dietary PL promotes re-acylation of digested lipids, increasing intracellular PL availability for lipoprotein synthesis in gilthead seabream (Caballero *et al*., 2003; Liu *et al*., 2002).

It is known that lipids are generally well digested by fish (Olsen and Ringo, 1997). In general, the digestibility of dietary lipids in fish is high, about 95%,

although the digestibility of different fatty acids is different depending on the degree of unsaturation and chain length. Thus, saturated and monounsaturated are less digestible than polyunsaturated fatty acids (Lied and Lambertsen, 1982, Olsen and Ringo, 1997). Therefore, it has been suggested that the absorption of some fatty acids is more efficient than that of others (Olsen *et al.*, 1998).

Dietary lipids are hydrolyzed in the digestive tract lumen by lipolytic enzymes producing mainly free fatty acids, monoacylglycerols and diacylglycerols. While in higher vertebrates the hydrolysis of dietary lipids is mainly catalyzed by a specific pancreatic lipase, which hydrolyzes the fatty acids sn-1 and sn-3 releasing 2-monoacylglycerols, in fish, the main enzyme responsible for digestion of neutral lipid is a neutral non specific lipase dependent bile salts (bile salt activated lipase, BAL) (Iijima *et al.*, 1998; Izquierdo and Henderson, 1998), which hydrolyzes the ester bonds of various fatty acids with glycerol molecule. Despite this enzyme is characterized by acting on a wide range of substrates, it also appears to have a greater specificity for polyunsaturated fatty acids (Lied and Lambrtsen, 1982; Iijima *et al.*, 1998; Izquierdo *et al.*, 2000).

Several types of lipases have been found in the digestive tract of juvenile or adult fish. Among them, the non-specific neutral lipase activated by bile salts (BAL) appears to play a major role in digestion of neutral lipids in most fish species such as seabass, anchovy, rainbow trout, red seabream, turbot or gilthead seabream (Patton, *et al.*, 1975; Ijima *et al.*, 1990, Iijima *et al.*, 1998; Izquierdo and Henderson, 1998; Caballero *et al.*, 2002). This enzyme catalyzes the hydrolysis not only of acylglycerides, but also of other dietary lipids including cholesterol esters and esters of vitamins. There is also evidence of the presence of specific pancreatic lipase (MPL) in several species, it is activated in the presence of bile salts and is specific for triacylglycerols (lijima *et al.*, 1998). Lipids containing polyunsaturated fatty acids of the n-3 series, which are essential for marine fish, are more resistant to hydrolysis by the MPL. On the contrary, it has been demonstrated that the BAL is specific for these fatty. Thus, although the MPL preferentially hydrolyzes esters of 18:1n-9 than 18:2 n-6 or 18:3n-3, the BAL shows a preference for 20:5 n-3 and 20:4 n-6 than 18:2 n-6 and 18:1n-9 (lijima *et al.*, 1998).

The distribution patterns of lipase activity along the digestive tract of juvenile and adult fish appear to differ between species (Izquierdo and Henderson, 1998). This difference in lipolytic activity distribution in adult fish, almost disappears in juveniles

and larvae, suggesting less functional differentiation in the digestive tract in the early stages of development.

Digestion of dietary phospholipids is relatively unstudied in fish, but it is presumed that the mechanisms are generally similar to those in mammals, whereby phospholipids are digested by intestinal phospholipase A2, secreted by the pancreas, in the intestine and specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond resulting in the formation of 1-acyl lyso-phospholipids and free fatty acids (Figure 1.2) that are absorbed by the intestinal mucosal cells (Henderson and Tocher, 1987; Sargent *et al.*, 1989; Izquierdo and Henderson, 1998). There are two isoforms of phospholipase A2 that have been purified in red seabream hepatopancreas (Ono and Iijima, 1998). The activity of phospholipase A2 was detected in marine fish larvae several days after first feeding (Izquierdo *et al.*, 2000).

Lipolytic enzymes of pancreas, lipase and phospholipase A2, increase with development of marine fish larvae (Izquierdo *et al.*, 2000) and are stimulated by the increase of their respective substrates, triglycerides and phospholipids in the diet. Phospholipase A2 activity increases with the level of its corresponding mRNA in sea bass larvae (Zambonino-Infante and Cahu, 1999) suggesting a transcriptional regulation of this enzyme that has been elevated when diets incorporated more than 4.5% phospholipids.

1.3.3.2 Phospholipid absorption and transport

The dietary lipid absorption in fish is simillar to that of mammals (Sire *et al.*, 1981; Tocher *et al.*, 2008). After intraluminal hydrolysis, dietary lipid is incorporated into intestinal epithelial cells by diffusion in a form of micelles of monoglycerides and free fatty acids (Izquierdo *et al.*, 2000; Tocher *et al.*, 2008). Products of hydrolysis of dietary lipids, free fatty acids, monoacylglycerols, diacylglycerols and lysophospholipids, are grouped with bile salts to form small micelles that are transported into the enterocyte (Figure 1.2), and the content of these micelles, which also includes other lipids such as cholesterol and liposoluble vitamins, crosses the cell membrane by diffusion and is poured inside the enterocyte (Smith *et al.*, 1983). Reacylation occurs in the endoplasmic reticulum and its products are released into the submucosal as lipoproteins of very low density (chylomicrons). The activity of the

enterocytes during the absorption of lipids is the packaging of lipids together with proteins to constitute stable molecules to transport them through the blood to sites of metabolism and storage (Gurr and Harwood, 1991).

Inside the intestinal cells, as the short chain fatty acids (less than 12 atoms carbon) are sufficiently soluble in the plasma, they are released directly from the enterocyte to the portal circulation and metabolized in the liver (Green and Selivonchick, 1987). The absorbed long-chain fatty acids and monoacylglycerols migrate to the endoplasmic reticulum vesicles, where they are resynthesized into triglycerides or other lipids by a multienzyme complex (Gurr and Harwood, 1991). These fatty acids diffuse to the endoplasmic reticulum associated with the binding protein FABP (Fatty Acid Binding Protein), which plays an important role in intracellular transport of the absorbed fatty acids. In fish, this protein has a preference for unsaturated fatty acids in the same manner as it occurs in mammals (Gangl *et al.*, 1980). Sire and Vernier (1981), reported that this protein is more abundant in fish fed high dietary lipids, which could indirectly affect the activation and reacylation of fatty acids.

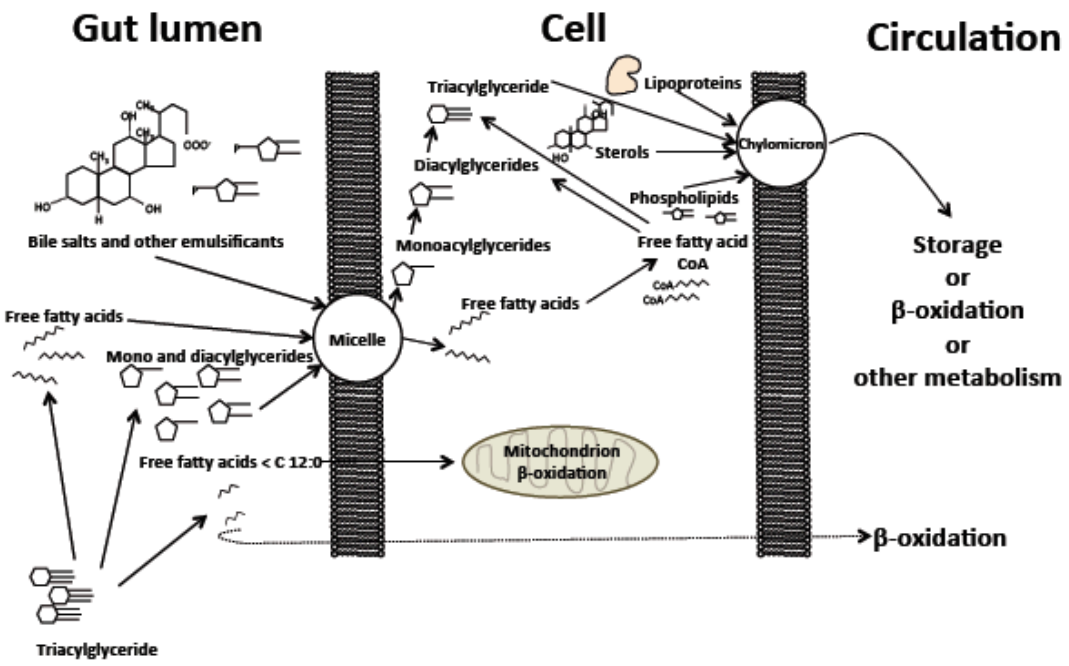

Figure 1.2 Absorption and metabolism of triacylglycerides (Adapted from Smith *et al.*, 1983).

The two main pathways of reacylation of fatty acids in the enterocytes are the monoacylglycerol and glycerol-3-phosphate pathway, both routes finally producing triacylglycerol and phospholipids (Iijima *et al.*,1983; Caballero *et al.*, 2006a) (Figure 3). Most enzymes involved in these pathways have been identified and studied in rats, but there are very few studies in fish that refer to them (Sire *et al.*,1981; Iijima *et al.*,1983; Caballero *et al.*, 2006a). Thus, Iijima *et al.,* (1983) showed that the intestine of carp (*Cyprinus carpio*) is able to esterify the palmitic acid in triglycerides and phospholipids. On the other hand, Sire *et al.*, (1981) found that linoleic acid is esterified more rapidly to triacylglycerols than palmitic acid in enterocytes of rainbow trout. Furthermore, these authors propose that while monoacylglycerol pathway in mammals is predominant due to the luminal hydrolysis products are the 2-monoacylglycerols, but in fish, the pathway glycerol-3-phosphate is the main due to free fatty acids and glycerol are the main products of hydrolysis intraluminal, so that the synthesis of glycerophospholipids in fish is more important than in mammals. Caballero *et al.,* (2006 a) isolating enterocytes microsomes demonstrated that in gilthead seabream phospholipid synthesis occurs mainly through the glycerol-3-phosphate pathway, whose activity is modulated through dietary lipids.

In the case of phospholipids, the mechanisms of absorption of their digestion products have not been studied deeply in fish, but are assumed to be generally similar to those in mammals. Thus, the hydrolysis products, 1-acyl-lysosome phospholipids and fatty acids free are associated with all other products of lipid digestion with bile salts in mixed micelles, which uptake in the intestinal mucosa, whose penetration occurs in the enterocytes by passive diffusion. Lyso-phospholipids concentration is very low in the intestinal mucosa, most of the 1-acyl lyso-phospholipids are reesterified with free fatty acids activated in microsomes prior to export from enterocytes to the circulatory system (Sargent *et al.*, 1989).

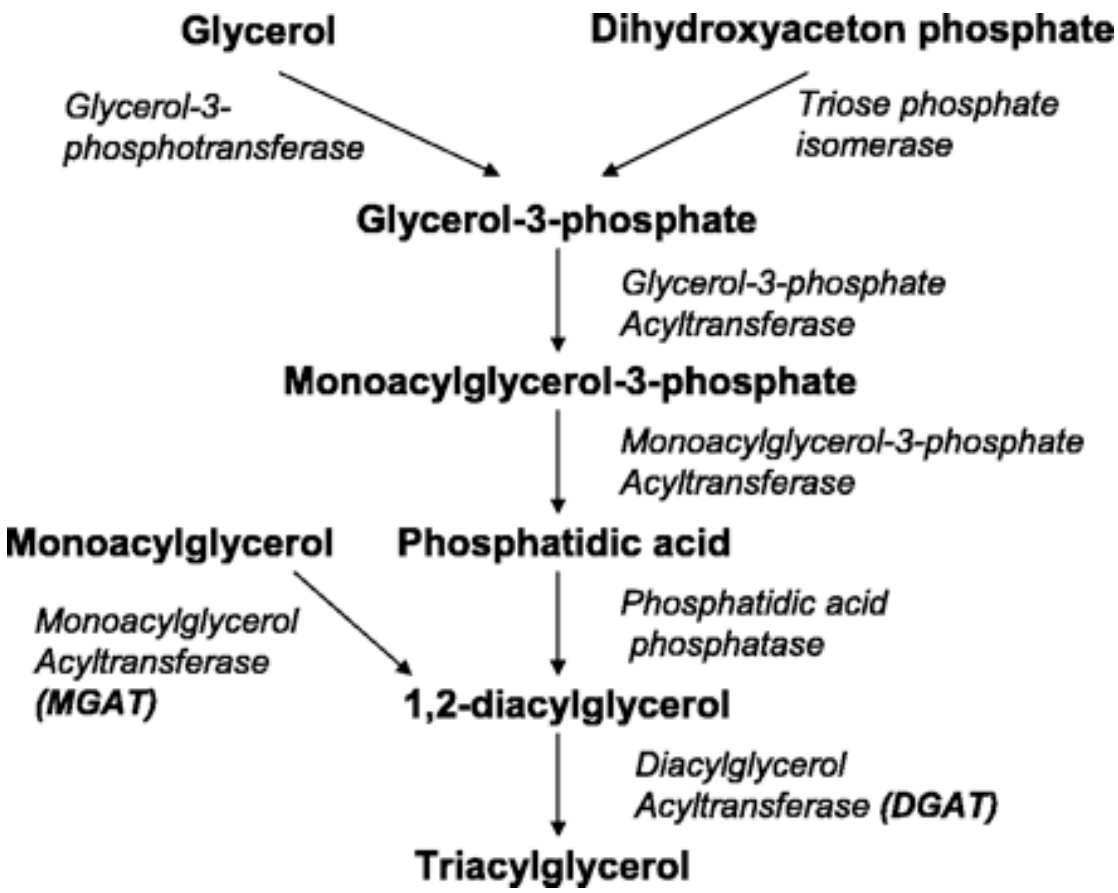

Figure 1.3 Schematic diagram of triacylglycerol biosynthetic pathway.

Transport of lipids and other lipid soluble components from the intestine to the tissues is predominantly by lipoproteins (Babin and Vernier, 1989; Izquierdo *et al.*, 2001; Liu *et al.,* 2002; Caballero *et al.,* 2003, 2006b). The lipoproteins are particles that differ in their proportion of lipids (triglycerides, cholesterol, esters of cholesterol and phospholipids) and proteins (Figure 1.4).

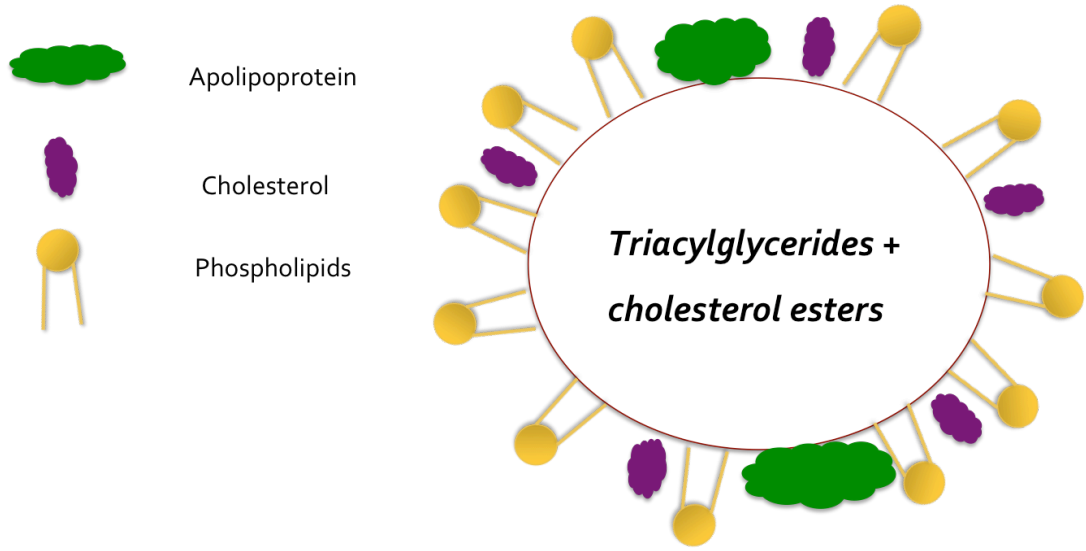

Figure 1.4 Schematic lipoprotein structure.

Lipoproteins are generally classified according to their density in six major classes: chylomicrons (CM), very low density lipoproteins (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL), high lipoprotein density lipoprotein (HDL) and very high density lipoprotein (VHDL) (Liu *et al.,* 2002; Caballero *et al*., 2003, 2006b). These lipoproteins have apolipoproteins as structural and functional components, which are cofactors for enzymes and ligands for cell-surface receptores. In particular, apoA1 is the major protein component of high-density lipoproteins; apoA4 is thought to act primarily in intestinal lipid absorption; and apoE is a blood plasma protein that mediates the transport and uptake of cholesterol and lipid by way of its high affinity interaction with different cellular receptors, including the low-density lipoprotein (LDL) receptor (Saito *et al.*, 2004).

VLDL carries mainly lipids and preferably contains endogenous triacylglycerol, similar to chylomicrons. Its main site of synthesis is the liver and its catabolism is basically the same as chylomicrons, acting upon them lipoprotein lipase, which hydrolyzes triacylglycerols (Figure 1.5). VLDL-lipase interaction is less efficient than the interaction chylomicron lipase, thus VLDL persist more time in the circulation (Sheridan, 1988). HDL is the principal store of cholesterol and this lipoprotein accepts the cholesterol from peripheral tissues and transports it to the liver for degradation and reutilization.

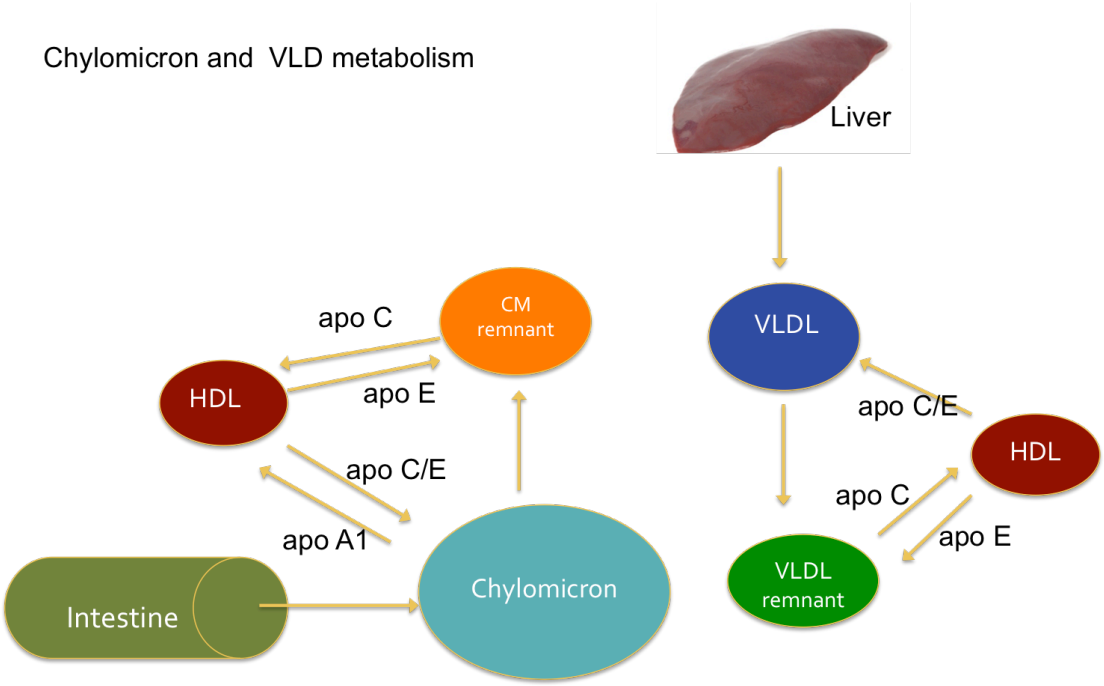

Figure 1.5 Schematic lipoproteins metabolism.

There is evidence that the transport of lipids from the enterocytes to the hepatocytes appears to be increased by supplementation dietary phospholipids (Kanazawa *et al.*, 1993). Feeding of seabream larvae on microdiets without supplementation of lecithin produced accumulation of lipid vacuoles in the enterocytes and steatosis in liver tissue, which is indicative of dietary-induced problems in lipid transport from the intestinal mucosa into the body circulation (Kanzawa *et al.*, 1993). Both effects are reduced by the addition of 2% lecithin to the diet which shows the increase of lipid transport in the intestine and liver (Kanzawa *et al.*, 1993). In agreement, insufficient levels of dietary PL (PL 23.7 g kg^{-1} dw, dietary lipids 178.5 g kg^{-1} dw), increase accumulation of lipid droplets in the enterocytes of marine larvae fed a PL deficient diet (Salhi *et al.*, 1999; Morais *et al.*, 2005), depending on the type and amount of dietary PL and its relation to the dietary NL content. The dietary phospholipid deficiency has been associated with the accumulation of fat droplets in the enterocytes of the foregut in larval carp, suggesting that this type of fat tend to have a specific role in the synthesis of VLDL as in mammals (Fontagné, 1996). This accumulation has led to the suggestion that intestinal lipid inclusions are a temporary storage form re-esterified fatty acids when the rate of lipid absorption exceeds the rate of lipoprotein synthesis or because of an inability to metabolize lipids (Kjørsvik, *et al.,* 1991).

The liver is an essential organ involved in the metabolism of substances absorbed by the intestine, also involved in processing these subsatnces to be used by other tissues, and it is the main lipid regulating place where involved in the synthesis and degradation of fatty acids (Cross, 1993). The liver is a metabolically sensitive organ that may be examined to determine potential side effects of lipidic metabolism because the hepatic energy storage responds sensitively and rapidly to nutritional changes in fish larvae (Hoehne-Reitan and Kjørsvik, 2004).

1.3.4 Phospholipid classes

The existing biochemical evidence suggests that pathways of phospholipids synthesis are essentially the same as in mammals (Tocher, 1995), where phospholipids can be synthesized de novo by two main mechanisms (Lykidis, 2007). One utilizes a cytidine diphosphate (CDP) activated polar head group for attachment to 1,2- diacylglycerol (DAG), and the other utilizes CDP-activated DAG and an inactivated polar head group (Figure 1.6) (Tocher *et al*., 2008). They both initially proceed by esterification of glycerol-3-phosphate with two activated fatty acids (acyl-CoA) to form phosphatidic acid (PA). Action of PA phosphatase on PA, results in the production of DAG, which can then react with CDP-choline or CDP-ethanolamine to produce PC and PE, respectively (Tocher *et al.*, 2008). Alternatively, CTP reacts with PA to form CDP–DAG, which can then combine with serine and inositol to form PS and PI, respectively (Figure 1.6).

Based on the few studies where single pure phosphoglycerides species have been used, the rank order for efficacy appears to be phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylserine (PS) (Izquierdo and Koven, 2010).

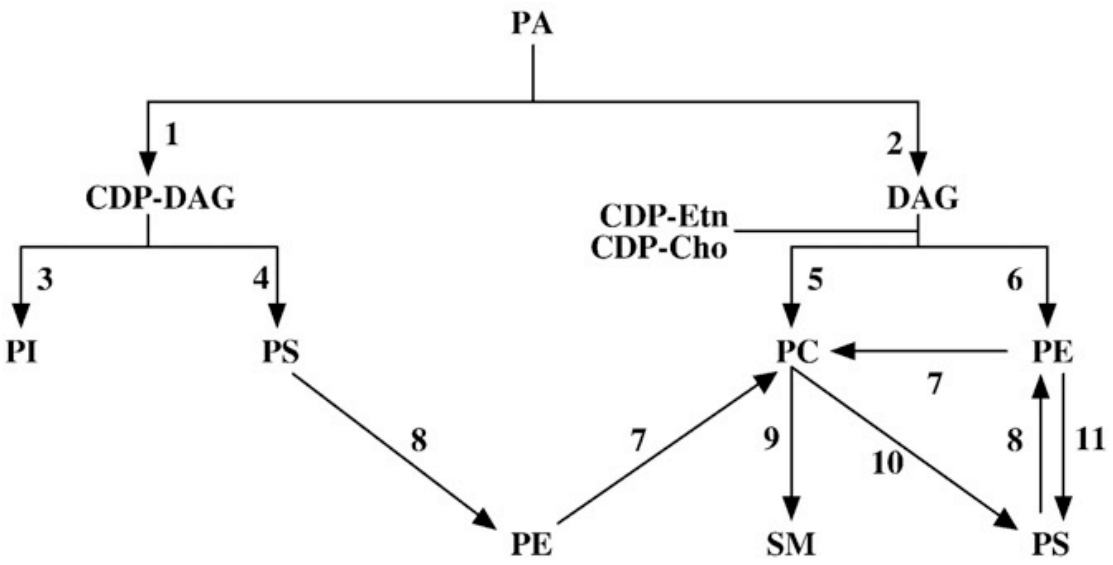

Figure 1.6 Biosynthetic pathways for the major phospholipid classes (Tocher *et al.*, 2008). CDP-Cho, CDP-choline; CDP–DAG, CDP-diacylglycerol; CDP-Etn, CDP-ethanolamine; DAG, diacylglycerol; PA, phosphatidic acid: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin. The enzymes are; 1, CDP– DAG synthases; 2, PA-phosphatases; 3, PI synthase; 4, PS synthase; 5, CDP-choline:DAG phosphotransferase; 6, CDP-ethanolamine:DAG phosphotransferase; 7, PE methyltransferase; 8, PS decarboxylase; 9, SM synthase; 10 and 11, PS synthases via base exchange.

Among the different phosphoglycerides , PC seems to be particularly important, being the main product of PL synthesis in fish enterocyte (Caballero *et al.*, 2006a) and comprising up to 95% of the PL found in VLDL (Lie *et al.*, 1993). Besides, PC induces the synthesis of apolipoprotein B in a higher extend than other phosphoglycerides (Field *et al.*, 1994; Field and Mathur, 1995). Enhanced lipoprotein synthesis can be responsible for the growth promotion effect by increasing energy flux from the intestinal mucosa into the blood (Seiliez *et al.*, 2006). Thus, PC has been found to promote growth better than other PL classes in several species (Takeuchi *et al.*, 1992; Kanazawa, 1993; Hadas, 1998; Geurden *et al.*, 1997a) and enhances feeding activity (Koven *et al.*, 1998). Phosphatidylinositol (PI) has a rich diversity of forms and functions within the cell besides being a structural component of biomembranes (Sargent *et al.*, 1989). Thus, PI acts as a precursor of second messengers (inositol 3 phosphate, IP3) regulating calcium ions' entry into the cell from the endoplasmatic reticulum (Cahu *et al.*, 2003a; Tocher *et al.*, 2008), as well as a membrane anchor for a great variety of cell surface proteins. Therefore, PI is

involved in a signalling system controlling biological processes in early development in vertebrates (Berridge and Irvine, 1989).

1.3.5 Effect of phospholipids on skeletal development

The skeletal system supports the body structural integrity during the development and provides sites for muscle attachment, protects vital organs (Lall and Lewis-McCrea, 2007). Teleost fish show a skeletal tissue that is described as a continuous spectrum ranging from connective tissue to cartilage and bone (Hall and Witten, 2007). The mature skeleton is a metabolically active organ that undergoes continuous remodeling by a process that replaces old bone with new bone (Figure 1.7). In healthy adults, bone resorption and formation are balanced and a constant level of bone mass is maintained (Kruger *et al.*, 2010). Osteoclasts responsible for bone resorption originate from haematopoetic stem cells know as monocytes, while osteoblasts responsible for bone formation originate from bone marrow stromal cells (Kruger *et al.*, 2010).

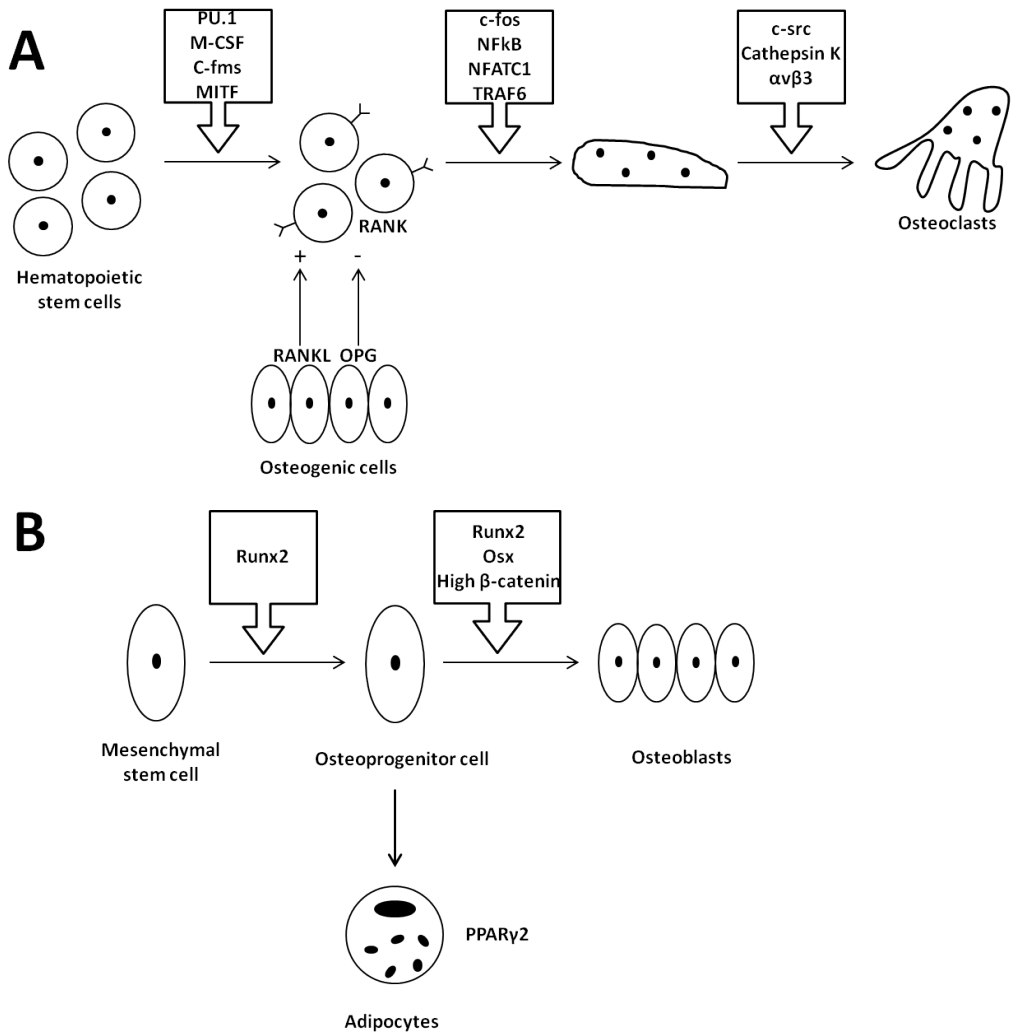

Figure 1.7 A simplified diagram of the differentiation of (A) osteoclast precursors into osteoclasts and (B) osteoblast precursors into osteoblasts. PU.1, Transcription factor PU.1; M-CSF, macrophage-colony stimulating factor; c-fms, c-fms genes; MITF, microphthalmia-associated transcription factor; c-fos, c-fos gene; NFαβ, nuclear factor αβ; NFATc1, nuclear factor of activated T cells 1; TRAF6, tumor necrosis factor receptor-associated factor-6; c-src, cellular-sarcoma gene; αvβ3, αvβ3-integrin; RANKL, receptor activator of nuclear factor αβ ligand; OPG, osteoprotegerin; Osx, osterix; PPARγ2, peroxisome proliferator activated receptor γ2 (Adapted from Kruger *et al.*, 2010).

Bone is a specialized vascularized connective tissue consisting of cells and a mineralized extracellular collagen matrix that then mineralizes by deposition of hydroxyapatite (Hall and Witten, 2007; Nordvick, 2007). During first feeding several dietary nutrients play a central role in bone development and the posterior appearance of skeletal malformations (Izquierdo *et al.*, 2010, 2012). Several studies dealing with the impact of first feeding on fish development showed that the dietary incorporation of total phospholipid reduced malformation, especially twist of jaw and scoliosis, in

ayu larvae, (*Plecoglossus altivelis*) (Kanazawa *et al*., 1981). Geurden *et al.,* (1998a) suggested that in carp larvae dietary PL, specially PI, reduces skeletal deformity. These findings were reinforced by Cahu *et al.,* (2003a) who found that dietary PC/PI ratio of 2.18 prevented deformities during larval development in European sea bass. Moreover, ossification of the vertebral column in cod larvae, first observed at 21dph in neural arches, occurred significantly earlier in groups fed diets containing PL than in those fed neutral lipids (NL), when measured both by larval size and larval age (Kjørsvik *et al.*, 2009). The PL has a role as a precursor of second messenger regulating calcium entry into the cells, and it is involved in a signaling system controlling biological processes in the early development of vertebrates (Berridge and Irvine, 1989). Sandel *et al.,* (2010) found a very high correlation between developmental age of gilthead seabream fed high PI and gene expression for osteocalcin, a protein used as a marker for bone and cartilage growth, and has been correlated with bone mineralization (Simes *et al.*, 2008). PI, as the second messenger inositol-3-phosphate (IP3), mobilizes calcium by regulating the entry of this ion into the cell from the endoplasmic reticulum (Cahu *et al.*, 2003a; Tocher *et al*., 2008). These authors argued that dietary PI may be increasing the availability of calcium for bone mineralization, which stimulates osteocalcin production and normal bone development. In support of this, Nishimoto *et al.,* (2003) found in carp that bone hydroxyapatite binding of bone Gla protein (BGP) is enhanced in the presence of calcium ions.

Changes in dietary PUFAs are reflected in the composition of various tissues, including bone cells such as the osteoblasts (Atkinson *et al.,* 1997; Moyad, 2005). Several of the long chain poly-unsturated fatty acids (LCPUFAs) have been shown to affect bone cells, i.e. osteoclasts and osteoblasts, via various cellular signaling pathways or growth factors, thereby affecting bone formation, resorption and bone density in animals or humans (Salari *et al.*, 2008). Changing the balance of PUFAs present in the membranes towards the n-3 fatty acids rather than n-6 fatty acids, shifts the ratio of n-6/n-3 in the membranes and affects production of several cytokines, which could affect bone resorption and formation. LCPUFAs have been shown to affect cellular proteins and the receptor activator of nuclear factor kβ (RANK), a receptor found on the osteoclast, which controls osteoclastogenesis (Sun *et al.*, 2003; Coetzee *et al.*, 2007).

A large number of studies have been done on animals that show that in growing

animals PUFAs affect bone mineral content, bone mass, femoral thickness and bone area, seemingly affecting bone formation and growth. n-3 PUFAs also increase alkaline phosphatase (ALP) activity in growing male rats as well as insulin-like growth factor I (IGF-1) and insulin-like growth factor binding protein (IGFBP) levels (Coetzee *et al.,* 2007; Li *et al.,* 1999).

Insulin-like growth factors (IGFs), especially IGF-I, are major bone-derived growth factors and are believed to function as both systemic and local growth factors for bone tissue. Once secreted and deposited in bone matrix, IGFs are released during osteoclastic bone resorptive activity, acting in an autocrine or paracrine fashion to stimulate new bone cell formation and matrix production (Watkins and Seifert, 2000). IGF-I acts as a regulator of bone cell function as it stimulates the proliferation of pre-osteoblasts, thereby increasing the number of cells capable of producing bone matrix. In addition, IGF-I increases collagen expression while decreasing collagen degradation, causing an anabolic effect in bone tissue (Schmid *et al.,* 1992; Delany *et al.,* 1994).

Dietary PUFAs may up-regulate or down-regulate IGF-I production in bone via their ability to modulate local concentrations of the arachidonic acid (AA) metabolite prostaglandin E2 (PGE2) (Li *et al.,* 1999; McCarthy *et al.,* 1991; Delany *et al.,* 1994). PGE2 is derived from the AA and is the major prostaglandin in bone. It has been shown to be a potent modulator of bone remodeling, affecting both bone resorption (Collins and Chambers*,* 1991) and formation (Jee and Ma, *et al.,* 1997; McCarthy *et al*., 1991). PGE2 also promotes the expression of various IGF-binding proteins (Schmid *et al.,* 1992; McCarthy *et al.,* 1994; DiBattista et al. 1996) suggesting that PGE2 could keep IGF-1 available for stimulation of osteoblasts at a later phase of bone remodeling (Schmid *et al*., 1992). McCarthy *et al.* (1991) suggested that the ability of PGE2 to enhance osteoblastic IGF-I synthesis could explain its anabolic potential, and furthermore suggests a role for PGE2 in coupled bone remodeling. The anabolic effects of PGE2 may occur through stimulation of endogenous IGF-I production by osteoblasts (Raisz *et al*., 1993) or by increased bone cell responsiveness to IGF-I (Hakeda *et al*., 1993)

In conclusion, enriching diets with n-3 LCPUFA-rich may elevate or preserve bone mass through increasing mesenchymal stem cell (MSC) number, through enhancing

expression of key transcription of factors required for bone formation such as core binding factor α-1 (Cbfα1) and osterix (Watkins *et al*., 2003; Zhang *et al*., 2002) that enhance differentiation of pre-osteoblasts into mature osteoblasts. Expression of both Cbfα1 and osterix is enhanced by IGF-1, transforming growth factor-β1 (TGF- β 1), bone morphogenic protein (BMP) and moderate amounts of PGE2 (Zhang *et al*., 2002), all of which result from diets rich in n-3 LCPUFA.

1.4 Oxidative stress: Antioxidant defense mechanism

Lipid peroxidation products such as hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons which are implicated in several pathological conditions damage cellular bio-membranes (Kanazawa, 1991, 1993) and subcellular membranes, such as those of mitochondria, causing several pathological conditions in fish (Kawatsu, 1969; Watanabe *et al*., 1970; Murai and Andrews, 1974; Sakai *et al*., 1989). Fish have an endogenous antioxidant defense system (Filho *et al*., 1993) with a wide range of antioxidant mechanisms to maintain an adequate oxidative balance. When this balance tilts in favor of the oxidant agents, oxidative stress arises with detrimental effects in molecules of great biological importance. Reactive oxygen species (ROS) are free radicals and/or oxygen derivatives produced during all aerobic biological systems and in normal cellular functions (Mates, 2000). The biological handicap associated with oxidative stress could be estimated by a physicochemical condition in which an increase in the steady-state levels of oxidative species e.g. H_2O_2, HO^-, O^{-2}, $ONNO^-$, R^- y ROO^- (Ansaldo *et al*., 2000)
increase in ROS leads to random cellular damages on the different cellular components. If continued along time will cause disease or even cell death.

1.4.1 Enzymatic defenses

The antioxidant defense system mostly formed by radical scavenging enzymes, play an important role in physiological antioxidant protection (Blazer, 1982). These antioxidants defense mechanisms to detoxify reactive oxygen species have evolved to counteract the potentially deleterious effects of activated oxygen (Yu, 1994). Among antioxidant enzymes, superoxide dismutase (SOD) catalyzes the superoxide anion dismutation to molecular oxygen and hydrogen peroxide; catalase (CAT) breaks

down hydrogen peroxide to molecular oxygen and water; glutathione peroxidase (GPX) detoxifies hydrogen peroxide into water, or organic peroxides into their corresponding stable alcohols, by oxidizing the reduced glutathione (GSH) into its oxidized form (GSSG); finally, glutathione reductase (GR) catalyzes the reduction of GSSG into GSH (Halliwell and Gutteridge, 2007). The SOD plays a crucial role since it is the first enzyme to respond to oxygen radicals, preventing initialization of the radical chain reaction that the superoxide anion produces (McCord and Fridovich, 1969; Winston and Di Giulio, 1991). All these enzymes, which are very important in antioxidant defense mechanisms, have been detected in most fish species (Rudneva, 1997). The activities of antioxidant defense enzymes showed variations in different organs of fish (Wdzieczak *et al*., 1982; Lemaire *et al*., 1993), depending upon feeding behavior (Radi and Markovics, 1988), feeding period (Mourente *et al*., 2002), ecological conditions (Winston and Di Giulio, 1991) and dietary antioxidant content. All these antioxidant mechanisms are responsible for maintaining an adequate "oxidative balance". When this balance tilts in favor of the oxidant agents, due to excessive generation of these compounds, or a weakening of antioxidant defenses, or both occurring simultaneously, "oxidative stress" arises (Sies, 1985; Tocher *et al*., 2002).

Antioxidants may be found in the cell plasma, mitochondria or cell membranes (Figure 1.8) and 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 O2 concentrations.

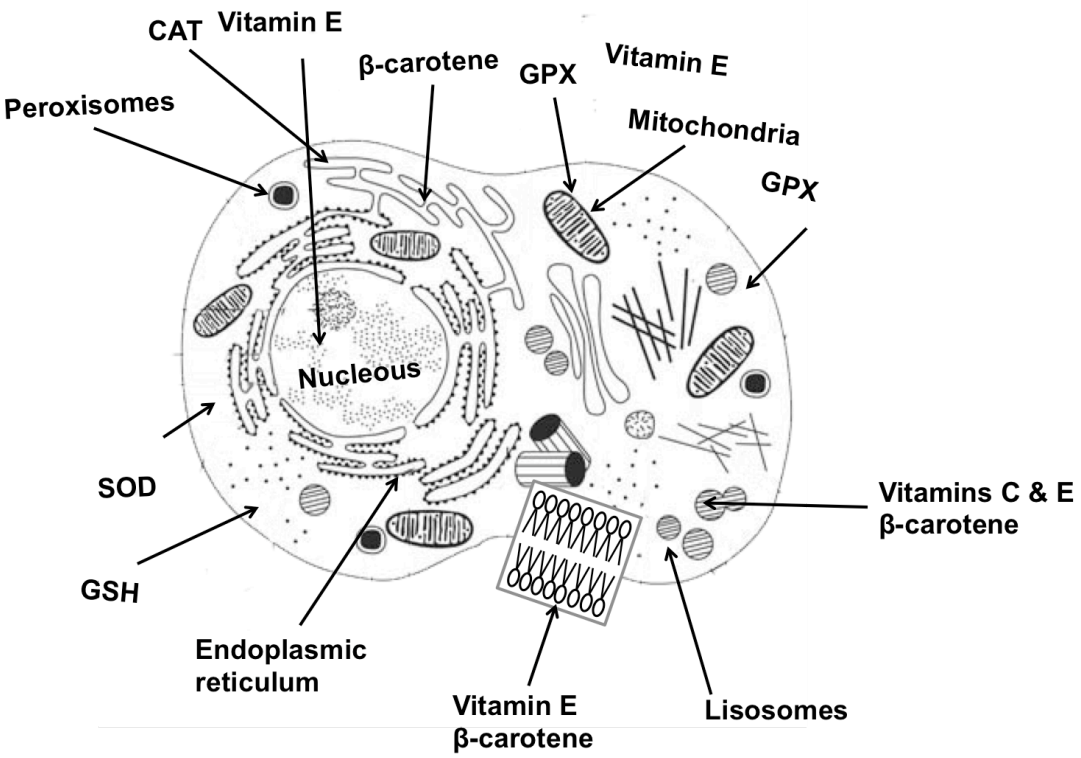

Figure 1.8 Antioxidants mechanisms within the cell. Adapted from Machlin and Bendich (1987). Catalase (CAT); Superoxide dismutase (SOD); Glutathione peroxidase; (GPX); Reduced glutathione (GSH).

1.4.2 Non enzymatic defenses

An antioxidant is a substance that, when present at low concentrations compared to those of a prone to oxidation substrate, is able to interact with free radicals to terminate the reaction (Halliwell and Gutteridge, 1990). Adequate levels of antioxidant compounds can be provided by nutritional supplements, including vitamins such as vitamin E and C or minerals such as selenium, that establish a second line of defense (Sen, 1995). These supplements will promote maximum benefits from these nutrients and avoid lipid oxidation problems that can cause pathologies (Sakai *et al*. 1989) including cellular damages (Halliwell and Gutteridge, 1996), muscles injuries (Betancor *et al*., 2011; 2012a), negative growth, bad feed intake and poor development, increased incidence of deformities (Lewis-McCrea and Lall, 2007), and, in general, oxidative damage to molecules of great biological importance such as lipids, proteins, carbohydrates and DNA causing alterations that can lead to cell death (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 2007).

1.4.2.1 Vitamin E (α-tocopherol)

The vitamin E has a great importance as exogenous component in the diet of aquaculture fish acting as an antioxidant defense in reduction of lipid peroxidation products and free radicals scavengers (Machlin, 1984; Betancor *et al*., 2011). Mourente *et al*. (2002) showed a reduction of peroxidation products in liver of gilthead seabream fed oxidized oil fed diets supplement with vitamin E in comparison with those fed diets lacking this vitamin.

Vitamin E is a lipid soluble vitamin that is absorbed from the digestive tract in association with fat molecules. Putnam and Comben (1987) showed that vitamin E is a structural component of cell membranes, where it acts as an antioxidant factor for phospholipids by chain-breaking antioxidant process (Sargent *et al*., 1997). Also, it has role in maintaining flesh 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), improving immunity, increasing the resistance of red blood corpuscles to haemolysis, and permeability of capillaries and heart muscle (Halver, 2002). Besides, it has been proposed to be the most important factor in maintaining the post-mortem membrane stability of fish fillet (Baker, 1997). Dietary requirement of vitamin E has been studied in different fish species (NRC, 1993) and recommendations include 120 mg kg^{-1} diet (Hamre and Lie, 1995) for Atlantic salmon, 30 to 50 mg kg^{-1} diet for channel catfish (*Ictalurus punctatus)* (Murai and Andrews, 1974; Wilson *et al*., 1984), 200 to 300 mg kg^{-1} diet for common carp (*Cyprinus carpio)* (Watanabe *et al*., 1977), 1200 mg kg^{-1} for gilthead seabream juveniles (Ortuño *et al*., 2000) and 99 mg kg^{-1} for mrigal fry (*Cirrhinus mrigala)* (Paul *et al*., 2004). The level of 250 mg α-tocopherol kg^{-1} diet is sufficient to meet the requirements for successful reproduction in gilthead seabream (Fernández-Palacios *et al*., 1998).

Little is known about the precise vitamin E requirements in marine fish larvae, but Atalah *et al.* (2008) used 1.5 and 3.0 g kg^{-1} dry diet of vitamin E for gilthead seabream and sea bass larvae, and suggested an optimal level of 3 g kg^{-1} because this high level reduced mortality after stress. In the same study, the high vitamin E level also alleviated muscular lesions caused by excessive dietary DHA (Betancor *et al*., 2011). The results of these studies are therefore in line with the general opinion that

vitamin E in larval diets should be above 1 g kg^{-1}, while the requirement given by NRC (1993) for juvenile fish is 50 mg kg^{-1}. However, when stress and immune responses are used to measure requirements of these vitamins, higher estimations of dietary optima are most often found, also in juvenile and adult fish (Hamre, 2011).

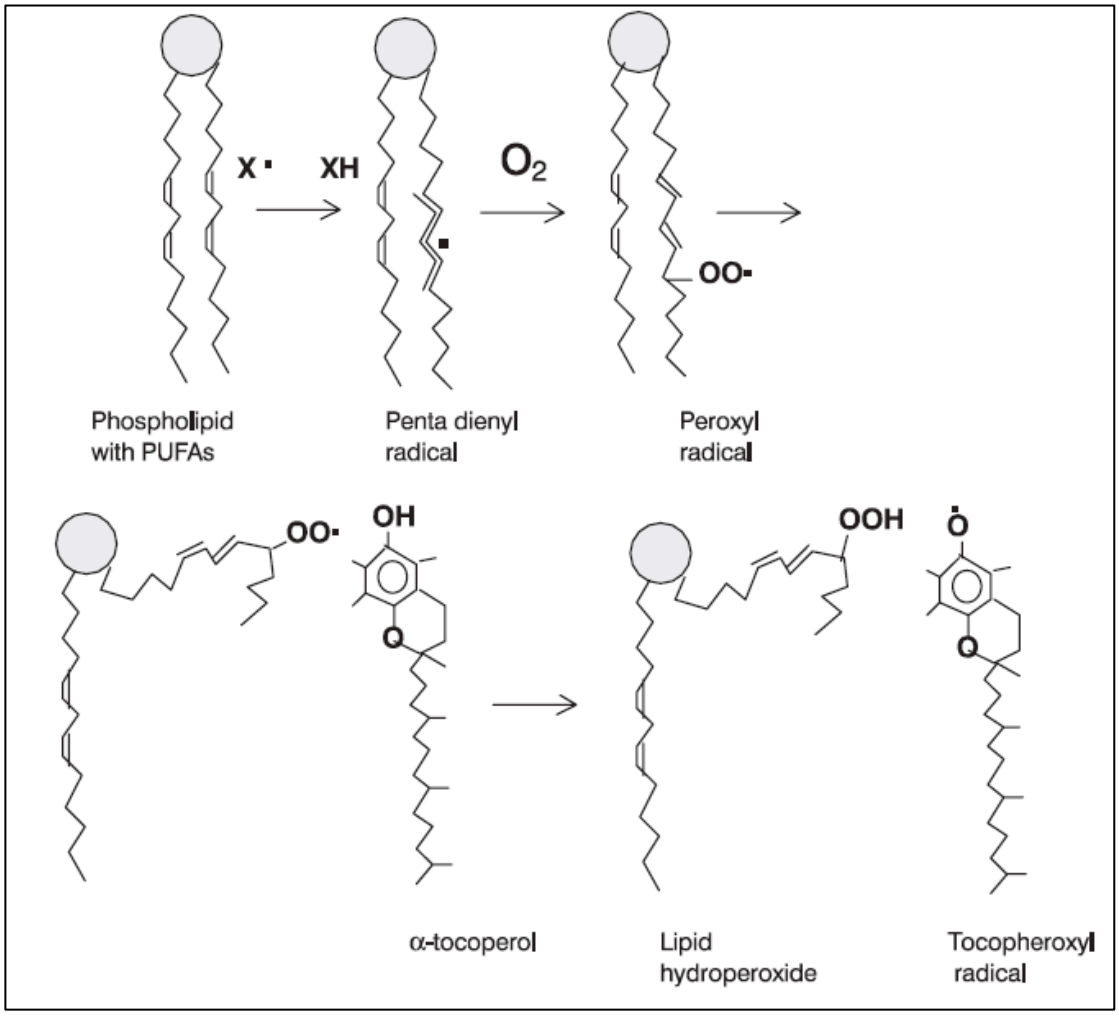

Figure 1.9 Proposed mechanism for the reaction of a-tocopherol with oxidizing lipids. The peroxyl radical group formed during lipid oxidation is polar and floats to the surface of the membrane where it can react with a- tocopherol, rendering a lipid hydroperoxyde and the tocopheroxyl radical (Buettner, 1993).

The effect of α-tocopherol in skeletal development 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) where it associates with the lipid bilayer of bone cells allowing it to be the first line of defense against free radicals (Arjmandi *et al*., 2002). The studies on the effect of ROS on fish bone development

are scarce. Lewis-McCrea and Lall (2007) found an increase in the incidence of scoliosis in Atlantic halibut juveniles feed increasing levels of oxidized oils. This may be a result of lipid peroxidation products effect, which stimulate osteoclastic differentiation and inhibit osteoblastic activity that may cause bone resorption and lead to improper bone formation/abnormalities (Tintut *et al*., 2002; Parhami, 2003; Kruger *et al*., 2010). The antioxidant mechanism of α-tocopherol intercepts the peroxyl radical (ROO^{-}) more rapidly than PUFA by donating its phenolic hydrogen atom to the radical and converting it to a hydroperoxide product (Figure 1.9), 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.

1.4.2.2 Selenium

Research on mineral requirements in fish larvae only started after 2005 and the number of publications is quite small. Selenium (Se) is a trace mineral and an essential micronutrient for vertebrates (Johansson *et al*., 2005). In fish, Se is also involved in thyroid hormone and insulin function, maintenance of fertility as well as regulation of cell growth (Lall, 2002; Kohlmeier, 2003). 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). 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 Penglase *et al*., (2010) fed rotifers enriched with up to 3 mg selenium kg^{-1} dry weight diet to cod larvae. They found only minor effects on growth and survival, but gene expression and activity of the glutathione peroxidases were enhanced by the enrichment, indicating a requirement of selenium above the control level.

The importance of Se to oxidative stress is due to its role in active sites of the antioxidant enzymes glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase (Felton *et al*., 1996), which catalyze the reduction of hydroperoxides and peroxides to less reactive alcohols and water (Felton *et al*., 1996). It must be taken into account that Se requirements of fish vary with the form of

selenium ingested, PUFA and vitamin E content of the diet (Lall, 2002).

As illustrated above, direct measurements of vitamin and mineral requirements in fish larvae are fragmented and scarce, as each nutrient is measured only in a few species or not at all. More work is therefore needed to conclude on whether micronutrient requirements in fish larvae are different from those in juvenile and adult fish

1.8 Objectives

Since good quality fry is essential for the further development of marine fish culture and the success of fry high production is greatly affected by the nutritional quality of the initial larval diets (Kolkovski *et al.,* 2009; Izquierdo *et al.,* 2000), a main objective in fish larval nutrition is to formulate an effective compound diet able to substitute live preys (Watanabe and Kiron, 1994). Dietary phospholipids are fundamental components of these weaning diets (Koven *et al*., 1995; Izquierdo, 1996; Fontagné *et al*., 2000; Izquierdo and Koven, 2010) due to their essential roles in larval development (Izquierdo and Koven, 2010). However, despite there are many studies denoting the importance of dietary PL, few of them have aimed to specifically determine the quantitative PL requirements testing at least 5 different levels. Moreover, the type of phospholipid used is determinant of its nutritional value, but few studies have been able to compare different sources at several levels. Finally, although increase of n-3 HUFA rich PL may increase the oxidative risk, these aspects have not been yet studied and no information is neither published about the potential interaction of PL with antioxidant nutrients. Therefore, the main objective of this thesis was to define the phospholipid requirements of gilthead seabream larvae and to improve the current knowledge on the effects of this nutrients on growth, survival, stress resistance, digestive enzymes activity, skeleton formation and mineralization and oxidative metabolism. To achieve this goal the following objectives were formulated:

1- To determine the optimum dietary levels of krill phospholipids (KPL) for seabream (*Sparus aurata*) larvae, and its influence on larval development and digestive enzymes activity.
2- To define the optimum dietary levels of soybean lecithin (SBL) for seabream (*Sparus aurata*) larvae, and its influence on larval development and digestive enzymes activity.

3- To compare the effectiveness of KPL vs SBL lecithin in early weaning microdiets for seabream (*Sparus aurata*) larvae on culture performance, skeleton malformation, bone mineralization, and molecular markers of oxidative status and bone development.
4- To evaluate the combined effect of graded levels of α-tocopherol with KPL or SBL in early weaning microdiets for seabream (*Sparus aurata*) larvae on culture performance, skeleton malformation, bone mineralization, and molecular markers of oxidative status and bone development.
5- To assess the effect of graded levels of Se derived yeast with dietary KPL in early weaning microdiets for seabream (*Sparus aurata*) larvae on culture performance and biomarkers of oxidative status and bone development.

Chapter 2: Materials and methods

2.1 Larvae

2.1.1 Gilthead seabream larvae

Gilthead seabream larvae were obtained from natural spawnings from Instituto Canario de Ciencias Marinas (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain). Larvae previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 16 dph, were randomly distributed in experimental tanks at a density of 2100 larvae tank^{-1} and fed one of the diets tested in triplicate for 15 days (Chapters 3 and 4) or 30 days (Chapters 5, 6 and 7).

2.2 Experimental conditions

The experimental tanks used had a volume of 200 L and a cylinder shape with a conical bottom and were made of light grey colour painted fibreglass. Tanks were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality. Temperature, pH and water quality were daily tested. Seawater was continuously aerated (125 ml min^{-1}). Photoperiod was kept at 12h light: 12h dark, by fluorescent daylights that provided a light intensity of 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia).

2.3 Diets and feeding

2.3.1 Rotifers

Rotifers (*Brachionus plicatilis*) were produced in 1700 L cylindrical containers provided with a central aeration through a porous stone. The culture medium included 80% seawater and 20% freshwater. Rotifers from the *Brachionus plicatilis* strain S-1 (150-250 µm lorica length) were inoculated at an initial density of 100 ind ml^{-1}. They were fed daily with 0.4 g fresh yeast extract for 10^6 ind divided in two doses. Every

four days rotifers were carefully filtered through a 64 µm mesh, rinsed in water, the container washed and the culture re-suspended in clean water at culture densities of 100-250 ind ml^{-1}. The rotifers fed to seabream larvae when green water was used in the larval rearing were previously enriched for 6 h at densities of 300 rotifers ml^{-1} with emulsified DHA Protein Selco® (INVE, Dendermond, Belgium) at a ration of 0.125 g l^{-1} ind divided in two daily doses. Rotifers were used to feed seabream larvae during green water culture and during the first two days of trial as co-feeding with the experimental microdiets.

2.3.2 Microdiets

2.3.2.1 Microdiets formulation

The experimental microdiets (pellet size 250-500 µm) included krill oil (Qrill, high PL, Aker BioMarine, Fjordalléen, Norway) or soybean lecithin (Acrofarma, Barcelona, Spain) as sources of PL. Vitamin E, in the α-tocopheryl acetate form, was obtained from Sigma-Aldrich (Madrid, Spain). 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). The protein source used was squid meal (Riber & Son, Bergen, Norway), which in some experimental diets was defatted (3 consecutive times with a chloroform:meal ratio of 3:1) to allow a better control of the microdiet lipid content.

2.3.2.2 Microdiets preparation

The microdiets were prepared by mixing squid powder, attractants mixture which prepared according to Kanazawa *et al.* (1989), and the hydro and lipo-soluble vitamins mixture and minerals mixture which prepared according to Teshima *et al.* (1982) (Table 2.1). The squid powder and water-soluble components of attractants and hydro-soluble vitamins were mixed well in a mortar, and then the lipids and fat-soluble vitamins were combined to obtain a homogeneous mix, which was afterwards merged with the powder mix. Finally, gelatine dissolved in warm water was added to the previously mixed ingredients to form a paste that was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra,

Barcelona, Spain) to obtain several particle sizes between 250 and 500 µm. Diets were prepared and analyzed for proximate and fatty acid composition at GIA laboratories.

Table 2.1 Mix of attractants, minerals and vitamins of the 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

2.3.3 Feeding

Diets were manually supplied every 45 min from 8:00 to 19:00. Larvae were fed with rotifers twice a day during the first two days of experimental feeding at a density of 1 individual ml^{-1}. To guarantee feed availability, daily dry supply was initially 2.5 g and increased 0.5 g each week. Larvae were observed under the binocular microscope to determine feed acceptance. If apparent feed intake differences were observed along different experimental diets, diet acceptance was determined calculating the percentage of gut occupation of the microdiet by image analysis in pictures of 30 larvae/tank. For such study, pictures of the, abdominal cavity of 30 larvae per tank were taken (Leica Wild M3Z, Optotek, California, USA).

2.4 Sampling

2.4.1 Biological parameters

At the beginning, middle and end of each experiment, samples of 30 live seabream larvae unfed for 12 h were taken to determine total length and whole body dry weight.

2.4.2 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, seabream larvae samples were taken to analyze initial biochemical composition.

2.4.3 Digestive enzymes

To analyze digestive enzymes activity, 15 fresh larvae $tank^{-1}$ were collected, washed with distilled water and kept at -80ºC in 2ml eppendorf tubes until analysis.

2.4.4 Histological studies

At the beginning, intermediate and final sampling points, 30 seabream larvae from each tank were collected and fixed in 10% buffered formalin.

2.4.5 Molecular biology

At the beginning and final sampling points, around 100 mg of unfed seabream larvae were collected, washed in Diethyl pyrocarbonate (DEPC) water and conserved in 500 µl of RNA Later (Sigma-Aldrich, Madrid, Spain) overnight at 4ºC, then RNA Later was removed and samples kept at -80ºC until RNA extraction.

2.4.6 Activity test and survival

At the end of the experiments an activity test was conducted by handling 20 larvae $tank^{-1}$ out of the water in a scoop net for 1-1.5 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 $tank^{-1}$ at the end of the experimental trials.

2.4.7 Growth

Growth was determined by measuring total length and dry body weight. 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. 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, for approximately 24 h, followed by 1 h periods until constant weight was reached.

2.5 Biochemical analysis

2.5.1 Proximate analysis

2.5.1.1 Moisture

Moisture content was determined by oven drying until constant weight at 110ºC, with a first 24 h drying period, followed by 1 h periods until weight was not reduced any further. Sample weight (approximately 100 mg) was recorded before drying and after each drying period, following the cooling until room temperature in a desiccator. 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

2.5.1.2 Ash

Ash content was determined in 200 mg samples by complete combustion in an oven at a temperature of 450ºC (A.O.A.C., 1995).

2.5.1.3. Proteins

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

2.5.1.4 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 butylated hydroxytoluene (BHT). The resulting solution was filtered by gravimetric 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 the watery phase was eliminated, the solvent was dried under nitrogen atmosphere and subsequently total lipids weighed.

2.5.1.4.1 Lipid classes

Separation of lipid classes was performed by double development high-performance thin layer chromatography (HPTLC) using the method described elsewhere (Olsen and Henderson, 1989). The lipid classes were visualized by charring at 160$_o$C for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC scanner (Version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS Planar Chromatography Manager. The identities of individual lipid classes were confirmed by comparison with reference to the Rf values of authentic standards run alongside samples on HPTLC plates and developed in the same conditions. Total glycerophospholipids, including lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS), were collectively isolated from HPTLC plates and subjected to acid-catalysed transesterification according to the method of Christie (1982). Extraction and purification was performed as described by Tocher and Harvie (1988).

2.5.1.4.2 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 NH2 Sep-pack cartridges (Waters S.A., Massachussets, USA) as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC-14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length:30 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).

2.5.2 Digestive enzymes activity

Enzymes activity was determined at the department of Applied Biology (Escuela Politécnica Superior, Universidad de Almería, España). The alkaline phosphatase, trypsine and lipase activities were expressed as relative fluorescence units (RFU) and PLA2 was expressed as units (U); one unit will hydrolyze 1.0 µmole of soybean L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a fatty acid per minute. The larvae were homogenized by a sonicator ultrasound (Misonix Microson XL2007 Ultrasonic Homogenizer) on ice in 110µl of high-purified water, centrifuged and the supernatant used as the sample stock solution. Alkaline phosphatase activities were quantified by fluorometric assay using a spectrofluorometer (Thermolab Systems; Helsinki, Finland) at excitation wavelengths of 358 nm and emission wavelengths of 455nm. After preparation of 140 µl of reaction buffer at pH 10.4 (100 mM Glycine, 1 mM $MgCl_2$, 1mM $ZnCl_2$), and 50 µl of substrate stock solution (200 µM of 6,8 Difluoro-4 methyllumbelliferyl phosphate (DiFMUP) solution in DMSO, the reaction was started by adding 10 µl of the sample stock solution and the kinetic curves were recorded for 20 min (Gee *et al.,* 1999).

Trypsine activities were quantified by a fluorometric assay using spectrofluorometer at excitation wavelengths of 380 nm and emission wavelengths of 440nm. After preparation of 195 µl of reaction buffer pH 8.0 (50mM Tris-HCl, 10 mM $CaCl_2$), and 5 µl of of substrate stock solution (20 µM of Boc-Gln-Ala-Arg-7 amido-4 methylcoumarin hydrochloride in DMSO), the reaction was initiated by adding 10 µl of the sample stock solution and the kinetic curves were recorded for 5 min (Rotllant *et al.,* 2008).

Neutral lipase activities were quantified by a fluorometric assay using spectrofluorometer at excitation wavelengths of 355 nm and emission wavelengths of 460nm. Firstly, 247.4 µl of 0.1 M phosphate reaction buffer pH 7.0 (19.5 ml of 0.2M phosphate monobasic (NaH_2PO_4 1 H_2O) solution and 30.5ml of 0.2M phosphate dibasic (NaH_2PO_4 7 H_2O) solution were mixed and completed up to 100 ml by adding 50 ml high-purified water), then 2.6µl of substrate stock solution (40 mM of 4Methylumbelliferol butyrate (MUB) in N,N Dimethyl formamide (DMSO) was added and then reaction was started by adding10 µl of the sample stock solution. Kinetics curves were recorded for 5 min (Rotllant *et al.,* 2008).

The PLA2 activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 377 nm and emission wavelengths of 450nm. First 160 µl of reaction buffer pH 8 (50mM Tris-HCl,100 mM NaCl, 2mM NaN_3, 5µg/ml bovine serum albumin, and 10 µm 1-anilinonaphthalene-8-sulfonate, and 20 µl of substrate stock solution (50 µl of 1,2-dimyristoyl-sn-glycero-3-phosphocholine solution in 40 mM, methanol mixed with 15 µl deoxycholic acid solution in 40 mM, methanol and quickly injected into 1ml high purified water, stirred for 1 min and sonicated for 2 min) and 10 µl of 100 mM $CaCl_2$ solution were incubated at 25 °C for 10 min. The reaction was started by adding 20 µl of the sample stock solution and the kinetic curves were recorded for 40 min (Huang *et al.,* 2006).

2.6 Measurement of thiobarbituric reactive substances (TBARS):

The thiobarbituric reactive substances **(**TBARS) were determined in triplicated samples using a method adapted from 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 homogenate 2.95 ml of freshly prepared 50mM thiobarbituric acid solution were 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:

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

2.7 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 in the Institute of Aquaculture, University of Stirling (Scotland, UK).

2.8 Whole mount staining for skeleton studies

Staining procedures with alizarin red (Table 2.2) were conducted to evaluate the skeletal anomalies and vertebral mineralization (Vandewalle *et al*.1998).

Table 2.2 Staining protocol according to Vandewalle *et al*. (1998)

STEP	DURATION	Significance of the step	Solutions
Alcian Blue (8GX)	2 hours	Proteoglycan (cartilage) Staining	For 100 mL: 80 mL alchol 95% 20 mL glacial acetic acid 10 mg Alcian blue
Ethanol 95%	1 hour	Hydratation	For 100 mL: 95 mL absolute ethanol 5 mL distilled water
Ethanol 95%	1 hour		
Ethanol 95%	1 hour		
Ethanol 75%	1 hour		For 100 mL: 75 mL absolute ethanol 15 mL distilled water
Ethanol 40%	1 hour		For 100 mL: 40 mL absolute ethanol 60 mL distilled water
Ethanol 15%	1 hour		For 100 mL: 15 mL absolute ethanol 75 mL distilled water
Distilled water	1 hour or all night		
Trypsine solution	1 hour	Tissue digestion	90 mg pancreas porcine trypsine; 70 mL distilled water 30 mL saturated solution of $Na_2B_4O_7$

Alizarine red	1 hour and a half	Calcium (bone) staining	1g L^{-1} alizarine red In a 0.5% KOH solution
Glycerin KOH 1:3	12 to 24 hours	Clearing	For 100 mL: 25 mL Glycerin 75 % KOH (0.5%)
Glycerin KOH 1:1	12 to 24 hours	Clearing	For 100 mL: 50 mL Glycerin 50 mL KOH (0.5%)
Glycerin KOH 3:1	12 to 24 hours	Clearing	For 100 mL: 75 mL Glycerin 25 mL KOH (0.5%)
Pure glycerine and some grains of thymol		Storage	

2.9 Molecular biology studies

Molecular biology analysis was carried out at the GIA laboratories in Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA, ULPGC, Las Palmas, Spain). Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30ml of RNase-free water.

The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of complementary desoxiribonucleic acid (cDNA) was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15,1:20 and 1:25). The product size of the real-time q PCR amplification was checked by electrophoresis

analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using β-actin as the house-keeping gene in a final volume of 20ml per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analysed once per gene. The PCR conditions were the following: 95ºC for 3min 30sec followed by 40 cycles of 95ºC for 15sec, 61ºC for 30sec, and 72ºC for 30sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalised and the Livak method ($2^{-\Delta\Delta Ct}$) used to determine relative mRNA expression levels. Gilthead seabream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in table 2.3.

Table 2.3 Sequences of forward and reverse primers (5´-3´) for real-time quantitative-PCR of seabream genes

Gene	Primers	Accession n^{o}
Catalase(CAT)	Forward primer: 5´-ATGGTGTGGGACTTCTGGAG-3´ Reverse primer: 3´-AGTGGAACTTGCAGTAGAAAC-5´	FJ860003
Superoxide dismutase (SOD)	Forward primer: 5´- AAGAATCATGGCGGTCCTACTGA-3´ Reverse primer: 3´- TGAGCATCTTGTCCGTGATGTCT -5´	AJ937872
Glutathione peroxidase (GPX)	Forward primer: 5´- TCCATTCCCCAGCGATGATGCC-3´ Reverse primer: 3´- TCGCCATCAGGACCAACAAGGA-5´	DQ524992
Osteocalcin	Forward primer: 5´-AGCCCAAAGCACGTAAGCAAGCTA-3´ Reverse primer: 3´- TTTCATCACGCTACTCTACGGGTT-5´	AF048703
Osteopontin	Forward primer: 5´- AAGATGGCCTACGACATGACAGAC- 3´ Reverse primer: 3´- CCTGAAGAGCCTTGTACACCTGC-5´	AY651247
Osteonectin	Forward primer: 5´- AAAATGATCGAGCCCTGCATGGAC-3´ Reverse primer: 3´- TACAGAGTCACCAGGACGTT-5´	AY239014
[a]RUNX2	Forward primer: 5´- GCCTGTCGCCTTTAAGGTGGTTGC-3´ Reverse primer: 3´- TCGTCGTTGCCCGCCATAGCTG-5´	AJ619023
Alkaline phosphatase	Forward primer: 5´- AGAACGCCCTGACGCTGCAA-3´ Reverse primer: 3´- TTCAGTATACGAGCAGCCGTCAC-5´	AY266359
Matrix Gla protein	Forward primer: 5´- GTGCCCTCCTTCATTCCAC-3´ Reverse primer: 3´- TATGACCACGTTGGATGCCT-5´	AY065652
[b]BMP4	Forward primer: 5´- CCACCAGGGCAGACACGTCC-3´ Reverse primer: 3´- GCGTAGCTGCTCCCAGTCCTC-5´	FJ436409
β-actin	Forward primer: 5´- TCTGTCTGG ATC GGAGGCTC-3´ Reverse primer: 3´- AAGCATTTG CGGTGGACG -5´	X89920

a RUNX2 (Runt-related transcription factor 2).
b BMP4 (Bone morphogenetic protein 4).

2.10 Statistical analysis

All data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation and were treated using one-way ANOVA. Means compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Chapter 3

Optimum krill phospholipids content in microdiets for gilthead seabream (*Sparus aurata*) larvae

This work was published in Saleh *et al.* (2012a) Aquaculture Nutrition, Doi: 10.1111/j.1365-2095.2012.00976.x

Abstract

The aim of the present study was to determine the optimum dietary levels of krill phospholipids (KPL) for seabream (*Sparus aurata*) larvae, and its influence on larval development and digestive enzymes activity. Larvae were fed five formulated microdiets with five different levels of KPL. Complete replacement of live preys with the experimental microdiets for seabream larvae produced high survival and growth rates, particularly in larvae fed the highest levels of KPL. In the present study, increase in dietary KPL up to 12% (10% total PL) significantly improved larval survival and growth, whereas further increase did not improved those parameters. An increase in alkaline phosphatase, trypsin and lipase activity with the elevation of KPL up to 12% was also found denoting a better functioning of digestive system. Besides, there was a linear substrate stimulatory effect of dietary KPL on phospholipase A2 activity. Finally, increasing dietary KPL lead to better assimilation of n-3 HUFA especially EPA, reflected in the higher content of these fatty acids in both neutral and polar lipids of the larvae. In summary, KPL were found to be an excellent source of lipids for seabream larvae. Optimum inclusion levels of this ingredient in microdiets to completely substitute live preys at this larval age were found to be 12% KPL.

Introduction

Dietary phospholipids (PL) improve culture performance of various freshwater and marine fish species (Izquierdo and Koven, 2011), enhancing growth and survival, reducing morphological alterations in larvae (Kanazawa 1993; Salhi *et al.,* 1999; Izquierdo *et al.,* 2001; Kjørsvik*,* 2009) and early juveniles (Coutteau *et al*., 1997), and increasing fish resistance to stress (Takeuchi *et al*., 1992; Kanazawa, 1993). Despite PL metabolic pathways, including those of *de novo* PL biosynthesis, are essentially the same in fish as in mammals (Caballero *et al*., 2006b), the fish larvae and early juvenile have a limited capacity to synthesise *de novo* (Salhi *et al*., 1999; Coutteau *et al*., 1997). Thus, addition of PL to diets for fish larvae contributes to assimilation of dietary lipid by increasing the enteric lipoprotein synthesis (Liu *et al*., 2002; Hadas *et al*., 2003) and release in lamina propria, significantly reducing lipoprotein size by promoting VLDL synthesis (higher in PL), rather than chylomicron production (higher in NL) (Liu *et al*., 2002; Caballero *et al*., 2003, 2006b). Indeed, young fish receive abundant of PL during embryo and larval development either from yolk sac lipids or from wild preys (Rainuzzo *et al*., 1997; van der Meeren *et al*., 2008). Therefore, PL seems to be essential for the adequate growth and development of fish larvae. Described phospholipid requirements are around 2–4% DW of diet for juvenile fish and they may be higher in larval fish. Frequently those requirements have used plant PL such as soybean lecithin, or egg yolk lecithin, whereas marine PL, rich in docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been more scarcely studied (Salhi *et al*., 1999; Cahu *et al*., 2003a). DHA and EPA present in the PL fraction of larval diets seem to be more beneficial than in the nuetral lipid (NL) fraction (Salhi *et al*., 1999; Cahu *et al*., 2003a; Gisbert *et al.,* 2005; Wold *et al*., 2007). Cahu *et al*. (2003a) ran a dose response study with sea bass larvae, using five levels of phospholipids at a constant dietary lipid level (PL, 27-116 g kg^{-1} dw). They found that the diet with the highest dietary PL gave the best larval performance and lower skeletal malformation rates. A similar result was found by Hamza et al. (2008) for pikeperch larvae, which also showed best growth with the diet highest in PL (9% of dry matter).

The digestive system of larvae is not fully developed at first feeding. The digestion of ingested food occurs in the larval intestine, where the pH remains alkaline and trypsin-like enzyme activity accounts for the proteolytic activity (Walford and Lam,

1993). At first feeding, the pancreatic and intestinal enzyme activities are generally low (Cousin *et al*., 1987). Digestive enzyme activity increase during the first 10 dph in *Solea senegalensis* (Ribeiro *et al*. 1999), whereas an increase in alkaline phosphatase activity has been found to reflect the development of the brush border membranes of enterocytes in Atlantic cod (*Gadus morhua*) (Wold *et al*., 2007). Moreover, the addition of dietary PL enhanced gut maturation index in this species, based on the relation between brush border alkaline phosphatase and cytosolic leucine-alanine aminopeptidase. Enhancement of gut maturation by dietary PL could be related with a higher intracellular availability of PL for cell membrane and cell organelles formation, since dietary PL promotes re-acylation of digested lipids, increasing intracellular PL availability for lipoprotein synthesis in gilthead seabream (Caballero *et al*. 2003; Liu *et al*. 2002).

In sea bass larvae, the response of phospholipase A2 to dietary phospholipid content was gradual and showed a great modulation range in expression. Also, amylase and alkaline phosphatase activities revealed a proper maturation of the digestive tract in the the larvae fed the highest dietary phospholipid levels (Cahu *et al*., 2003a).

Based on the few studies where single pure phospholipid species have been used, the rank order for efficacy appears to be phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine and phosphatidylserine (Izquierdo and Koven, 2011). Several studies also suggested that the phospholipid effect was not due to a general enhanced emulsification and digestion of lipids. The evidence rather led to the hypothesis that early developing stages of fish had impaired ability to transport dietary lipids away from the intestine possibly through limitations in lipoprotein synthesis. Thus, dietary PL increase the efficiency of transport of dietary fatty acids and lipids from the gut to the rest of the body (Coutteau *et al*., 1997; Fontagne *et al*., 1998; Salhi *et al*., 1999; Izquierdo *et al*., 2001).

However, despite the many studies available denoting the importance of dietary PL, few of them have intended to determine quantitative PL requirements testing diets with at least five different levels of this nutrient. Recently, krill PL have been found to constitute a suitable phospholipid source in diets for larval gilthead seabream (Betancor *et al*., 2012b). Thus, the aim of the present study was to determine the optimum requirements of krill PL for gilthead seabream (*Sparus aurata*) larvae, and its influence on larval production performance and digestive enzymes activity.

Materials and methods

Gilthead seabream larvae were obtained from natural spawnings from Instituto Canario de Ciencias Marinas (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain). Larvae (5.4 mm total length, 120 µg dry body weight) previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 16 dph, were randomly distributed in 15 experimental tanks at a density of 2100 larvae $tank^{-1}$ and fed one of the diets tested in triplicate. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality, which was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min^{-1}) attaining 6.1±1 ppm dissolved O_2. Average water temperature and pH along the trial were 19.1±1°C and 7.85, respectively. Photoperiod was kept at 12h light: 12h dark, by fluorescent daylights and the light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia).

Five experimental microdiets (pellet size < 250 µm) with increasing phospholipid contents were formulated using sardine oil (Agramar S.A., Spain) and krill oil (Qrill, high PL, Aker BioMarine, Fjordalléen, Norway) as sources of triglycerides and PL, respectively. Their formulation and proximate analysis are shown in Table 1. The fatty acid content is shown in Tables 2, 3 and 4. The desired lipid content (about 21% DW) was completed if necessary with a non-essential fatty acid source, oleic acid (Oleic acid, Merck, Darmstadt, Germany). The microdiets were prepared by mixing squid powder and water-soluble components, and then the lipids and fat-soluble vitamins and, finally, gelatine dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain a particle size below 250 µm. Diets were prepared and analyzed for proximate and fatty acid composition (Tables 3.1, 3.2, 3.3 and 3.4) at GIA laboratories (Las Palmas de Gran Canaria, Spain).

Table 3.1 Formulation and proximate composition of the experimental microdiets containing several levels of KPL

Ingredients (g/100 g diet)	0 KPL	3 KPL	6 KPL	12 KPL	17.5 KPL
Squid Powder[a]	75.6	75.5	75.5	71.5	66
Krill PL[b]	0	3	6	12	17.5
Sardine oil[c]	7.9	3	0	0	0
Oleic acid[d]	0	2	2	0	0
Basal premix	16.5	16.5	16.5	16.5	16.5
Proximate analysis % dry weight					
Total Lipids	21.33	20.82	20.97	22.01	24.33
Polar Lipids	6.00	7.00	9.00	10.00	11.00
Polar Lipid/Total lipid	0.28	0.34	0.43	0.45	0.45
Protein	71.26	72.36	72.26	70.68	66.12
Ash	6.9	6.84	7.04	7.06	7.26
Moisture	7.1	8.1	8.6	8	8.6

a Rieber and Son, Bergen, Norway.
b Qrill, high PL, Aker BioMarine, Fjordalléen, Norway.
c Agramar S.A., Spain.
d Merck KGaA, Darmstadi, Germany.

Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00 for 16 days. Non-enriched rotifers were co-fed during days 16th and 17th (1 rotifer ml^{-1}). To assure feed availability, daily feed supplied was maintained at 1.5 and 2.5 g tank-^{1} during the first and second weeks of feeding, respectively. Larvae were observed under the binocular microscope to determine feed acceptance. 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 living larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 h) and total length (Profile Projector V-12A Nikon, Tokyo, Japan) of 30 fish tank^{-1} at the beginning, in the middle and at the end of the trial. In addition, at the end of the trial and after 12 h of starvation, the all larvae in each tank were washed with distilled water, sampled and kept at -80 ºC for biochemical composition.

Moisture (A.O.A.C. 1995), protein (Kjeldhal) and crude lipid (Folch 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supercolvax-

10-fused silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC/min and then held at 215 ºC for 10 min. Fatty acid methyl esters were quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) following the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

For enzymes activity determination, the alkaline phosphatase, trypsine and lipase activities are expressed as relative fluorescence units (RFU) and PLA2 is expressed as units (U). The larvae were homogenized by a sonicator ultrasound (Misonix Microson XL2007 Ultrasonic Homogenizer) on ice in 110µl of high-purified water, centrifuged and the supernatant used as the sample stock solution.

The alkaline phosphatase activities were quantified by fluorometric assay using a spectrofluorometer (Thermolab Systems; Helsinki, Finland) at excitation wavelengths of 358 nm and emission wavelengths of 455nm. After preparation of 140 µl of reaction buffer at pH 10.4 (100 mM Glycine, 1 mM $MgCl_2$, 1mM $ZnCl_2$), and 50 µl of substrate stock solution (200 µM of 6,8 Difluoro-4 methyllumbelliferyl phosphate (DiFMUP) solution in DMSO, the reaction was started by adding 10 µl of the sample stock solution and the kinetic curves were recorded for 20 min (Gee *et al.,*1999).

Trypsine activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 380 nm and emission wavelengths of 440nm. After preparation of 195 µl of reaction buffer pH 8.0 (50mM Tris-HCl, 10 mM $CaCl_2$), and 5 µl of of substrate stock solution (20 µM of Boc-Gln-Ala-Arg-7 amido-4 methylcoumarin hydrochloride in DMSO), the reaction was started by adding 10 µl of the sample stock solution and the kinetic curves were recorded for 5 min (Rotllant *et al.,* 2008).

Neutral lipase activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 355 nm and emission wavelengths of 460nm. Firstly, 247.4 µl of 0.1 M phosphate reaction buffer pH 7.0 (19.5 ml of 0.2M phosphate monobasic (NaH_2PO_4 1 H_2O) solution and 30.5ml of 0.2M Phosphate dibasic (NaH_2PO_4 7 H_2O) solution were mixed and completed up to 100 ml by adding 50 ml high-purified water), then 2.6µl of substrate stock solution (40 mM of

4Methylumbelliferol butyrate (MUB) in N,N Dimethyl formamide (DMSO) was added and then reaction was started by adding10 µl of the sample stock solution. Kinetics curves were recorded for 5 min (Rotllant *et al.,* 2008).

The PLA2 activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 377 nm and emission wavelengths of 450nm. First 160 µl of reaction buffer pH 8 (50mM Tris-HCl,100mM NaCl, 2mM NaN_3, 5µg/ml bovine serum albumin, and 10 µm 1-anilinonaphthalene-8-sulfonate, and 20 µl of substrate stock solution (50 µl of 1,2-dimyristoyl-sn-glycero-3-phosphocholine solution in 40 mM, methanol mixed with 15 µl deoxycholic acid solution in 40 mM, methanol and quickly injected into 1ml high purified water, stirred for 1 min and sonicated for 2 min) and 10 µl of 100 mM $CaCl_2$ solution were incubated at 25 °C for 10 min. The reaction was started by adding 20 µl of the sample stock solution and the kinetic curves were recorded for 40 min (Huang *et al.,* 2006).

All data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation and were treated using one-way ANOVA. Means compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results

All the experimental diets were well accepted by gilthead seabream larvae according to the microscopic observations. Generally, survival was very high for this type of studies and it was significantly correlated to the increase in dietary marine PL. Thus, average survivals of larvae fed diet 12 KPL (10% dietary PL content) and diet 17.5 KPL (11% dietary PL content) were significantly higher than the other treatments (Figure 3.1). The resistance to stress also increased by dietary PL levels, larvae fed the17.5 KPL diet showing higher survival after the activity test, although not significantly different from that of larvae fed diets 12 KPL and 6 KPL (9% dietary PL content) (Figure 3.2). On the contrary, larvae fed diet 0 KPL (6% dietary PL content) had a marked drop in survival after stress.

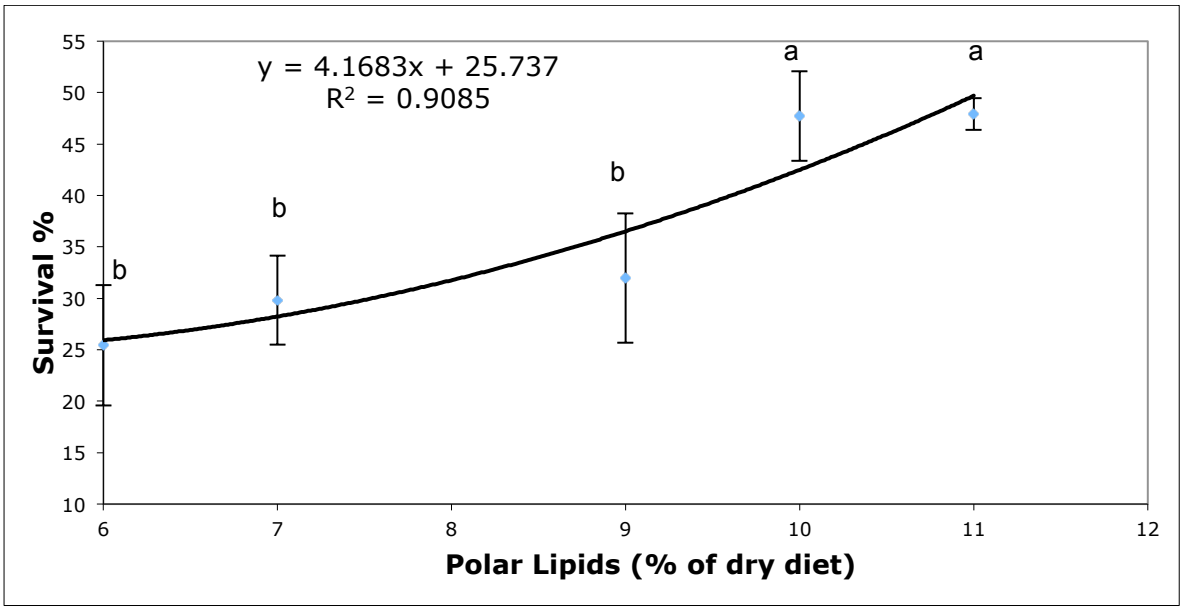

Figure 3.1 Survival rate (% of population) of larvae reared from 16 to 31 dph on five dietary KPL levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

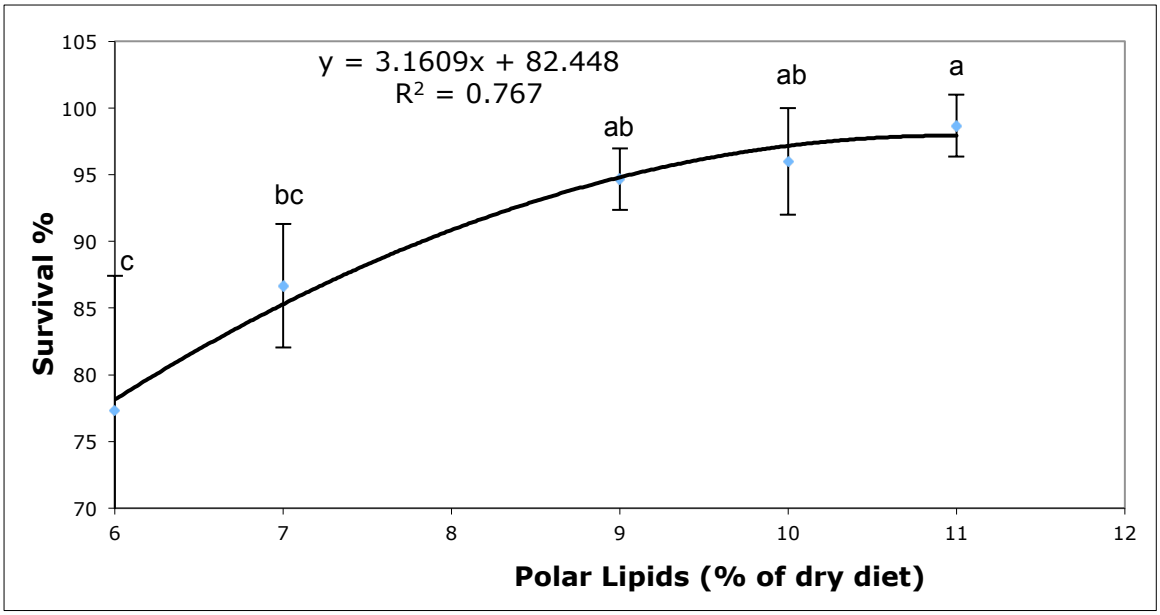

Figure 3.2 Survival 24 h after activity test of larvae (31 dph) fed five dietary KPL levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Larval growth was also improved by dietary PL and after 15 days of feeding, growth of larvae fed 17.5 KPL(11% dietary PL content) diet had significantly higher total length than those fed 0 KPL, 3 KPL, and 6 KPL, but did not differed from those fed 12 KPL(10% dietary PL content) (Figure 3.3). In terms of body weight, larvae fed 17.5 KPL were bigger than larvae fed 0 KPL and 3 KPL but did not differed significantly from larvae fed 6 KPL or 12 KPL diets (Figure 3.4).

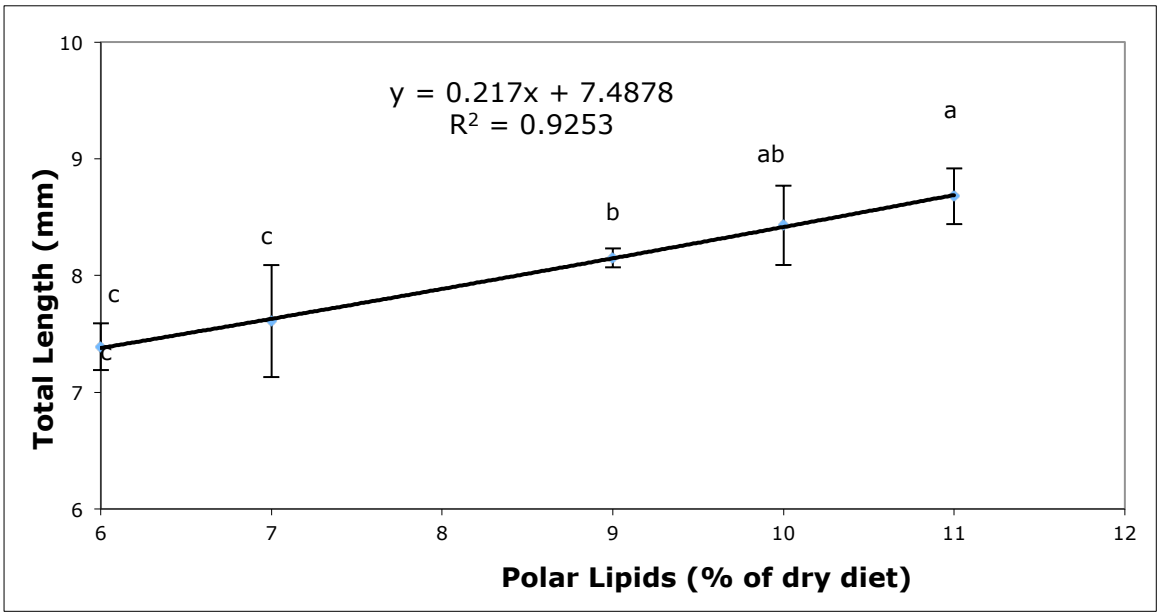

Figure 3.3 Total length of larvae (31 dph) fed five dietary KPL levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

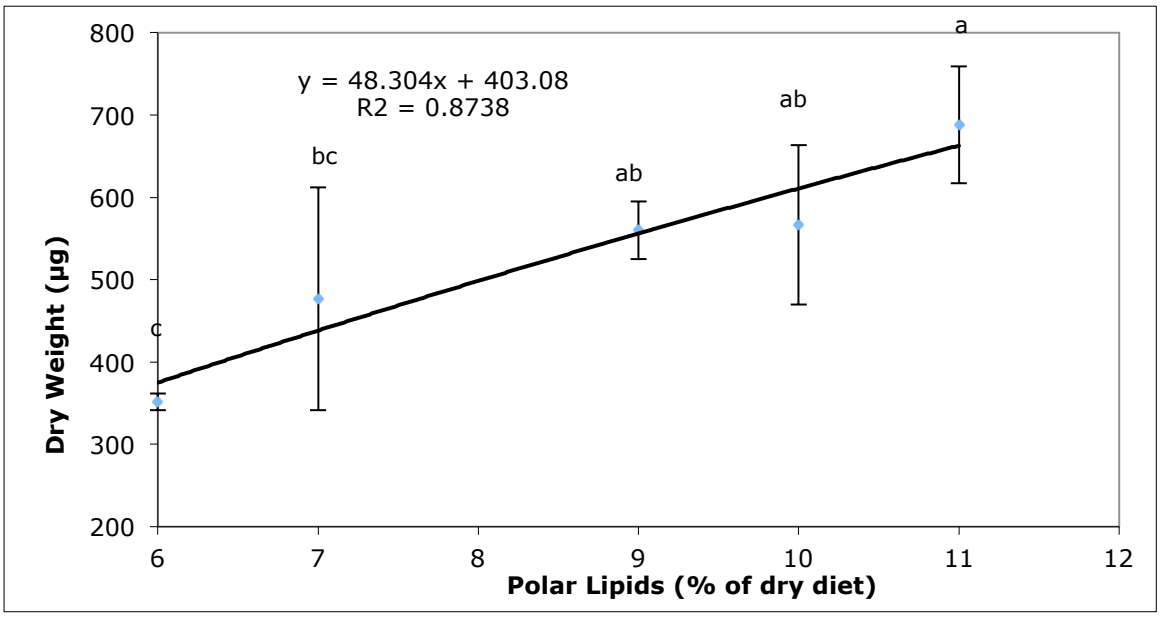

Figure 3.4 Dry weight of larvae (31 dph) fed five dietary KPL levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

In general digestive enzyme activity was increased by the elevation of dietary marine PL. Thus, alkaline phosphatase was lowest in 0 KPL larvae and significantly increased with the elevation of dietary PL up to 9 % (diet 6 KPL), whereas trypsine activity was significantly lowest in both 0 KPL and 3 KPL larvae (Figure 3.5 and 3.6). Neutral lipase activity was lowest in 0 KPL and 3 KPL larvae and significantly higher in 12 KPL and 17.5 KPL larvae (Figure 3.7). PLA2 activity was significantly higher in larvae fed 17.5 KPL than in larvae fed 0 KPL, 3 KPL or 6 KPL, but did not differed from that of 12 KPL larvae (Figure 3.8).

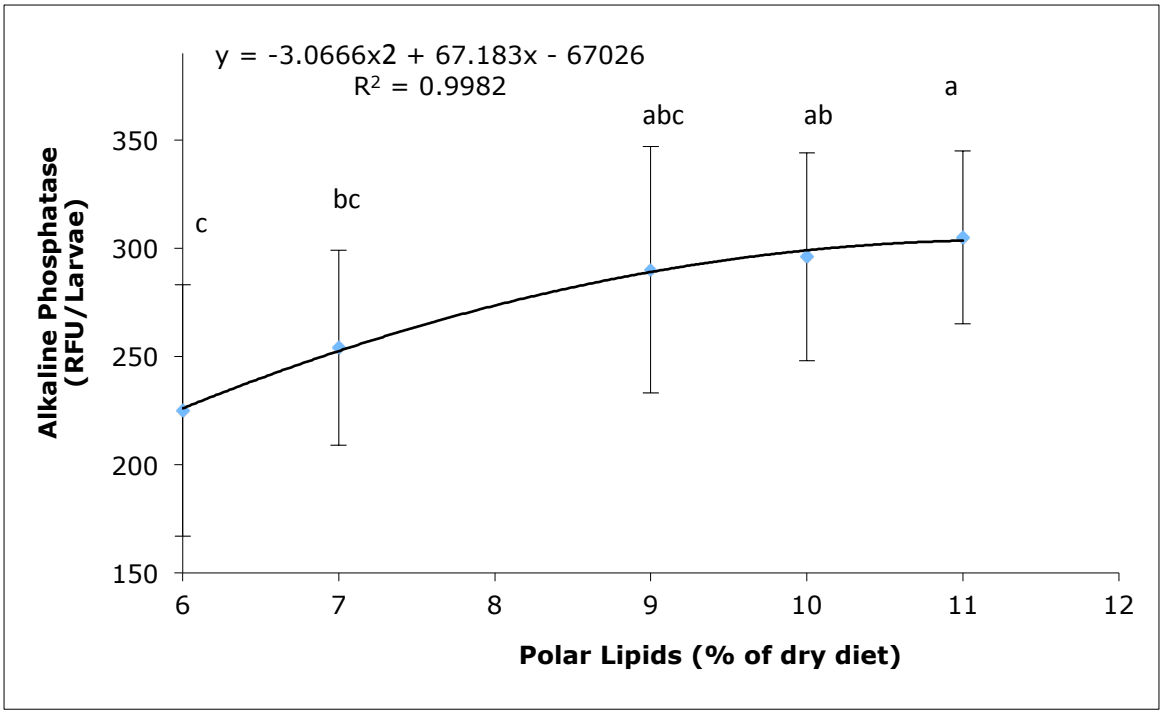

Figure 3.5 Alkaline phosphatase activity in seabream larvae (31 dph) fed five dietary KPL. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

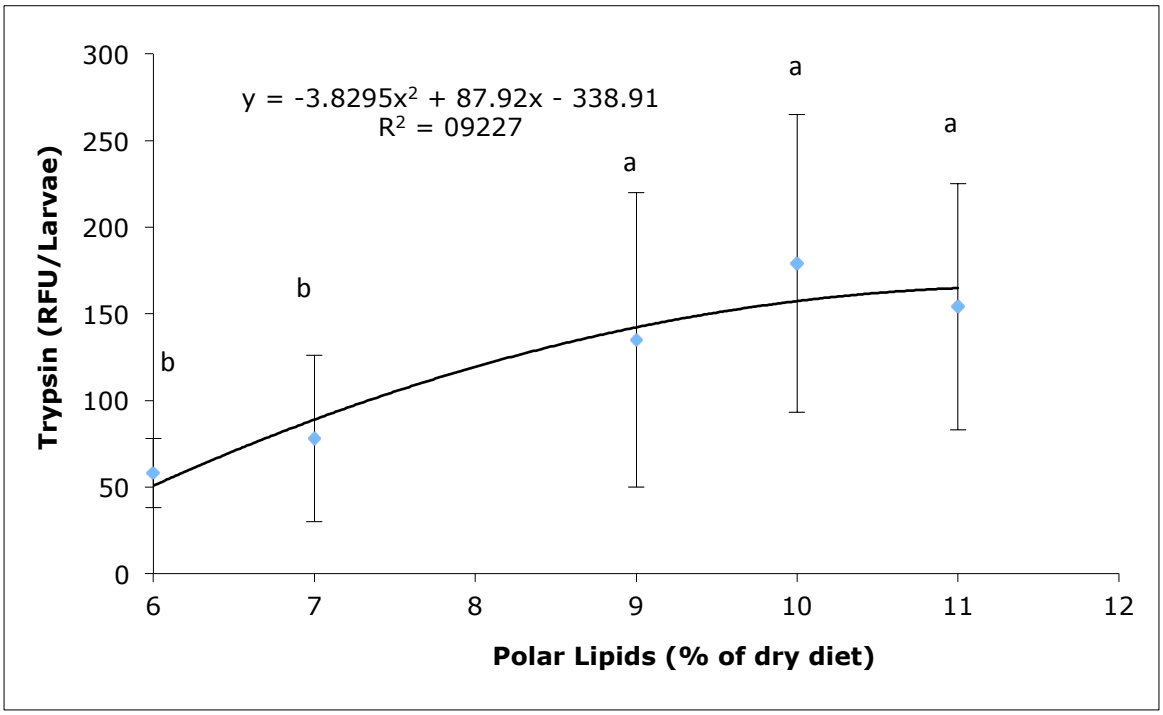

Figure 3.6 Trypsin activity in seabream larvae (31 dph) fed five dietary KPL. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

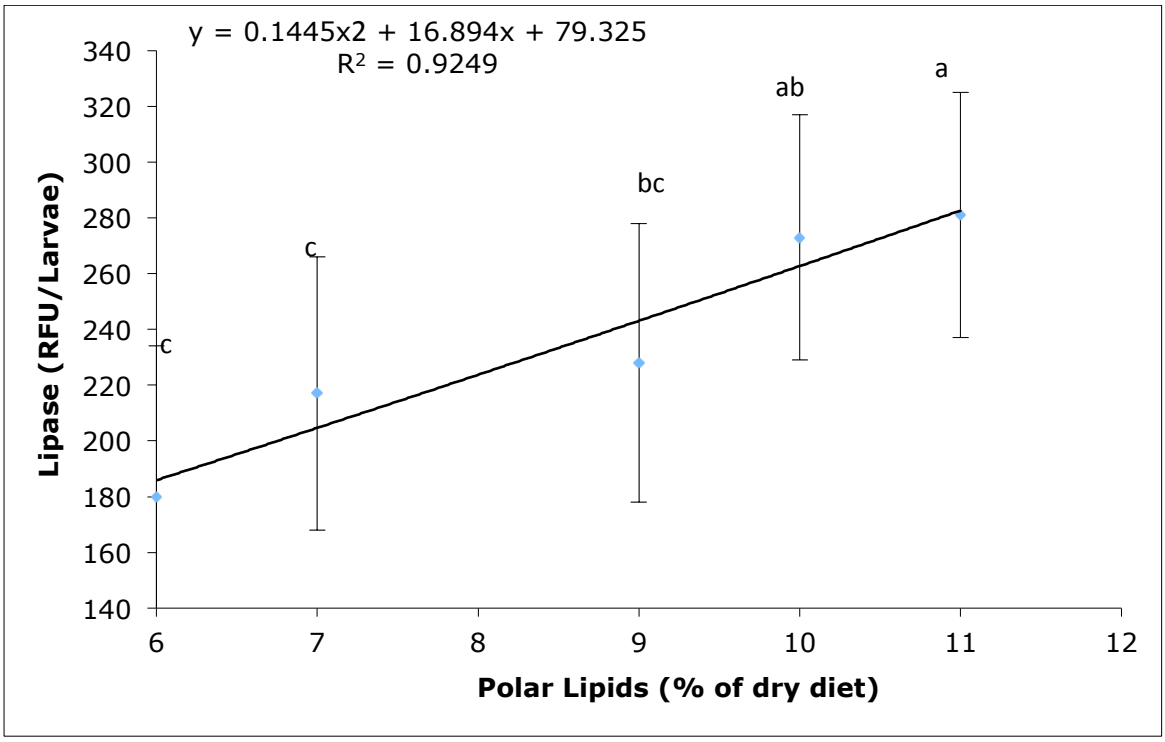

Figure 3.7 Lipase activity in seabream larvae (31 dph) fed five dietary KPL. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

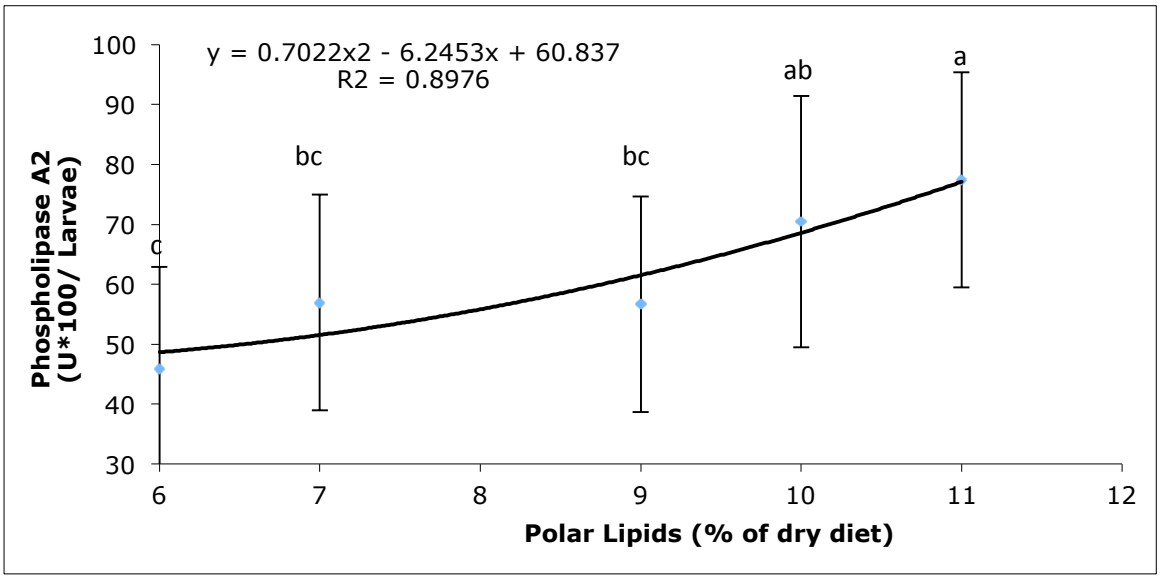

Figure 3.8 Phospholipase A2 activity in seabream larvae (31 dph) fed five dietary KPL. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Fatty acid composition of total lipids of the microdiets showed that gradual inclusion of PL lead to an increase in n-3 fatty acids, particularly due to the increase in EPA and DHA, together with 14:0. On the contrary, oleic acid and n-6 fatty acids, particularly 18:2n-6, were reduced by the inclusion of dietary PL (Table 3.2). However, fatty acid composition of PL from microdiets was much more stable and only showed a slight reduction in 20:1n-9+n-7 and increase in EPA (Tables 3.3 and 3.4).

Table 3.2 Fatty acids (% dry weight) composition in total lipids of diets containing five KPL levels

	0 KPL	3 KPL	6 KPL	12 KPL	17.5 KPL
14:0	0.54	0.77	0.86	1.45	2.00
14:1n-7	0.01	0.01	0.01	0.03	0.07
14:1n-5	0.01	0.01	0.01	0.04	0.11
15:0	0.09	0.09	0.08	0.10	0.19
15:1n-5	0.00	0.00	0.00	0.01	0.06
16:0ISO	0.01	0.01	0.01	0.02	0.07
16:0	4.78	4.72	4.16	4.98	5.73
16:1n-7	0.52	0.58	0.58	1.05	1.38
Me 16:0	0.03	0.04	0.04	0.07	0.11
16:1n-5	0.04	0.05	0.05	0.07	0.12
16:2n-6	0.06	0.04	0.04	0.07	0.13
16:2n-4	0.12	0.12	0.11	0.14	0.19
17:0	0.06	0.04	0.03	0.05	0.08

16:3n-4	0.01	0.00	0.01	0.01	0.03
16:3n-3	0.03	0.04	0.04	0.06	0.10
16:3n-1	0.07	0.07	0.06	0.06	0.07
16:4n-3	0.02	0.02	0.04	0.08	0.12
16:4n-1	0.00	0.00	0.01	0.01	0.02
18:0	0.99	0.80	0.55	0.50	0.53
18:1n-9	4.12	5.83	3.93	1.59	2.01
18:1n-7	0.46	0.47	0.55	1.01	1.31
18:1n-5	0.05	0.05	0.04	0.05	0.06
18:2n-9	0.00	0.00	0.01	0.01	0.02
18:2n-6	4.07	2.23	0.70	0.31	0.41
18:2n-4	0.02	0.01	0.00	0.01	0.01
18:3n-6	0.01	0.01	0.01	0.04	0.05
18:3n-4	0.03	0.01	0.01	0.01	0.01
18:3n-3	0.52	0.24	0.08	0.13	0.16
18:4n-3	0.05	0.06	0.13	0.27	0.36
18:4n-1	0.01	0.00	0.00	0.01	0.02
20:0	0.04	0.03	0.01	0.01	0.01
20:1n-9	1.06	0.77	0.50	0.53	0.52
20:1n-5	0.04	0.04	0.03	0.05	0.06
20:2n-9	0.01	0.00	0.00	0.00	0.00
20:2n-6	0.09	0.06	0.04	0.04	0.04
20:3n-6	0.02	0.01	0.01	0.01	0.02
20:4n-6	0.13	0.14	0.18	0.21	0.22
20:3n-3	0.09	0.08	0.08	0.08	0.07
20:4n-3	0.06	0.04	0.04	0.08	0.10
20:5n-3	0.83	1.12	2.52	3.72	4.53
22:1n-11	0.45	0.17	0.08	0.14	0.19
22:1n-9	0.06	0.06	0.00	0.02	0.03
22:4n-6	0.02	0.02	0.05	0.08	0.10
22:5n-6	0.02	0.02	0.03	0.03	0.03
22:5n-3	0.11	0.07	0.08	0.11	0.13
22:6n-3	1.56	1.82	4.25	4.66	4.76
Total Saturated	6.51	6.45	5.70	7.08	8.54
Total Monounsaturated	14.08	14.57	5.82	4.62	5.79
Total n-3	3.28	3.50	7.27	9.19	10.34
Total n-6	4.42	2.54	1.05	0.79	1.00
Total n-9	4.15	5.85	3.98	1.67	2.11
Total n-3HUFA	2.57	3.06	6.90	8.57	9.52
EPA/ARA	6.35	8.09	14.1	18.08	20.87
DHA/EPA	1.87	1.64	1.69	1.25	1.05
DHA/ARA	11.88	13.23	23.78	22.62	21.94
n-3/n-6	0.74	1.38	6.91	11.55	10.39

Table 3.3 Fatty acids (% dry weight) composition in neutral lipids of diets containing five KPL levels

	0 KPL	3 KPL	6 KPL	12 KPL	17.5 KPL
14:0	0.46	0.83	1.48	2.81	3.66
14:1n-7	0.01	0.02	0.03	0.06	0.08
14:1n-5	0.01	0.01	0.01	0.03	0.04
15:0	0.06	0.07	0.10	0.15	0.12
15:1n-5	0.00	0.00	0.01	0.02	0.02
16:0ISO	0.01	0.01	0.01	0.02	0.02
16:0	2.49	2.51	2.92	4.02	4.74
16:1n-7	0.59	0.70	1.05	2.22	2.72
Me 16:0	0.03	0.04	0.05	0.10	0.14
16:1n-5	0.02	0.02	0.04	0.07	0.08
16:2n-6	0.07	0.06	0.07	0.15	0.20
16:2n-4	0.06	0.10	0.18	0.33	0.38
17:0	0.07	0.05	0.04	0.08	0.09
16:3n-4	0.01	0.00	0.01	0.01	0.02
16:3n-3	0.01	0.01	0.03	0.06	0.06
16:4n3	0.03	0.04	0.07	0.16	0.23
18:0	0.69	0.54	0.51	0.48	0.41
18:1n-9	4.93	7.57	7.46	2.93	3.54
18:1n-7	0.36	0.34	0.47	1.13	1.53
18:1n-5	0.04	0.03	0.04	0.05	0.06
18:2n-6	5.73	3.49	1.38	0.73	0.47
18:2n-4	0.02	0.01	0.00	0.01	0.01
18:3n-6	0.01	0.01	0.02	0.02	0.06
18:3n-4	0.03	0.02	0.01	0.01	0.01
18:3n-3	0.87	0.43	0.09	0.16	0.18
18:4n-3	0.11	0.13	0.17	0.41	0.57
18:4n-1	0.03	0.02	0.00	0.01	0.01
20:0	0.07	0.05	0.04	0.04	0.03
20:1n9+n7	0.75	0.50	0.35	0.38	0.40
20:1n-5	0.03	0.03	0.03	0.06	0.08
20:2n-9	0.01	0.00	0.02	0.01	0.00
20:2n-6	0.08	0.05	0.02	0.03	0.03
20:3n-6	0.02	0.01	0.01	0.02	0.02
20:4n-6	0.07	0.06	0.06	0.09	0.11
20:3n-3	0.05	0.04	0.04	0.05	0.04
20:4n-3	0.13	0.07	0.04	0.06	0.09
20:5n-3	0.84	0.91	1.24	2.33	3.03
22:1n-11	0.54	0.28	0.09	0.16	0.14
22:1n-9	0.09	0.07	0.08	0.02	0.02
22:4n-6	0.04	0.03	0.03	0.05	0.07
22:5n-6	0.02	0.01	0.01	0.02	0.02
22:5n-3	0.29	0.15	0.04	0.06	0.08
22:6n-3	1.58	1.49	1.72	2.37	2.70
Total Saturated	3.82	4.05	5.10	7.57	9.04

Total Monounsaturated	7.36	9.57	9.63	7.12	8.69
Total n-3	3.90	3.28	3.43	5.66	6.96
Total n-6	6.04	3.72	1.60	1.12	0.98
Total n-9	5.03	7.65	7.57	2.97	3.57
Total n-3HUFA	2.88	2.66	3.07	4.87	5.94
EPA/ARA	12.19	15.84	20.95	24.52	26.66
DHA/EPA	1.88	1.63	1.39	1.02	0.89
DHA/ARA	22.91	25.89	29.18	24.89	23.75
n-3/n-6	0.65	0.88	2.14	5.04	7.11

Table 3.4 Fatty acids (% dry weight) composition in polar lipids of diets containing five KPL levels

	0 KPL	3 KPL	6 KPL	12 KPL	17.5 KPL
14:0	0.33	0.34	0.35	0.43	0.51
14:1n-7	0.01	0.01	0.01	0.01	0.01
14:1n-5	0.01	0.01	0.01	0.00	0.00
15:0	0.14	0.13	0.12	0.12	0.12
15:1n-5	0.00	0.00	0.00	0.00	0.00
16:0ISO	0.01	0.01	0.01	0.01	0.02
16:0	5.78	5.61	5.23	5.86	6.52
16:1n-7	0.10	0.20	0.23	0.32	0.41
Me 16:0	0.04	0.04	0.04	0.05	0.06
16:1n-5	0.05	0.05	0.05	0.07	0.08
16:2n-6	0.01	0.01	0.01	0.02	0.02
16:2n-4	0.16	0.14	0.12	0.11	0.10
17:0	0.03	0.03	0.03	0.03	0.03
16:3n-4	0.00	0.00	0.00	0.00	0.01
16:3n-3	0.05	0.05	0.05	0.06	0.07
16:3n-1	0.11	0.10	0.08	0.07	0.06
16:4n-3	0.02	0.02	0.01	0.01	0.02
16:4n-1	0.03	0.02	0.00	0.00	0.01
18:0	0.94	0.81	0.67	0.61	0.57
18:1n-9	0.63	0.65	0.66	0.82	1.02
18:1n-7	0.27	0.43	0.51	0.77	1.04
18:1n-5	0.05	0.05	0.04	0.05	0.06
18:2n-9	0.00	0.01	0.00	0.01	0.01
18:2n-6	0.47	0.33	0.29	0.33	0.35
18:2n-4	0.01	0.01	0.01	0.01	0.01
18:3n-6	0.01	0.01	0.01	0.01	0.03
18:3n-4	0.01	0.01	0.01	0.01	0.01
18:3n-3	0.05	0.06	0.08	0.12	0.17
18:4n-3	0.02	0.06	0.10	0.16	0.26
18:4n-1	0.03	0.02	0.02	0.01	0.01
20:0	0.04	0.03	0.02	0.02	0.01
20:1n-9+n7	0.86	0.75	0.63	0.58	0.55
20:1n-5	0.03	0.03	0.03	0.05	0.05

20:2n-9	0.01	0.01	0.01	0.01	0.00
20:2n-6	0.07	0.06	0.05	0.05	0.04
20:3n-6	0.00	0.00	0.01	0.01	0.02
20:4n-6	0.30	0.28	0.25	0.25	0.28
20:3n-3	0.13	0.12	0.10	0.09	0.09
20:4n-3	0.02	0.04	0.05	0.08	0.11
20:5n-3	2.54	3.06	3.42	4.31	5.99
22:1n-11	0.07	0.14	0.17	0.23	0.31
22:1n-9	0.02	0.00	0.00	0.04	0.05
22:4n-6	0.03	0.05	0.07	0.09	0.14
22:5n-6	0.07	0.06	0.05	0.05	0.05
22:5n-3	0.10	0.10	0.11	0.13	0.17
22:6n-3	7.68	6.89	6.35	5.96	6.88
Total Saturated	7.26	6.95	6.41	7.06	7.76
Total Monounsaturated	2.09	2.31	2.35	2.94	3.59
Total n-3	10.60	10.39	10.27	10.91	13.76
Total n-6	0.96	0.80	0.73	0.80	0.91
Total n-9	0.66	0.67	0.68	0.87	1.08
Total n-3HUFA	10.47	10.21	10.03	10.56	13.24
EPA/ARA	8.6	11.1	13.5	17.0	21.5
DHA/EPA	3.0	2.3	1.9	1.4	1.1
DHA/ARA	26.0	25.0	25.0	23.5	24.7
n-3/n-6	11.0	12.9	13.9	13.4	14.8

Analysis of fatty acids composition of larvae showed significant differences between the five dietary PL treatments in both neutral and polar lipids (Tables 3.5 and 3.6). Neutral lipids were more markedly affected by dietary lipids and showed increased myrisitc acid (14:0), 18:1n-7, 18:4n-3, n-3 fatty acids, particularly EPA, and reduced stearic (18:0), oleic (18:1n-9), 20:1n-9 and linoleic (18:2n-6) acids with increased dietary PL. Thus, 17.5 KPL larvae were significantly highest in EPA while the 0 KPL has the lowest. Larvae fed diets 12 KPL and 17.5 KPL diets showed higher n-3/n-6 and EPA/ARA ratios than 0 KPL, 3 KPL and 6 KPL larvae. Larval polar lipids were more conservative than the neutral lipids and only showed a marked increased in n-3 fatty acids and EPA, together with a reduction in n-6 fatty acids due to the lower linoleic acid contents. On the contrary, regardless dietary levels, ARA and DHA contents were not significantly different among larvae fed different levels of dietary PL. Larvae fed 12 KPL and 17.5 KPL diets showed the highest total n-3, n-3HUFA, and EPA content of polar lipids.

Table 3.5 Fatty acids (% total identified fatty acids) composition in neutral lipids of larvae fed diets containing five KPL levels. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	0 KPL	3 KPL	6 KPL	12 KPL	17.5 KPL
14:0	1.42	1.62	2.84	5.62	5.21
14:1n-5	0.02	0.02	0.02	0.03	0.03
14:1n-7	0.13	0.13	0.12	0.15	0.14
15:0	0.35	0.31	0.39	0.62	0.41
15:1n-5	0.02	0.02	0.03	0.03	0.05
16:0iso	0.07	0.06	0.07	0.09	0.08
16:0	14.81	15.12	17.73	29.54	18.44
16:1n-7	2.28	2.16	3.11	4.31	5.19
Met 16:0	0.17	0.18	0.22	0.30	0.31
16:1n-5	0.29	0.31	0.33	0.39	0.37
16:2n-6	0.70	0.63	0.69	0.69	0.63
16:2n-4	0.71	0.97	1.51	2.17	2.08
17:0	0.31	0.30	0.28	0.24	0.25
16:3n-4	0.03	0.03	0.04	0.04	0.04
16:3n-3	0.12	0.13	0.21	0.29	0.27
16:3n-1	0.10	0.12	0.08	0.05	0.04
16:4n-3	0.09	0.09	0.13	0.16	0.20
16:4 n-1	0.00	0.00	0.00	0.00	0.00
18:0	7.26	7.22	6.28	6.37	4.78
18:1n-9	14.55	17.25	16.88	8.06	9.07
18:1n-7	2.77	2.74	3.47	4.61	5.43
18:1n-5	0.37	0.40	0.37	0.41	0.34
18:2n-9	0.08	0.17	0.10	0.08	0.10
18:2n-6	13.41	7.17	2.65	1.33	1.52
18:2n-4	0.06	0.06	0.06	0.07	0.07
18: 3n-6	0.18	0.23	0.19	0.22	0.29
18:3n-4	0.12	0.12	0.19	0.10	0.08
18:3n-3	1.54	0.71	0.30	0.40	0.52
18:4n-3	0.24	0.24	0.44	0.85	1.13
18:4n-1	0.04	0.02	0.01	0.02	0.01
20:0	0.47	0.32	0.25	0.34	0.19
20:1n-9	3.46	2.53	2.58	2.42	1.96
20: 1n-5	0.32	0.30	0.34	0.36	0.36
20: 2n-9	0.06	0.08	0.03	0.03	0.02
20:2n-6	0.72	0.50	0.31	0.21	0.20
20:3n-9+n-	0.02	0.02	0.00	0.00	0.00
20:3n-6	0.08	0.09	0.06	0.06	0.09
20:4n-6	1.79	2.13	1.48	1.06	1.10
20: 3n-3	0.38	0.33	0.37	0.33	0.29
20:4n-3	0.32	0.24	0.25	0.35	0.44
20:5n-3	5.7	6.84	8.63	10.32	12.8
22:1n-11	1.33	0.95	1.42	1.47	1.34
22:1n-9	0.80	0.63	0.21	0.23	0.23
22:4n-6	0.12	0.13	0.20	0.30	0.37

22:5n-6	0.26	0.29	0.22	0.17	0.17
22:5n-3	1.59	1.47	1.33	1.48	1.71
22:6n-3	20.35	24.64	23.61	20.16	21.69
Total Saturated	24.62±0.85^{bc}	24.9±0.17^{bc}	27.77±0.51^{bc}	32.73±4.83^{a}	29.28±0.28^{b}
Total Monounsaturated	32.06+3.05^{a}	32.38+2.05^{a}	34.1+2.65^{a}	27.93+2.05^{b}	29.21+2.01^{b}
Total n-3	30.34±1.76^{c}	34.7±2.15^{b}	35.27±1.82^{b}	34.34±3.08^{b}	39.05±0.82^{a}
Total n-6	17.26±0.07^{a}	11.17±0.16^{b}	5.8±0.05^{c}	4.03±0.56^{d}	4.36±0.02^{d}
Total n-9	18.98±1.13^{a}	20.68±0.82^{a}	19.81±1.13^{a}	11.31±0.9^{b}	11.38±0.27^{b}
Total n-3HUFA	28.35±1.84^{b}	33.52±2.19^{a}	34.18±1.84^{a}	34.64±2.87^{a}	36.93±1.04^{a}
ARA	1.79±0.06^{b}	2.13±0.31^{a}	1.48±0.15^{b}	1.06±0.14^{c}	1.1±0.12^{c}
EPA	5.71±0.26^{e}	6.84±0.21^{d}	8.63±0.54^{c}	10.32±0.94^{b}	12.8±0.16^{a}
DHA	20.35±1.44^{c}	24.64±1.97^{a}	23.61±1.32^{ab}	20.16±1.78^{c}	21.69±0.97^{bc}
EPA/ARA	3.2±0.03^{d}	3.26±0.37^{d}	5.85±0.24^{c}	9.78±0.41^{b}	11.79±1.37^{a}
DHA/EPA	3.56±0.09^{a}	3.6±0.18^{a}	2.74±0.02^{b}	1.95±0.07^{c}	1.69±0.08^{d}
DHA/ARA	11.38±0.42^{c}	11.65±0.75^{c}	15.94±0.76^{b}	19.11±1.01^{a}	19.91±1.4^{a}
n-3/n-6	1.76±0.11^{d}	3.11±0.24^{c}	6.08±0.37^{b}	8.57±0.46^{a}	8.96±0.15^{a}

Table 3.6 Fatty acids (% total identified fatty acids) composition in polar lipids of larvae fed diets containing five KPL levels. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	0 KPL	3 KPL	6 KPL	12 KPL	17.5 KPL
14:0	0.57	0.69	0.86	1.05	1.15
14:1n-5	0.01	0.01	0.01	0.01	0.00
14:1n-7	0.05	0.06	0.04	0.04	0.04
15:0	0.32	0.33	0.35	0.31	0.30
15:1n-5	0.01	0.02	0.01	0.00	0.00
16:0iso	0.05	0.05	0.04	0.05	0.05
16:0	23.76	24.82	27.00	26.45	28.13
16:1n-7	1.19	1.32	1.38	2.10	2.16
Met 16:0	0.13	0.15	0.17	0.22	0.25
16:1n-5	0.26	0.29	0.26	0.25	0.28
16:2n-6	0.55	0.52	0.53	0.47	0.34
16:2n-4	0.64	0.70	0.74	0.70	0.72
17:0	0.29	0.32	0.27	0.24	0.22
16:3n-4	0.06	0.01	0.01	0.01	0.02
16:3n-3	0.36	0.11	0.13	0.16	0.19
16:3n-1	0.45	0.67	0.62	0.55	0.49
16:4n-3	0.21	0.38	0.36	0.19	0.19
16:4n-1	0.13	0.12	0.11	0.13	0.13
18:0	9.30	7.09	7.49	6.32	5.52
18:1n-9	6.80	13.22	11.65	7.69	7.66
18:1n-7	1.19	2.29	2.34	3.08	3.58

18:1n-5	0.16	0.20	0.20	0.20	0.20
18:2n-9	0.13	0.14	0.12	0.06	0.08
18:2n-6	7.19	4.27	1.75	0.88	0.96
18:2n-4	0.04	0.03	0.03	0.03	0.03
18: 3n-6	0.05	0.10	0.07	0.06	0.06
18:3n-4	0.10	0.07	0.08	0.07	0.03
18:3n-3	0.40	0.20	0.08	0.12	0.13
18:4n-3	0.05	0.04	0.04	0.08	0.09
18:4n-1	0.01	0.01	0.00	0.01	0.02
20:0	0.17	0.15	0.15	0.10	0.08
20:1n-9	1.15	0.94	0.82	0.73	0.70
20: 1n-5	0.13	0.13	0.13	0.15	0.16
20: 2n-9	0.03	0.03	0.03	0.02	0.02
20:2n-6	0.55	0.38	0.22	0.16	0.14
20:3n-6	0.10	0.09	0.07	0.08	0.09
20:4n-6	1.63	1.52	1.60	1.67	1.51
20: 3n-3	0.19	0.15	0.14	0.13	0.11
20:4n-3	0.16	0.12	0.12	0.17	0.19
20:5n-3	5.87	6.87	8.99	12.52	14.85
22:1n-11	0.10	0.07	0.06	0.06	0.14
22: 1n-9	0.05	0.06	0.03	0.03	0.04
22:4n-6	0.08	0.08	0.11	0.16	0.19
22:5n-6	0.38	0.33	0.29	0.30	0.21
22:5n-3	1.41	1.18	1.03	1.19	1.11
22:6n-3	29.50	29.71	29.47	30.98±0.52	28.43
Total saturated	57.86±1.48^{b}	57.87±1.35^{b}	62.77±4.00^{a}	60.62±1.15^{ab}	63.23±1.73^{a}
Total Monounsaturated	11.0+1.12^{c}	19.0+1.09^{a}	17.09+1.98^{ab}	14.19+1.09^{b}	15.1+1.08^{b}
Total n-3	38.15±0.44^{c}	38.7±1.12^{c}	40.36±0.57^{b}	45.55±0.53^{a}	44.28±1.12^{a}
Total n-6	10.53±0.04^{a}	7.28±0.22^{b}	4.65±0.33^{c}	3.78±0.07^{d}	3.51±0.14^{d}
Total n-9	7.03±0.88^{c}	13.45±0.21^{a}	11.84±1.23^{ab}	7.81±0.25^{bc}	7.81±0.41^{bc}
Total n-3HUFA	37.13±0.31^{c}	38.04±1.14^{c}	39.75±0.55^{b}	45±0.54^{a}	43.69±1.13^{a}
ARA	1.63±0.07^{a}	1.52±0.07^{a}	1.6±0.11^{a}	1.67±0.11^{a}	1.51±0.14^{a}
EPA	5.87±0.21^{e}	6.87±0.49^{d}	8.99±0.29^{c}	12.52±0.32^{b}	14.85±0.38^{a}
DHA	29.5±0.52^{a}	29.71±1.61^{a}	29.47±0.25^{a}	30.98±0.52^{a}	28.43±1.23^{a}
EPA/ARA	3.62±0.29^{d}	4.56±0.53^{cd}	5.64±0.33^{c}	7.54±0.65^{b}	9.89±0.98^{a}
DHA/EPA	5.04±0.26^{a}	4.36±0.54^{b}	3.28±0.08^{c}	2.48±0.09^{d}	1.85±0.12^{e}
DHA/ARA	18.13±0.51^{a}	19.58±0.15^{a}	18.49±1.21^{a}	18.64±0.93^{a}	18.2±0.78^{a}
n-3/n-6	3.62±0.03^{e}	5.33±0.31^{d}	8.71±0.51^{c}	12.05±0.25^{b}	12.64±0.24^{a}

Discussion

Complete replacement of both rotifers and *Artemia* with the experimental microdiets for gilthead seabream larvae produced high survival and growth rates, particularly in fish fed the two highest levels of krill PL. High survival and fast growth rates are required to be able to determine optimum ingredient contents and nutrient requirements in fish diets. In the present study, final average survival (48%) and total length (8.6 mm) in seabream fed diets containing 12% and 17.5% krill PL was higher than the best obtained by other authors (30-38% survival) feeding only microdiets for a similar period in this (Seiliez *et al*., 2006) or other species (Cahu *et al*., 1998; Zambonino-Infante and Cahu 1999). Moreover, it was even higher than that obtained feeding live preys under similar experimental conditions (41%) (Salhi *et al*., 1997) or in commercial hatcheries (about 20 %) (Roo *et al*. 2005). However, a much higher survival (73%) was obtained by Cahu and coworkers (2003a) feeding high PL microdiets to European seabass larvae (*Dicentrarchus labrax*), a species with a faster development of the digestive system than gilthead seabream.

Fatty acid composition in larvae reflected that of corresponding diet, where the supplimentation of dietary PL led to an increase in larval n-3 HUFA fatty acids. Best results of growth and survival in 12 KPL and 17.5 KPL larvae could hence be due to higher levels of PUFA which are essential for marine fish larvae, particularly EPA and DHA, which are increased with Krill PL inclusion in the diets. An increase in both dietary DHA and EPA improves larval performance, in terms of survival, stress resistance and growth (Liu *et al*., 2002; Izquierdo *et al*., 2005). Moreover, a higher n-3/n-6 fatty acid ratio was observed in the 12 KPL and 17.5 KPL diets, which resulted in the best growth and survival in those groups. This was also observed by (Izquierdo *et al*., 2000, 2001; Caballero *et al*., 2002; Caballero *et al*., 2006a) who showed that a well-balanced n-3/n-6 fatty acid ratio in the diet improved lipid metabolism such as digestion, absorption and transport.

Despite the large amount of bibliography regarding the effect of dietary PL, few studies have been conducted to determine optimum dietary levels with a wide range (≥ 5) of dietary PL contents. In the present study, increase in dietary krill PL up to 12% (about 10% total PL in diet) significantly improved larval survival and promoted growth, leading to high final total length and body weight, whereas further increase

did not affected those parameters. These levels are lower than those reccommended for European seabass (12% total PL) (Cahu *et al*., 2003a) for maximum survival and growth when soybean lecithin was used as a source of PL. These results can be related to a higher effectiveness of marine PL in the present study in comparison to soybean lecithin (Salhi *et al*., 1999; Izquierdo *et al*., 2001; Wold *et al*., 2007), or to different requirements among species, since they are higher than those recommended for larval rock bream (*Oplegnathus fasciatus*) (5% total PL) (Kanazawa *et al*., 1983a). Larval growth and survival have been found to be increased by dietary PL in several fish species (Kanazawa *et al*., 1983a; Izquierdo *et al*., 2001; Cahu *et al.*, 2003a; Gisbert *et al*., 2005). On the contrary, survival was not improved by elevation of dietary PL in other species such as pikeperch (*Sander lucioperca*) larvae (Hamza *et al*., 2008). The promoting effect of PL on larval growth is probably related to their importance as components of biomembranes (Tocher, 2003) and it has been mainly associated with their content in PC (Takeuchi *et al*., 1992; Kanazawa, 1993), the main PL class in cell membrane. In relation to improved growth, other authors also found an increase in feed intake that has been associated to the stimulation of gustatory response by the trimethyl group of the choline base of PC (Izquierdo and Koven, 2010). However, in the present study no differences were found in feed intake and all diets were supplemented with the same amount of choline in the vitamin mixture.

The growth improvement obtained with increased dietary PL contents could be at least partly related to the higher maturation of the gut suggested by the significant increase in alkaline phosphatase activity. This enzyme, an important component of brush-border membrane enzymes, increases during development of marine fish larvae in association to enterocyte development and denoting intestine maturation In the present study, increased dietary krill PL up to 12% (about 10% total PL) significantly increased alkaline phosphatase activity, whereas higher PL levels did not further enhanced it. Similar PL values (9%) were required to increase alkaline phosphatase activity in European seabass when soybean lecithin was used as the PL source (Cahu *et al*., 2003) and cod larvae (Wold *et al*., 2007). Enhanced enterocyte maturation by PL could be related with to a higher intracellular availability of PL for cell membrane and cell organules formation, since dietary PL promotes re-acylation of digested lipids increasing intracellular PL availability for lipoprotein synthesis in gilthead seabream (Caballero *et al*., 2003; Liu *et al*., 2002). In fact, membrane cell organelles

such as mitochondria have been found to be affected by dietary PL (Macqueen Leifson *et al*., 2003).

On the contrary to the saturation effect of dietary PL levels on alkaline phosphatase activity, there was a linear substrate stimulatory effect of dietary PL on PLA2 activity. PLA2 activity has been found in larvae of several species such as striped bass (*Morone saxatilis*) (Ozkizilcik *et al*., 1996) or European seabass (*Dicentrarchus labrax*) (Cahu and Zambonino-Infante, 2001). The steady increase in PLA2 activity by dietary PL is in agreement with the transcriptional regulation of PLA2 by dietary PL found in European seabass (Zambonino-Infante and Cahu, 1999). These authors also suggested a post-transcriptional regulation of endocrine origin. In concordance with this hypothesis, in the present study, the increase trypsine and lipase activity by elevation of krill dietary contents up to 6%, regardless dietary protein and lipid content, denotes a general effect of dietary PL on pancreatic enzymes. Endocrine modulation of the pancreatic digestive function in fish is regulated by cholecystokinine (CCK) whose secretion in turn is stimulated by the presence of several nutrients such as free amino acids (Rojas-García and Rønnestad, 2002). However, free fatty acids, and particularly n-3 polyunsaturated fatty acids, are also potent stimulators of CCK secretion (Little *et al*., 2007) by mediation of the G-protein-coupled receptor GPR40 (Liou *et al*. in press). In the present study, increased PLA2 activity (as well as lipase activity) by elevation of dietary PL would be responsible for a more effective digestion of dietary lipids and, therefore, higher concentrations of free polyunsaturated fatty acids in intestine lumen that could stimulate CCK secretion as it occurs in mammals. Moreover, increase in dietary PL increased the polyunsaturated fatty acid content in biomembranes of larval tissues, as denoted by the fatty acid composition of larval PL, could modulate CCK function responsible for the higher pancreatic enzymes activity. In mammals, fatty acid composition of cell membranes has been found to be determinant for specific recognition of CCK receptor (Romano *et al*., 2003) and modulation of CCK function in pancreatic tissue (Chang *et al*., 1984). In the present study, increasing dietary PL lead to better assimilation of n-3HUFA especially EPA, reflected in the higher content of these fatty acids in both neutral and polar lipids of the larvae. This could be partly due to a better digestion efficiency of dietary lipids by enhanced PLA2 and lipase activity, but also to an improvement in lipid transport. Izquierdo *et al*. (2001) found a

better incorporation of fatty acids from dietary polar lipids in larval seabream fed dietary PL in comparison to larvae fed triglycerides.

In summary, the results of the present study have shown that dietary krill PL are an excellent source of lipids for gilthead seabream larvae. Optimum inclusion levels of this ingredient in microdiets to completely substitute live preys at this larval age were found to be 12% krill PL, providing about 10% total PL. Lower levels of this ingredient markedly reduced culture performance, gut development, digestive enzymes acitivity, dietary lipid utilization, growth, survival and stress resistance in seabream larvae.Further studies are required to determine the optimum content of other phospholipid sources such as soyben lecithin in early weanning diets for gilthead seabream.

Acknowledgements

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Chapter 4

Optimum soybean lecithin contents in microdiets for gilthead seabream (*Sparus aurata*) larvae

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Abstract

The aim of the present study was to determine the optimum dietary levels of soybean lecithin (SBL) for seabream (*Sparus aurata*) larvae, and its influence on production performance and digestive enzymes activity. Larvae were fed five formulated microdiets with five levels of SBL. Complete replacement of live preys with the experimental microdiets for seabream larvae at 16 dph produced over 55% survival rates, particularly in fish fed the highest levels of SBL. Moreover, increase in dietary SBL up to 80 g kg^{-1} significantly improved larval growth, leading to high final total length and body weight. An increase in alkaline phosphatase activity with the elevation up to 80 g kg^{-1} SBL was also found denoting a better maturation of the digestive system. Besides, there was a stimulatory effect of dietary SBL on PLA2 activity. Finally, increasing dietary SBL lead to better utilization of dietary HUFA, as it was reflected in their higher content in both neutral and polar lipid of the larvae. In summary, elevation of dietary SBL up to 80 g kg^{-1} in microdiets for seabream significantly improved digestive enzymes activities, enterocyte maturation, utilization and deposition of dietary essential fatty acids and larval growth, as a consequence of a better digestion, absorption, transport and deposition of dietary nutrients.

Introduction

Phospholipids (PL) are particularly important in fish larvae production for their essential function as necessary components for cellular bio-membranes and organelles formation, as well as for being an endogenous energy source during early development (Izquierdo and Koven, 2011). Indeed, young fish contain abundant PL received during embryo and larval development either from endogenous yolk sac or exogenous live preys lipids (Rainuzzo *et al.,* 1997; van der Meeren *et al.,* 2008). Studies on dietary PL were initiated by Kanazawa *et al*. (1981, 1983a, b), who showed that despite their significant roles in fish metabolism, developing fish possess a limited ability for phospholipids synthesis (Kanazawa, 1985) and, consequently, they must be included in young fish diets.

Thus, PL inclusion in young fish diets has been found to enhance growth, survival and stress resistance, and reduce the incidence of morphological alterations in fish larvae (Takeuchi *et al.,* 1992; Kanazawa, 1993; Salhi *et al.,* 1999; Izquierdo *et al.,* 2001; Cahu *et al*., 2003a; Kjørsvik *et al.,* 2009). PL are necessary as emulsifiers in the formation of mixed micelles in the digestive tract (Olsen and Ringø, 1997) and appear to be crucial for lipid transport (Fontagné *et al.,* 1998; Salhi *et al.,* 1999; Caballero *et al.,* 2006a).

At hatching, the larval digestive system is not completely developed, lacks a functional stomach and main digestion of ingested food occurs in the intestine, where the pH remains alkaline and trypsin-like enzyme activity accounts for the proteolytic activity (Walford and Lam, 1993; Morais *et al*., 2005). Lipolytic activity in fish larvae is leaded by a nonspecific neutral lipase dependent on the presence of bile salts (bile salt activated lipase, BAL) (Iijima *et al.,* 1998; Izquierdo and Henderson, 1998), whereas PL are specifically digested by the intestinal phospholipase A2 (PLA2) secreted by the pancreas (Sargent *et al.,* 1989). The activity of all these luminal enzymes tends to increase with gut development (Moyano *et al.,* 1996; Izquierdo *et al.,* 2000; Zambonino-Infante and Cahu, 2001; Lazo *et al.,* 2007). Intestinal brush border membrane enzymes such as alkaline phosphatases are lowest at first feeding and subsequently increase with gut development (Cahu and Zambonino-Infante, 1995b; Lazo *et al.,* 2007). Nevertheless, during early development certain dietary ingredients, such as PL may promote digestive enzymes activities and induce

digestive system maturation (Zambonino-Infante and Cahu, 2001), playing an important role on larval digestive physiology and the metabolic pathways of the assimilated nutrients (Kolkovski *et al.,* 2009; Morais *et al.,* 2006). PL have been also found to be required for the formation of very low-density lipoproteins during the intestinal absorption of lipids (Salhi *et al*., 1999; Liu *et al*., 2002; Caballero *et al.,* 2006b). Feeding high lipid diets low in PL causes the accumulation of lipidic vacuoles in the basal zone of the enterocyte and esteatosis in the hepatic tissue, both of them being markedly reduced by a 2 % addition of soybean lecithin, denoting an enhancement in the lipid transport activity in gut and liver (Liu *et al*., 2002) and emphasizing the limited ability of fish larvae to synthesize PL. Moreover, this fact may reduce the absorption of different ingested nutrients from the lumen (Morais *et al*., 2006).

Therefore, dietary PL are required for the appropriate development and biological performance of young fish (Kanazawa, 1993). The quantitative requirements of PL for larval fish range from about 2–12% of diet, being lower in the larvae of common carp (*Cyprinus carpio*) (2% chicken egg) (Geurden *et al.,* 1995a) and in red seabream (*Pagrus major*) larvae was 5% SBL and knife jaw (*Oplegnathus fasciatus*) larvae (7.4 % SBL) (Kanazawa *et al.* 1983a) and Japanese flounder (*Paralichthys olivaceus*) larvae (7 % SBL) (Kanazawa 1993), with the highest reported value being for Gilthead seabream (*Sparus aurata*) (15 % SBL) (Seiliez *et al.,* 2006). However, for the later species, our recent studies have found an optimum inclusion of about 4% using krill PL as a PL source (Saleh *et al.,* 2012a).

Being gilthead seabream one of the most important species for Mediterranean aquaculture, it is necessary to quantify its optimum dietary PL requirements to optimize weaning diets, particularly since this species does not develop a functional stomach until 35-40 dah (Moyano *et al.,* 1996).

As soybean lecithin is a readily available and cost effective PL source in fish diets, the objective of the present study was to determine the optimum quantity of soybean lecithin required in weaning diets to support better growth, survival and development of gilthead seabream larvae.

Materials and methods

Gilthead seabream larvae were obtained from natural spawnings from Instituto Canario de Ciencias Marinas (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain). Larvae (initial total length 5.4±0.6 mm, mean±SD; dry body weight 120±37 µg) previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until they reached 15 day old, were randomly distributed in 15 experimental tanks at a density of 2100 larvae $tank^{-1}$ and fed one of the experimental diets tested in triplicate for 15 days. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 mg L^{-1} salinity) at an increasing rate of 0.4-1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality, which was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min^{-1}) attaining 6.0±1 ppm dissolved O^2. Average water temperature and pH along the trial were 21.0±1°C and 7.85±0.1, respectively. Photoperiod was kept at 12h light: 12h dark, by fluorescent daylights and the light intensity was 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Osborne Park City, Australia) at the water surface.

Five experimental microdiets (pellet size < 250 µm) with increasing phospholipid contents were formulated using sardine oil (Agramar S.A., Spain) and soybean lecithin oil (Acofarma, Barcelona, Spain) as sources of triglycerides and PL, respectively. Their formulation and proximate analysis are showed in Table 1. The fatty acids profiles are shown in Tables 2, 3 and 4. The desired lipid content (about 180 g kg^{-1}) was completed if necessary with a non-essential fatty acid source, oleic acid (Oleic acid vegeTable, Merck, Darmstadt, Germany). The microdiets were prepared by mixing squid meal and water-soluble components, and then the lipids and fat-soluble vitamins and, finally, gelatine dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain a particle size below 250 µm. Diets were prepared and analyzed for proximate and fatty acid composition (Tables 4.1, 4.2, 4.3 and 4.4) at GIA laboratories.

Table 4.1 Formulation and proximate composition of the experimental microdiets containing several levels of SBL

Ingredients (g kg^{-1} diet)	0 SBL	2 SBL	4 SBL	8 SBL	12 SBL
Squid meal[a]	756	755	755	755	715
Soybean lecithin[b]	0.0	20	40	80	120
Sardine oil[c]	79	40	20	0.0	0.0
Oleic acid[d]	0.0	20	20	0.0	0.0
Basal premix	165	165	165	165	165
Proximate analysis (g kg^{-1} diet)					
Lipid	180.2	175	179.2	183.4	200.2
Polar Lipids	49.4	56.5	65.4	88.1	95.9
Protein	712.2	710.3	714.3	720.4	671.3
Ash	68.2	68.8	68.7	70.3	70.5
Moisture	81.0	80.0	78.8	80.3	78.7

a Rieber and Son, Bergen, Norway.
b Acofarma, Barcelona, Spain.
c Agramar S.A., Las Palmas, Spain.
d Merck KGaA, Darmstadi, Germany.

Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00 for 16 days. Non-enriched rotifers were co-fed only during days 16th and 17th (1 rotifer ml^{-1}). To assure feed availability, daily feed supplied was maintained at 1.5 and 2.5 g L^{-1} $tank^{-1}$ during the first and second week of feeding, respectively. Larvae were observed under the binocular microscope to determine feed acceptance.

Before the end of the experiment an activity test was conducted by handling 20 larvae $tank^{-1}$ out of the water in a blotted 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 living larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 h) and total length (Profile Projector V-12A Nikon, Tokyo, Japan) of 30 fish $tank^{-1}$ at the beginning, in the middle and at the end of the trial. In addition, at the end of the trial and after 12 h of starvation, the all larvae in each tank were washed with distilled water, sampled and kept at -80 ºC for biochemical composition.

Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and crude lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for

the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC min^{-1} and then held at 215 ºC for 10 min. Fatty acid methyl esters were quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) following the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

For enzymes activity determination, the alkaline phosphatase, trypsine and lipase activities are expressed as relative fluorescence units (RFU) and PLA2 is expressed as units (U). The larvae were homogenized by a sonicator ultrasound (Misonix Microson XL2007 Ultrasonic Homogenizer) on ice in 110µl of high-purified water, centrifuged and the supernatant used as the sample stock solution.

The alkaline phosphatase activities were quantified by fluorometric assay using a spectrofluorometer (Thermolab Systems; Helsinki, Finland) at excitation wavelengths of 358 nm and emission wavelengths of 455nm. After preparation of 140 µl of reaction buffer at pH 10.4 (100 mM Glycine, 1 mM $MgCl_2$, 1mM $ZnCl_2$), and 50 µl of substrate stock solution (200 µM of 6,8 Difluoro-4 methyllumbelliferyl phosphate (DiFMUP) solution in DMSO., the reaction was started by adding 10 µl of the sample stock solution and the kinetic curves were recorded for 20 min (Gee *et al.,*1999).

Trypsine activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 380 nm and emission wavelengths of 440nm. After preparation of 195 µl of reaction buffer pH 8.0 (50mM Tris-HCl, 10 mM $CaCl_2$), and 5 µl of of substrate stock solution (20 µM of Boc-Gln-Ala-Arg-7 amido-4 methylcoumarin hydrochloride in DMSO), the reaction was started by adding 10 µl of the sample stock solution and the kinetic curves were recorded for 5 min (Rotllant *et al.,* 2008).

Neutral lipase activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 355 nm and emission wavelengths of 460nm. Firstly, 247.4 µl of 0,1 M phosphate reaction buffer pH 7.0 (19.5 ml of 0.2M phosphate monobasic (NaH_2PO_4 1 H_2O) solution and 30.5ml of 0.2M Phosphate dibasic (NaH_2PO_4 7 H_2O) solution were mixed and completed up to 100 ml by adding 50 ml high-purified water), then 2.6µl of substrate stock solution (40 mM of 4Methylumbelliferol butyrate (MUB) in N,N Dimethyl formamide (DMSO) was added and then reaction was started by adding 10 µl of the sample stock solution. . Kinetics curves were recorded for 5 min (Rotllant *et al.,* 2008).

The PLA2 activities were quantified by fluorometric assay using spectrofluorometer at excitation wavelengths of 377 nm and emission wavelengths of 450nm. First 160 µl of reaction buffer pH 8 (50mM Tris-HCl,100mM NaCl, 2mM NaN_3, 5µg ml^{-1} bovine serum albumin, and 10 µm 1-anilinonaphthalene-8-sulfonate, and 20 µl of substrate stock solution (50 µl of 1,2-dimyristoyl-sn-glycero-3-phosphocholine solution in 40 mM, methanol mixed with 15 µl deoxycholic acid solution in 40 mM, methanol and quickly injected into 1ml high purified water, stirred for 1 min and sonicated for 2 min) and 10 µl of 100 mM $CaCl_2$ solution were incubated at 25 °C for 10 min. The reaction was started by adding 20 µl of the sample stock solution and the kinetic curves were recorded for 40 min (Huang *et al.,* 2006).

All data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation and were treated using one-way ANOVA. Means compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results

All the experimental diets were well accepted by the larvae as early as 16 days post hatching (dph) as observed in the microphotography. Regardless the diet fed survival was high 52-58 % (Figure 4.1) and did not significantly differed (P>0.05) among larvae fed different diets. Dietary treatments did neither affected survival 24 h after the handling test, being always over 88 % (Figure 4.2). Larval growth in terms of total length significantly (P>0.05) increased by the elevation of dietary SBL up to 80 g kg^{-1}, whereas further elevation of SBL dietary levels to 120 g kg^{-1} did not further improved larval growth (Figure 4.3). Similarly, elevation of SBL dietary contents up to 40-80 g kg^{-1} significantly (P>0.05) raised dry body weight, whereas elevation to 120 g kg^{-1} did not further increased weight (Figure 4.4).

In general digestive enzymes activity was increased by the elevation of dietary marine phospholipids. Thus, alkaline phosphatase was lowest in 0 SBL larvae and significantly increased with the elevation of dietary SBL up to 8 SBL and 12 SBL larvae, whereas trypsine activity was significantly lowest in both 0 SBL and 2 SBL larvae (Figure 4.5 and 4.6). Neutral lipase activity was lowest in 0 SBL 2 SBL larvae and significantly increased in 8 SBL and 12 SBL larvae (Figure 4.7). PLA2 activity

was significantly higher in larvae fed 12 SBL than in larvae fed 0 SBL, 2 SBL or 4 SBL, but did not differed from that of 8 SBL larvae (Figure 4.8).

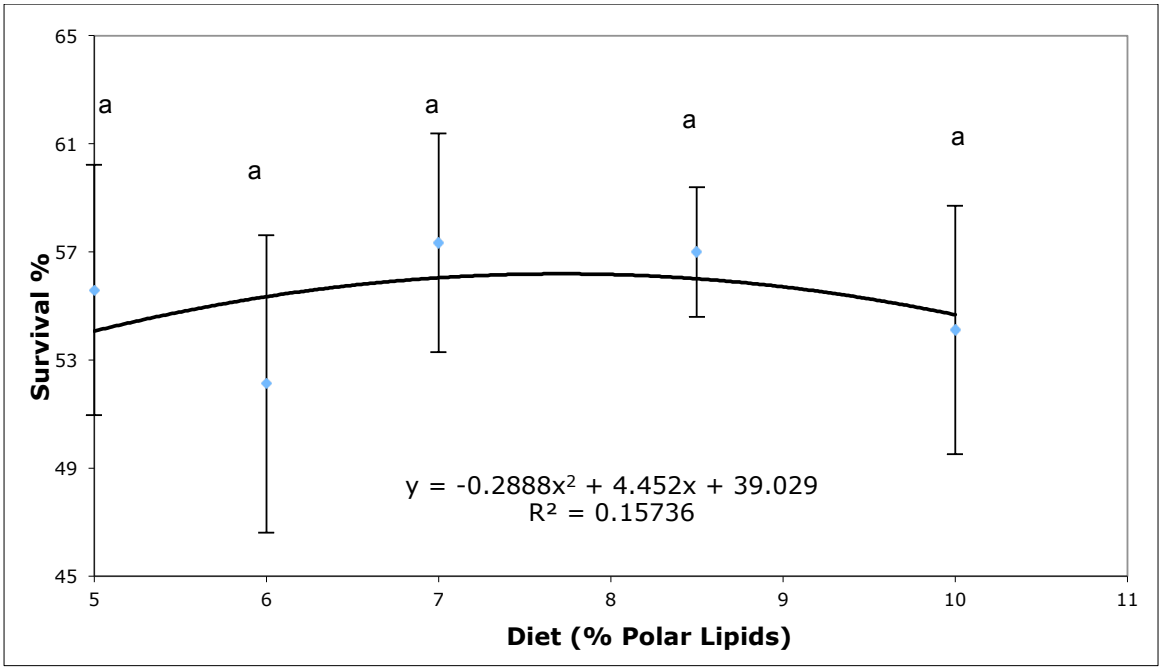

Figure 4.1. Survival rate (% of population) of larvae reared from 16 to 31 dph on five levels of SBL. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

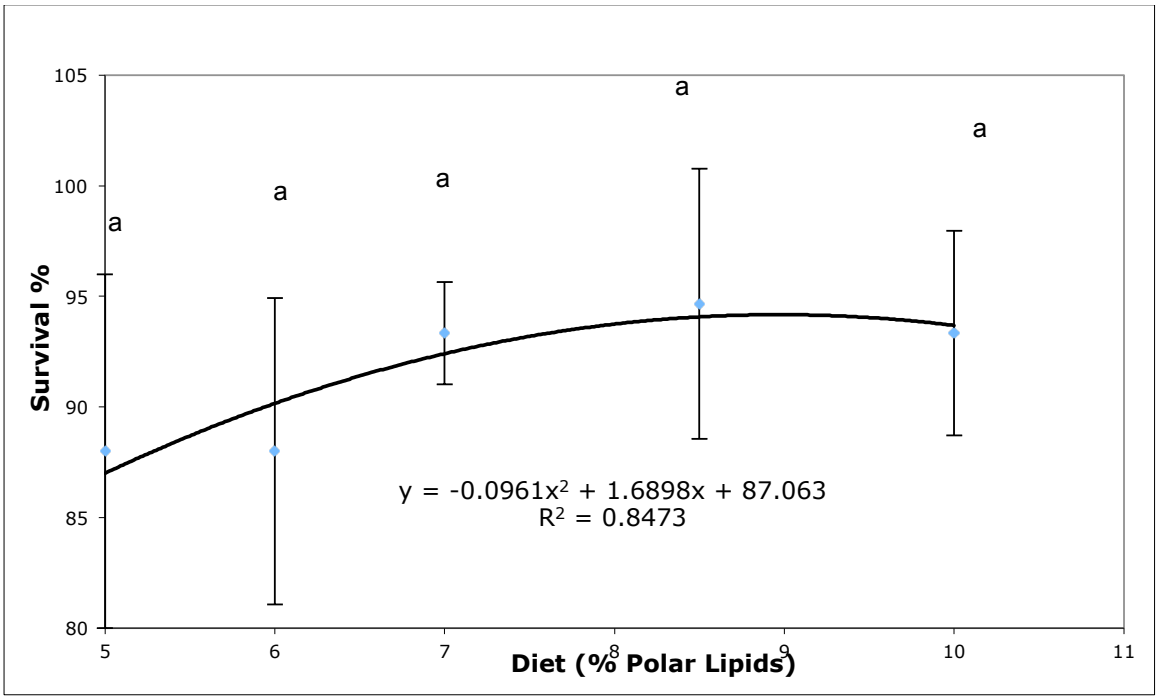

Figure 4.2 Survival 24 h after activity test of larvae (30 dph) fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

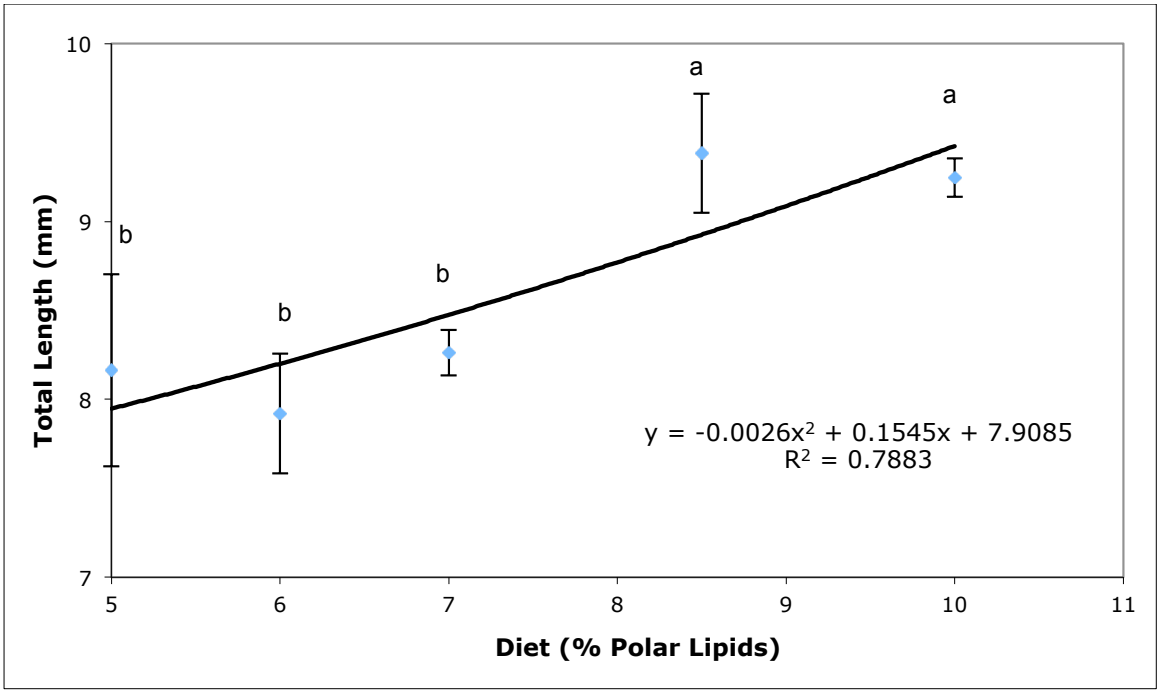

Figure 4.3 Total length of larvae (30 dph) fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

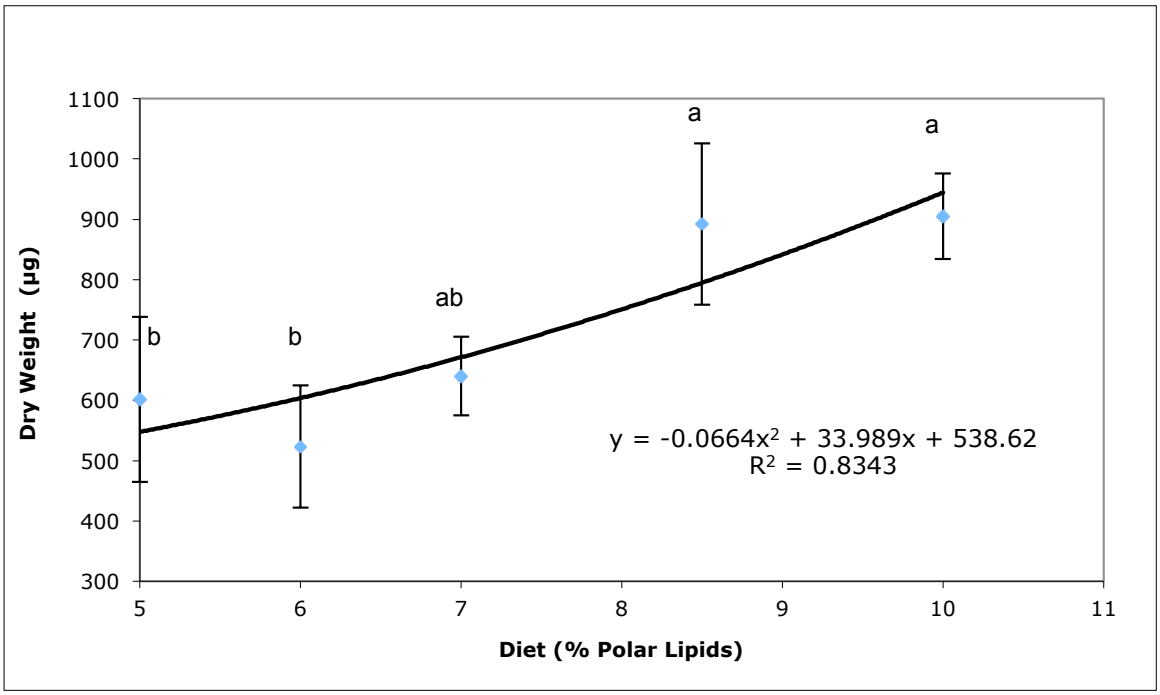

Figure 4.4 Dry body weight of larvae (30 dph) fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

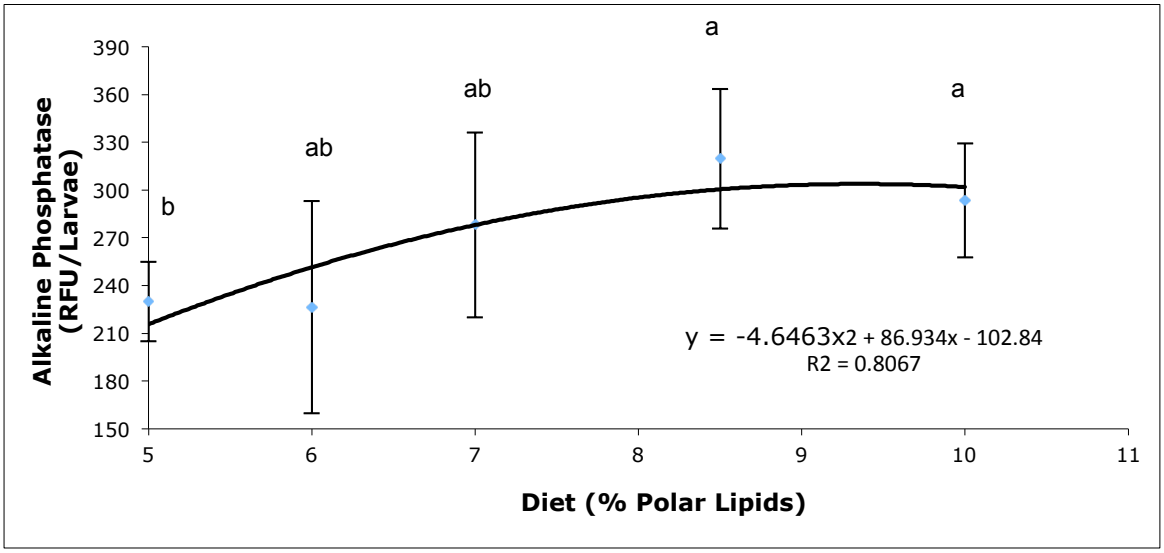

Figure 4.5 Alkaline phosphatase activity in seabream larvae (30 dph) fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

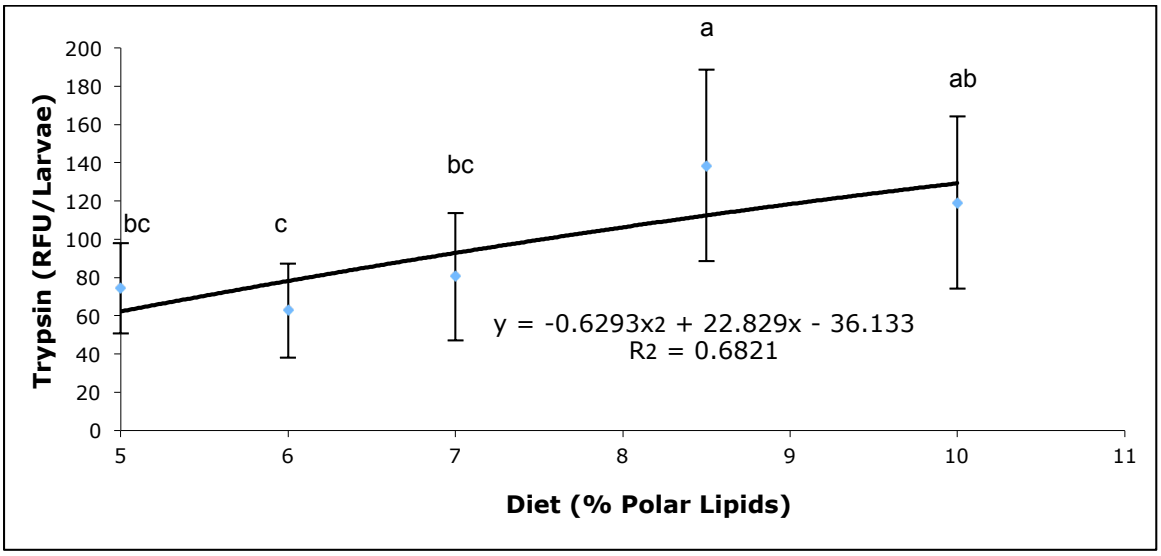

Figure 4.6 Trypsin activity in seabream larvae (30 dph) fed increased levels of SBL for 15 days. (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

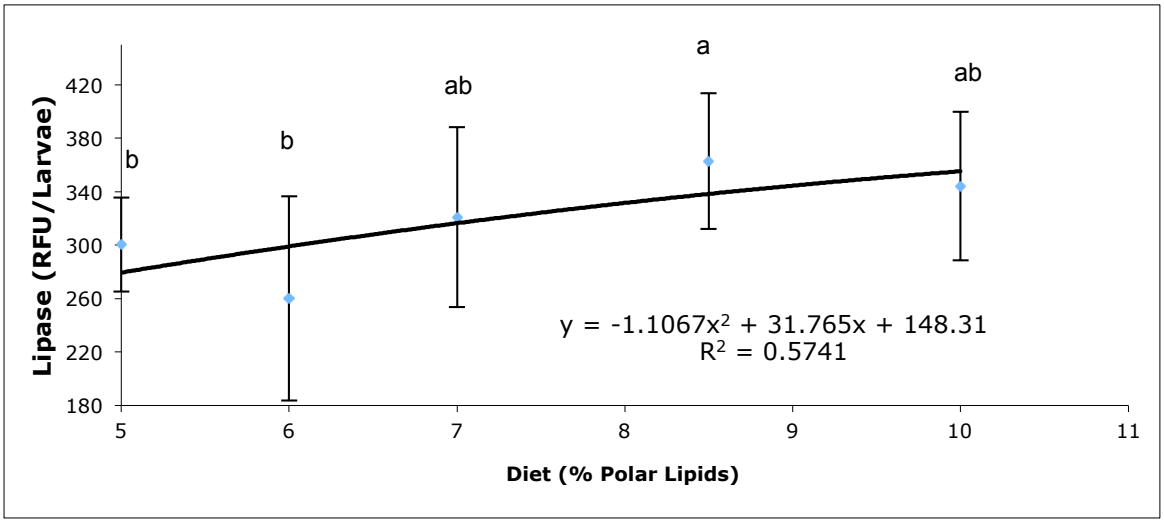

Figure 4.7 Lipase activity in seabream larvae (30 dph) fed increased levels of SBL for 15 days. (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

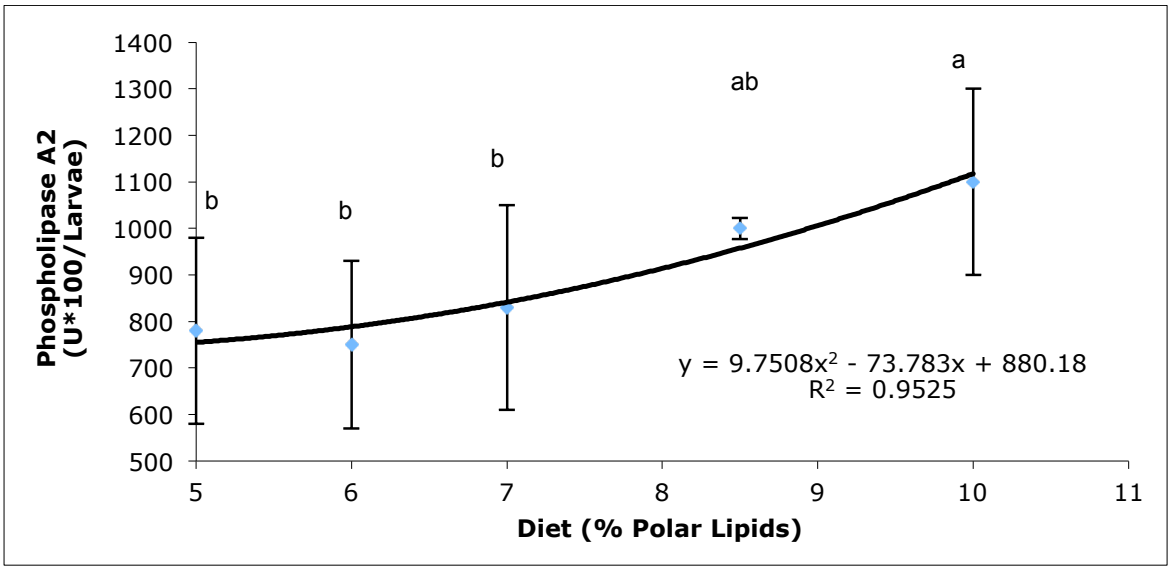

Figure 4.8 Phospholipase A2 activity in seabream larvae (30 dph) fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Fatty acid composition of total lipids, neutral lipids and polar lipids from the experimental microdiets showed that gradual inclusion of SBL lead to an increase in n-6 fatty acids, particularly 18:2n-6 fatty acid (Tables 4.2 and 4.3). The 12 SBL diet has the highest content of saturated fatty acids of total lipids and neutral lipids particularly 16:0 and 18:0 fatty acids, and has the lowest monounsaturated fatty acids particularly 18:1n-9 fatty acid. The diets 0 SBL, 2 SBL and 4 SBL have higher n-9 fatty acids of total lipids and neutral lipids compared to 8 SBL and 12 SBL. Fatty acid

composition of polar lipids from the microdiets showed a reduction of n-3 fatty acids, particularly DHA and EPA, by the elevation of SBL (Table 4.4).

Table 4.2 Fatty acids (% dry weight) composition in total lipids of five dietary SBL

	0 SBL	2 SBL	4 SBL	8 SBL	12 SBL
14:0	0.39	0.27	0.26	0.14	0.16
14:1n-5	0.00	0.00	0.00	0.00	0.00
14:1n-7	0.01	0.01	0.00	0.00	0.00
15:0	0.07	0.06	0.07	0.06	0.07
15:1n-5	0.00	0.00	0.00	0.00	0.00
16:0ISO	0.01	0.01	0.01	0.00	0.00
16:0	3.51	3.17	3.72	3.62	6.36
16:1n-7	0.39	0.24	0.18	0.09	0.03
Me 16:0	0.02	0.02	0.02	0.02	0.01
16:1n-5	0.03	0.02	0.02	0.02	0.02
16:2n-6	0.04	0.03	0.02	0.01	0.00
16:2n-4	0.08	0.07	0.08	0.07	0.12
17:0	0.04	0.03	0.03	0.02	0.01
16:3n-4	0.00	0.00	0.00	0.00	0.00
16:3n-3	0.03	0.02	0.02	0.02	0.02
16:3n-1	0.05	0.05	0.00	0.06	0.07
16:4n-3	0.02	0.01	0.06	0.01	0.00
18:0	0.70	0.63	0.89	0.71	1.36
18:1n-9	3.18	4.93	5.17	1.92	1.81
18:1n-7	0.34	0.00	0.00	0.24	0.22
18:1n-5	0.04	0.02	0.03	0.03	0.03
18:2n-6	3.37	3.16	3.75	5.21	6.80
18:2n-4	0.02	0.01	0.01	0.00	0.01
18:3n-6	0.01	0.01	0.00	0.00	0.01
18:3n-4	0.01	0.01	0.01	0.01	0.01
18:3n-3	0.48	0.34	0.33	0.53	0.54
18:4n-3	0.05	0.03	0.02	0.01	0.01
18:4n-1	0.01	0.01	0.00	0.00	0.00
20:0	0.03	0.03	0.03	0.03	0.06
20:1n9+n7	0.81	0.58	0.56	0.43	0.28
20:1n-5	0.03	0.02	0.02	0.01	0.01
20:2n-9	0.01	0.00	0.00	0.00	0.00
20:2n-6	0.07	0.05	0.04	0.03	0.02
20:3n-6	0.01	0.01	0.01	0.00	0.00
20:4n-6	0.12	0.11	0.10	0.13	0.09
20:4n-6	0.12	0.11	0.10	0.13	0.09
20:3n-3	0.08	0.06	0.06	0.07	0.05
20:4n-3	0.07	0.04	0.02	0.01	0.01
20:5n-3	1.00	0.88	0.66	0.78	0.81
22:1n-11	0.40	0.20	0.11	1.21	0.10
22:1n-9	0.00	0.01	0.02	0.01	0.00
22:4n-6	0.03	0.02	0.01	0.01	0.01

22:5n-6	0.03	0.02	0.02	0.02	0.02
22:5n-3	0.15	0.09	0.05	0.03	0.03
22:6n-3	2.26	2.22	1.49	1.94	2.37
Total Saturated	8.20	7.29	8.65	8.21	14.31
Total Monounsaturated	5.23	6.02	6.11	2.77	2.50
Total n-3	4.13	3.70	2.72	3.42	3.83
Total n-6	3.68	3.41	3.94	5.43	7.01
Total n-9	3.19	4.93	5.19	1.93	1.81
Total n-3HUFA	3.56	3.30	2.29	2.85	3.26
EPA/ARA	8.28	8.07	6.87	5.90	9.24
ARA/EPA	0.12	0.12	0.15	0.17	0.11
DHA/EPA	2.26	2.51	2.26	2.50	2.93
DHA/ARA	18.68	20.23	15.54	14.75	27.11
n3/n6	1.12	1.09	0.69	0.63	0.55

Table 4.3 Fatty acids (% dry weight) composition in neutral lipids of five dietary SBL

	0SBL	2 SBL	4 SBL	8 SBL	12 SBL
14:0	4.7	3.2	2.4	1.5	1.3
14:1n-7	0.1	0.1	0	0	0
15:0	0.6	0.6	0.6	0.9	0.6
15:1n-5	0	0.1	0.1	0.1	0.1
16:0iso	0.1	0.1	0.1	0.1	0.1
16:0	26.9	22.9	22.3	32.1	31.6
16:1n-7	5.4	3.2	2.4	0.9	1.1
Met 16:0	0.2	0.2	0.2	0.2	0.2
16:1n-5	0.1	0.2	0.2	0.2	0.2
16:2n-6	0.6	0.3	0.1	0.1	0.1
16:2n-4	0.6	0.7	0.6	0.8	1
17:0	0.5	0.4	0.3	0.3	0.3
16:3n-3	0.1	0.1	0.1	0.1	0.1
16:4n-3	0.1	0.1	0	0.1	0.2
18:0	8	3.3	7	10	5
18:1n-9	48.3	73.9	77.7	38.4	43.5
18:1n-7	3.6	0	0	2.5	0
18:1n-5	0.4	0.1	0.1	0.3	0.1
18:2n-6	47.3	40.6	44.1	71.6	85.8
18:2n-4	0.2	0.2	0.1	0.3	0.8
18: 3n-6	0	0.4	0.2	0.2	0.2
18:3n-4	0.1	0.1	0.1	0.1	0.1
18:3n-3	5.4	4.2	4	6.4	8.7
18:4n-3	0.4	0.4	0.3	0.1	0.2
18:4n-1	0.1	0.1	0.1	1	0.9
20:0	0.7	0.6	0.7	3	2.4
20:1n-9	7.6	4.7	3.9	0.1	0.1
20:1n-5	0.3	0.2	0.2	0	0

20:2n-6	0.7	0.4	0.3	0.3	0.3
20:3n-6	0.2	0.4	0.4	0.5	0.6
20:4 n-6	0.4	0.3	0.3	0.3	0.3
20:3n-3	0.4	0.3	0.3	0.4	0.3
20:4 n-3	0.6	0.4	0.3	0	0.1
20:5n-3	2.8	2.8	2.4	2.5	3.1
22:1n-11	6.5	3.4	1.8	0.3	0.2
22:1n-9	0.1	0.1	0.5	0.5	0.4
22:4n-6	0.1	0.1	0.2	0.3	0.4
22:5n-6	0.1	0.1	0	0	0
22:5n-3	0.9	0.6	0.4	0.1	0.1
22:6n-3	4.8	5.1	4.6	6.5	9.7
Total Saturated	67.8	53.2	55	78.9	72.1
Total Monounsaturated	72.5	85.9	86.4	42.9	45.2
Total n-3	15.5	14	12.4	16.2	22.5
Total n-6	49.3	42.6	45.6	73.5	87.8
Total n-9	48.5	74	78.2	38.9	43.9
Total n-3HUFA	9.4	9.2	8	9.5	13.4
EPA/ARA	7.29	9.03	9.16	7.43	8.91
ARA/EPA	0.14	0.11	0.11	0.13	0.11
DHA/EPA	1.70	1.84	1.91	2.58	3.13
DHA/ARA	12.36	16.65	17.52	19.16	27.86
n3/n6	0.31	0.33	0.27	0.22	0.26

Table 4.4 Fatty acids (% dry weight) composition in polar lipids of five dietary SBL

	0SBL	2 SBL	4 SBL	8 SBL	12 SBL
14:0	0.46	0.71	0.33	0.27	0.23
14:1n-5	0.01	0.01	0.01	0.00	0.00
14:1n-7	0.01	0.03	0.01	0.00	0.00
15:0	0.18	0.31	0.14	0.12	0.11
15:1n-5	0.00	0.00	0.00	0.00	0.01
16:0iso	0.02	0.03	0.01	0.01	0.01
16:0	7.56	6.48	6.76	6.22	6.40
16:1n-7	0.15	0.16	0.11	0.14	0.13
Met 16:0	0.04	0.02	0.03	0.03	0.03
16:1n-5	0.06	0.08	0.05	0.04	0.04
16:2n-6	0.01	0.06	0.04	0.03	0.02
16:2n-4	0.21	0.42	0.19	0.14	0.13
17:0	0.04	0.05	0.03	0.03	0.03
16:3n-4	0.00	0.00	0.00	0.00	0.00
16:3n-3	0.06	0.09	0.04	0.04	0.03
16:3n-1	0.00	0.00	0.00	0.00	0.08
16:4n-3	0.13	0.22	0.11	0.09	0.01
16:4n-1	0.00	0.03	0.02	0.00	0.00
18:0	1.10	2.15	1.08	1.01	1.01
18:1n-9	0.68	1.54	0.99	1.27	1.56
18:1n-7	0.31	0.50	0.27	0.29	0.32

18:1n-5	0.06	0.09	0.04	0.04	0.04
18:2n-9	0.01	0.01	0.00	0.00	0.00
18:2n-6	0.24	2.22	2.28	3.82	5.27
18:2n-4	0.01	0.01	0.00	0.01	0.02
18:3n-6	0.02	0.10	0.01	0.00	0.01
18:3n-4	0.02	0.04	0.02	0.02	0.02
18:3n-3	0.03	0.17	0.18	0.28	0.39
18:4n-3	0.01	0.01	0.01	0.01	0.01
18:4n-1	0.03	0.01	0.00	0.00	0.02
20:0	0.02	0.15	0.07	0.03	0.03
20:1n-9	1.02	1.55	0.75	0.65	0.57
20:1n-5	0.03	0.04	0.02	0.02	0.02
20:2n-9	0.01	0.01	0.00	0.00	0.00
20:2n-6	0.07	0.10	0.05	0.05	0.04
20:3n-6	0.00	0.07	0.03	0.01	0.01
20:4n-6	0.22	0.29	0.18	0.15	0.13
20:3n-3	0.12	0.17	0.09	0.08	0.07
20:4n-3	0.02	0.02	0.01	0.01	0.01
20:5n-3	1.36	1.68	1.12	0.93	0.85
22:1n-11	0.12	0.18	0.01	0.03	0.07
22:1n-9	0.03	0.05	0.09	0.08	0.01
22:4n-6	0.01	0.02	0.01	0.01	0.01
22:5n-6	0.05	0.05	0.04	0.03	0.03
22:5n-3	0.05	0.06	0.04	0.03	0.03
22:6n-3	3.49	3.95	2.64	2.30	2.23
Total Saturated	16.73	15.93	15.04	13.78	14.10
Total Monounsaturated	2.44	4.19	2.26	2.49	2.76
Total n-3	5.25	6.38	4.25	3.77	3.62
Total n-6	0.61	2.93	2.64	4.11	5.52
Total n-9	0.72	1.61	1.09	1.34	1.58
Total n-3HUFA	5.03	5.88	3.91	3.35	3.18
EPA/ARA	6.20	5.79	6.29	6.36	6.60
ARA/EPA	0.16	0.17	0.16	0.16	0.15
DHA/EPA	2.57	2.34	2.36	2.46	2.64
DHA/ARA	15.96	13.57	14.85	15.67	17.41
n3/n6	8.58	2.17	1.61	0.92	0.66

Analysis of fatty acids composition of the larvae reflected the fatty acids composition of the diets. It had shown significant difference between the five dietary SBL treatments in both neutral and polar lipids (Tables 4.5 and 4.6). There are significant difference ($P<0.05$) between the 12 SBL and 0 SBL treatments in total n-3, n-6 and n-3 HUFA particularly DHA content in both neutral and polar lipids where the larvae fed 0 SBL treatment has the highest total n-3 and n-3 HUFA particularly DHA, while the larvae fed diet 12 SBL has the highest total n-6, particularly 18:2n-6. Also the

larvae fed 2, 4 and 8 SBL diets have higher total n-3 and n-3HUFA, DHA and EPA content of neutral and polar lipid than larvae fed 12 SBL, but the last one has the highest total n-6 fatty acids particularly linoleic acid content in both neutral and polar lipids. It was not found significant difference (*P*>0.05) in EPA content between all of them in both neutral and polar lipids.

Table 4.5 Fatty acids (% total area) composition in neutral lipids of larvae fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	0SBL	2 SBL	4 SBL	8 SBL	12 SBL
14:0	1.71	1.29	1.20	1.10	0.97
14:01	0.00	0.00	0.00	0.00	0.00
14:1n-5	0.02	0.02	0.02	0.01	0.02
14:1n-7	0.11	0.12	0.09	0.08	0.10
15:0	0.39	0.38	0.33	0.39	0.39
15:1n-5	0.02	0.04	0.04	0.04	0.07
16:0iso	0.06	0.05	0.05	0.04	0.03
16:0	14.57	14.54	14.26	15.85	16.16
16:1n-9	0.00	0.00	0.00	0.00	0.00
16:1n-7	2.32	1.69	1.55	1.39	1.03
Met 16:0	0.13	0.11	0.11	0.10	0.09
16:1n-5	0.26	0.26	0.22	0.23	0.18
16:2n-6	0.62	0.56	0.54	0.54	0.43
16:2n-4	0.69	0.70	0.65	0.72	0.63
16:2n-3	0.00	0.00	0.00	0.00	0.00
17:0	0.28	0.26	0.21	0.20	0.14
16:3n-4	0.02	0.01	0.00	0.01	0.00
16:3n-3	0.14	0.12	0.11	0.11	0.09
16:3n-1	0.05	0.03	0.03	0.03	0.04
16:4n-3	0.06	0.01	0.00	0.00	0.00
16:4n-1	0.00	0.00	0.00	0.00	0.00
18:0	6.42	6.46	6.23	7.14	7.13
18:1n-9	16.58	21.13	23.25	14.90	13.57
18:1n-7	2.33	1.99	1.62	1.83	1.64
18:1n-5	0.34	0.34	0.33	0.32	0.28
18:2n-9	0.08	0.07	0.05	0.00	0.00
18:2n-6	15.63	18.36	17.49	23.43	31.09
18:2n-4	0.09	0.06	0.05	0.04	0.02
18:3n-9	0.00	0.00	0.00	0.00	0.00
18:3n-6	0.22	0.20	0.20	0.17	0.12
18:4n-6	0.00	0.00	0.00	0.00	0.00
18:3n-4	0.11	0.00	0.05	0.08	0.07
18:3n-3	1.95	1.78	1.65	2.14	2.80
18:3n-1	0.00	0.05	0.00	0.00	0.03

18:3n-1	0.00	0.05	0.00	0.00	0.03
18:4n-3	0.27	0.15	0.13	0.09	0.02
18:4n-1	0.05	0.02	0.02	0.01	0.00
20:0	0.39	0.34	0.33	0.33	0.32
20:1n-9	4.24	3.28	3.01	2.74	2.13
20:1n-7	0.00	0.00	0.00	0.00	0.00
20:1n-5	0.33	0.27	0.24	0.23	0.18
20:2n-9	0.05	0.06	0.04	0.04	0.02
20:2n-6	0.74	0.75	0.65	0.91	0.92
20:3n-9+n-	0.00	0.00	0.00	0.00	0.00
20:3n-6	0.06	0.04	0.03	0.03	0.02
20:4n-6	1.09	0.90	0.88	0.83	0.65
20:3n-3	0.44	0.43	0.41	0.47	0.41
20:4n-3	0.35	0.19	0.15	0.11	0.06
20:5n-3	4.95	4.16	4.01	4.14	3.29
22:1n-11	1.84	0.77	0.57	0.25	0.29
22:1n-9	1.34	1.07	0.96	0.89	0.30
22:4n-6	0.16	0.10	0.10	0.09	0.06
22:5n-6	0.19	0.16	0.14	0.16	0.12
22:5n-3	1.50	1.02	0.90	0.86	0.55
22:6n-3	16.87	16.64	15.55	14.92	13.53
Total Saturated	24.13±1.98^{a}	23.27±1.72^{ab}	22.57±0.38^{b}	25.01±0.96^{a}	25.10±0.67^{a}
Total Monounsaturated	29.02±1.67^{b}	27.50±1.99^{b}	33.26±2.01^{a}	29.10±1.92^{b}	19.72±1.58^{c}
Total n-3	26.95±1.3^{a}	24.64±0.58^{b}	22.92±0.49^{c}	22.83±0.28^{c}	20.76±0.20^{d}
Total n-6	18.29±1.13^{c}	21.08±3.15^{cb}	20.03±0.73^{c}	26.16±3.02^{b}	33.40±0.47^{a}
Total n-9	21.98±0.24^{bc}	25.61±5.21^{ab}	27.31±0.45^{a}	18.56±3.86^{bc}	16.02±0.01^{c}
Total n-3HUFA	24.58±1.18^{a}	22.44±0.67^{ab}	21.03±0.46^{b}	22.50±0.11^{b}	17.84±0.19^{c}
ARA	1.09±0.02^{a}	0.90±0.06^{b}	0.88±0.01^{b}	0.83±0.10^{b}	0.65±0.00^{c}
EPA	4.95±0.22^{a}	4.16±0.03^{b}	4.01±0.07^{b}	4.14±0.07^{b}	3.29±0.02^{c}
DHA	16.87±0.82^{a}	16.64±0.86^{a}	15.55±0.36^{b}	14.92±0.31^{b}	13.53±0.18^{c}
EPA/ARA	4.54±0.32^{a}	4.59±0.31^{a}	4.56±0.05^{a}	4.99±0.53^{a}	5.06±0.01^{a}
DHA/EPA	3.49±0.01^{b}	3.75±0.24^{ab}	3.87±0.02^{a}	4.08±0.15^{a}	4.10±0.03^{a}
DHA/ARA	15.92±1.18^{b}	17.28±2.27^{ab}	17.65±0.30^{ab}	20.43±2.95^{ab}	20.78±0.21^{a}
n-3/n-6	1.47±0.01^{a}	1.04±0.12^{b}	1.14±0.01^{b}	0.95±0.1^{b}	0.62±0.01^{c}

Table 4.6 Fatty acids (% total area) composition in polar lipids of larvae fed increased levels of SBL for 15 days. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	0SBL	2 SBL	4 SBL	8 SBL	12 SBL
14:0	0.47	0.40	0.39	0.33	0.27
14:01	0.00	0.00	0.00	0.00	0.00
14:1n-5	0.01	0.01	0.01	0.02	0.00
14:1n-7	0.04	0.04	0.04	0.03	0.02
15:0	0.29	0.28	0.29	0.27	0.24
15:1n-5	0.01	0.02	0.01	0.00	0.01

16:0	22.87	23.09	22.70	23.13	23.63
16:1n-9	0.00	0.00	0.00	0.00	0.00
16:1n-7	0.90	0.79	0.73	0.70	0.55
Met 16:0	0.11	0.10	0.09	0.08	0.08
16:1n-5	0.23	0.23	0.22	0.20	0.17
16:2n-6	0.57	0.52	0.54	0.48	0.37
16:2n-4	0.61	0.62	0.61	0.60	0.54
16:2n-3	0.00	0.00	0.00	0.00	0.00
17:0	0.27	0.27	0.25	0.25	0.19
16:3n-4	0.01	0.00	0.00	0.00	0.00
16:3n-3	0.09	0.09	0.09	0.09	0.08
16:3n-1	0.67	0.63	0.66	0.66	0.58
16:4n-3	0.39	0.40	0.46	0.30	0.24
16:4n-1	0.10	0.09	0.09	0.09	0.06
18:0	7.82	7.74	7.87	7.79	7.88
18:1n-9	11.79	11.97	12.93	9.82	8.61
18:1n-7	1.77	1.74	1.59	1.65	1.53
18:1n-5	0.18	0.16	0.16	0.15	0.12
18:2n-9	0.10	0.08	0.08	0.07	0.02
18:2 n-6	8.22	9.15	9.28	11.80	16.65
18:2n-4	0.03	0.02	0.02	0.01	0.00
18:3n-9	0.00	0.00	0.00	0.00	0.00
18:3n-6	0.08	0.10	0.10	0.10	0.10
18:4 n-6	0.00	0.00	0.00	0.00	0.00
18:3n-4	0.08	0.06	0.08	0.10	0.06
18:3n-3	0.42	0.38	0.33	0.46	0.60
18:3n-1	0.00	0.00	0.00	0.00	0.00
18:4n-3	0.02	0.01	0.01	0.01	0.00
18:4n-1	0.01	0.01	0.00	0.00	0.00
20:0	0.15	0.14	0.15	0.13	0.12
20:1n-9	1.10	0.94	0.95	0.84	0.69
20:1n-7	0.00	0.00	0.00	0.00	0.00
20:1n-5	0.12	0.10	0.10	0.09	0.08
20:2n-9	0.02	0.04	0.01	0.04	0.03
20:2n-6	0.52	0.54	0.53	0.63	0.68
20:3n-9+n-	0.01	0.01	0.00	0.00	0.00
20:3n-6	0.08	0.07	0.07	0.06	0.05
20:4n-6	1.82	1.79	1.76	1.65	1.48
20:3n-3	0.20	0.19	0.19	0.21	0.19
20:4n-3	0.14	0.10	0.09	0.06	0.04
20:5n-3	5.72	5.96	5.57	6.03	5.62
22:1n-11	0.13	0.06	0.05	0.03	0.03
22:1n-9	0.08	0.03	0.07	0.03	0.02
22:4n-6	0.07	0.06	0.06	0.05	0.04
22:5n-6	0.36	0.35	0.34	0.33	0.29
22:5n-3	1.34	1.16	1.09	0.98	0.79

22:5n-6	0.36	0.35	0.34	0.33	0.29
22:5n-3	1.34	1.16	1.09	0.98	0.79
22:6n-3	29.47	29.41	29.32	29.62	27.18
Total Saturated	32.24±0.12[a]	31.90±1.22[a]	31.64±0.04 [a]	31.88±0.49 [a]	32.32±0.07 [a]
Total Monounsaturated	16.35±1.10[a]	16.09±1.17[a]	16.85±1.19[a]	13.57±1.04[b]	11.84±1.02[c]
Total n-3	37.65±1.47[a]	37.71±1.43[a]	37.14±0.36[a]	37.77±0.54[a]	34.76±0.40[b]
Total n-6	11.86±0.83[c]	12.60±1.50[bc]	12.67±0.02[bc]	15.09±1.57[b]	19.66±0.07[a]
Total n-9	13.40±1.12[ab]	13.06±1.11[ab]	14.04±0.26[a]	10.79±1.54[bc]	9.37±0.19[c]
Total n-3HUFA	36.76±1.45[a]	36.84±1.34[a]	36.25±0.37[a]	36.91±0.51[a]	33.83±0.39[b]
ARA	1.82±0.11[a]	1.79±0.20[a]	1.76±0.02[ab]	1.65±0.05[ab]	1.48±0.054[b]
EPA	5.72±0.49[a]	5.96±0.01[a]	5.57±0.24[a]	6.03±0.11[a]	5.62±0.01[a]
DHA	29.47±0.62[a]	29.41±1.15[a]	29.32±0.12[a]	29.62±0.50[a]	27.18±0.38[b]
EPA/ARA	3.12±0.06[b]	3.35±0.38[ab]	3.15±0.09[b]	3.65±0.20[ab]	3.80±0.14[a]
DHA/EPA	5.16±0.33[a]	4.93±0.19[a]	5.26±0.20[a]	4.91±0.01[a]	4.83±0.08[a]
DHA/ARA	16.16±0.72[b]	16.50±1.22[ab]	16.60±0.14[ab]	17.95±0.95[ab]	18.38±0.42[a]
n-3/n-6	3.18±0.34[a]	3.02±0.47[a]	2.93±0.03[a]	2.51±0.22[a]	1.767±0.02[b]

Discussion

Regardless the dietary PL levels tested in the present study, the larvae were early weand at 16 dph on the experimental microdiets without rotifers or *Artemia* and produced very strong larvae (over 90% survival rate after a handling test) and high survival rates (52-57 %). Indeed, survival rates were higher than those previously obtained for marine fish larvae of this or other species fed only microdiets (Zambonino-Infante and Cahu, 1999; Roo *et al.,* 2005; Seiliez *et al.,* 2006). Under this conditions, increase of PL (49.4-95.9 g kg^{-1} in dry weight of diet) by the inclusion of SBL from 0 to 120 g kg^{-1} did not affected larval survival or stress resistance, suggesting that about 50 g kg^{-1} PL in the form of SBL is enough to maintain good larval survival. In agreement, the increase from 100 to 120 g kg^{-1} PL in the form of marine PL did neither improved larval survival in Atlantic cod (*Gadus morhua*) (Wold *et al*., 2007) or pikerperch (*Sander lucioperca*) (Hamza *et al.,* 2008) fed ranged levels of SBL (up to 12%). Nevertheless, increased dietary PL up to 88.1 g kg^{-1} PL (80 g SBL kg^{-1} diet) significantly improved gilthead seabream larvae growth both in terms of total length and dry body weight.

Dietary polar lipids have been found to improve microdiet ingestion (Izquierdo and Koven, 2010), that resulted in significant enhancement of digestive enzymes activities, particularly, PLA2 and alkaline phosphatase, was found by the elevation of

dietary PL up to 88.1 g kg^{-1}. Although marine fish larvae may have a wide range of digestive enzymes for an efficient digestion (Kolkovski *et al*., 2009), the presence of certain nutrients such as protein hydrolysates or short peptides can markedly contribute to enhance digestive enzymes activity as well as intestine maturation (Zambonino-Infante *et al*., 1999). Indeed, nutritional changes in the intestinal lumen are well known to affect directly or indirectly (via hormones, growth factors and cytokines) the epithelial cell function and differentiation in other vertebrates (Sanderson and Naik, 2000).

Intestinal PLA2 activity has been determined in several fish species and catalyses the hydrolysis of dietary PL, producing free fatty acids and lysoPL (Izquierdo and Henderson, 1998). Despite dietary PL can be also hydrolysed by other lipolytic enzymes such as bile salt activated non specific lipase, PLA2 has been found to be more effective hydrolysing these type of lipids (Izquierdo and Henderson, 1998). Indeed, in the present study, increase in dietary PL only slightly increased lipase activity, whereas a linear substrate stimulatory effect of dietary SBL was found on PLA2 activity. These results are in agreement with those obtained in larval seabass, where PLA2 rather than lipase activity was increased in larvae fed increased PL levels, regulation occurring mainly at the transcriptional level (Cahu *et al.,* 2003a). Lipase activity was markedly increased by dietary PL of marine origin in our previous studies (Saleh *et al*., 2012a), denoting the high affinity of this enzyme for n-3 HUFA esterified lipids (Izquierdo *et al*., 2000). Increased PLA2 activity in the present study would imply a better digestion of dietary PL, both from SBL and from the marine ingredients (squid meal and sardine oil), suggesting a better digestive utilization of ARA, EPA and DHA, high in the later lipid sources. Trypsin activity was only slightly affected by dietary SBL, being significantly higher only in larvae fed 88.1 g PL kg^{-1} diet, in agreement with previous studies feeding marine PL in this (Saleh et al. 2012a) or other species (Wold *et al*., 2007). This moderated effect on trypsin activity could be expected in these diets with similar protein contents, since this enzyme is the most important proteolytic enzyme in the early life stage of marine fish larvae (Ueberschär, 1993). Thus, the increase in trypsin could be more related to a general improvement in gut maturation or the stimulation of nervous or hormonal control of pancreatic secretion.

The intestinal alkaline phosphatase is an intestinal specific isozyme, localized in the

apical brush border of the enterocyte and enriched in surfactant-like particles, which is involved in nutrition processes, intestinal transport and intestinal inflammation in vertebrates, including fish (Bates *et al*., 2007). Since, this enzyme is intimately associated with the hydrophobic core of the intestinal microvillus membrane, its functioning is directly dependant on the development of this structure and it shows a sharp increase in activity during the post-embryonic development of mammals and fish (Bates *et al*. 2007). Consequently, the intestinal alkaline phosphatase has been used as a marker for enterocyte maturation in vertebrates (Zambonino-Infante and Cahu, 2001; Cahu *et al*., 2003; Bates *et al*., 2007; Saleh *et al*., 2012a). Nutritional factors, such as dietary lipids, have been found to affect alkaline phosphatase activity in mammals, as a result of the increase in the number of enterocytes that can express these enzymes (Sanderson and Naik, 2000). Indeed, increase in dietary PL improved the activity of this enzyme and enterocyte maturation in larvae fed increased PL (MacQueen Leifson *et al*., 2003; Wold *et al*., 2007; Saleh *et al*., 2012a). However, the phospholipid source used in these three studies was of marine origin, rich in n-3HUFA, known to markedly affect cell differentiation and proliferation in several tissues (Izquierdo and Koven, 2010) and particularly in intestine (Sanderson and Naik, 2000). The present study shows that n-3 HUFA free source of PL, the SBL rich in linoleic acid (LA), also increases alkaline phosphatase activity in gilthead seabream larvae reflecting an improvement in enterocyte maturation.

Dietary PL are hydrolysed mainly to free fatty acids and 1-acyl lyso-glyceroPL that are absorbed by the intestinal mucosal cells (Izquierdo and Henderson, 1998), where they are re-acylated to form PL mainly by the glycerol-3-phosphate pathway as demonstrated by Caballero *et al*. (2006a). In gilthead seabream, phospholipid synthesis is mainly regulated by two enzymes of this pathway diacylglycerol choline phosphotransferase and diacylglycerol ethanolamine phosphotransferase, whose activity can be modulated through dietary lipids (Caballero *et al*., 2006a). Thus, elevation of dietary soybean oil, rich in linoleic acid, increases PL synthesis, particularly PC, in gilthead seabream enterocytes (Caballero *et al*., 2006a). In agreement, in the present study, elevation of dietary SBL and, consequently, in PLA2 activity, would have increased the presence of substrates for these reacilation enzymes promoting PL synthesis in the enterocyte, which in turn would have stimulated membrane formation for both cell organelles and cell membrane

promoting enterocyte and brush border maturation. Indeed, larvae of this study (15-30 dph) were in the most critical phase of digestive tract development (phase IV) in gilthead seabream, when connective tissue starts to develop and the first mucosal folds appear (Elbal *et al*., 2004).

The improved gut maturation caused by the increased dietary SBL contents would improve digestive and absorptive utilization of dietary nutrients from the experimental microdiets, what could contribute to the improved growth rates. Besides, nutrient transport could have been also improved contributing to dietary nutrients utilization. For instance, dietary linoleic acid, high in SBL, has been found to increase lipoprotein, particularly VLDL, synthesis and composition, (Caballero *et al*., 2003, 2006b) in gilthead seabream juveniles. Moreover, in gilthead seabream larvae dietary SBL markedly increases lipoproteins synthesis in larval gut (Liu *et al*., 2002), promoting dietary lipids and proteins transport. In agreement, addition of SBL in microdiets for gilthead seabream improves enterocytes morphology and reduces lipid droplets accumulation (Salhi *et al.,* 1999; Izquierdo *et al*., 2000). Indeed, lipid transport may be a more important limiting factor for utilization of dietary lipids in marine fish larvae than lipid digestion according to the results of Morais *et al*. (2006).

Improved lipid digestion, absorption and transport can be also responsible for the high incorporation of ARA, EPA and DHA into polar lipids of larvae fed increased levels of SBL in the present study. Thus, despite the levels of this fatty acids were lower in both total and polar lipids of the experimental diets containing increased levels of SBL, the contents of these fatty acids in the larvae fed up to 88.1 g PL kg^{-1} diet remained unchanged in larval PL, and almost constant in larval NL. However, further increase in dietary PL up to 95.9 g kg^{-1} diet (120 g SBL kg^{-1} diet) markedly reduced the contents of these three essential fatty acids in both larval NL and PL. This reduction can be related to the high content in linoleic acid in SBL, since this fatty acid not only promotes PL synthesis, particularly PC (Caballero *et al*., 2006a), but is also specifically incorporated into gilthead seabream PL (Izquierdo *et al*., 2003; Ganga *et al*., 2005) and it is highly retained in this fraction even after months of being removed from the diet (Izquierdo *et al*., 2005). Therefore, the high linoleic acid content (52.1 g kg^{-1} in dry weight basis) of diets containing 95.9 g PL kg^{-1} diet (120 g SBL kg^{-1}) could have compete for incorporation of ARA, EPA and DHA into larval lipids, in turn reducing the increase in larval growth caused by dietary SBL addition.

From these results, an 80 g kg^{-1} SBL inclusion in microdiets for gilthead seabream larvae could be recommended, since further increase does not improve larval growth and survival and could reduce the DHA incorporation into the different larval tissues. Thus, a 50 % increase in final dry weight was obtained by the increase in SBL from 0 to 80 g kg^{-1} SBL, as it occurs in pikerperch larvae when SBL is increased from 15 to 95 g kg^{-1} (Hamza *et al.,* 2008), being the requirements similar for both fish species. However, higher SBL levels (120 g kg^{-1}) are required in microdiets for European sea bass (Cahu *et al.,* 2003a). Our former studies in larval gilthead seabream fed microdiets with a similar formulation to those used in the present study, showed a higher requirement for PL (100 g kg^{-1}) when a marine PL source was used (Saleh *et al*., 2012a). Finally, Further studies are being conducted to compare the effectiveness of the different sources of dietary PL.

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Chapter 5

Effect of krill phospholipids vs soybean lecithin in microdiets for gilthead seabream (*Sparus aurata*) larvae on molecular markers of antioxidative metabolism and bone development

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Abstract

The objective of the present study was to compare the effectiveness of dietary marine phospholipids (MPL) obtained from krill and soybean lecithin (SBL) on the rearing performance and development of seabream (*Sparus aurata*) larvae. Larvae were fed from 16 to 44 day post hatching (dph) five formulated microdiets with three different levels (5, 7 or 9 %) of phospholipids (PL) obtained either from a MPL or a SBL source. Larvae fed MPL showest a higher survival, stress resistance and growth than those fed SBL, regardless the dietary PL level. Overall, the increase in MPL up to 7% total PL in diet was enough to improve larval gilthead seabream performance, whereas even the highest SBL inclusion level (9% PL) was not able to provide a similar success in larval growth or survival. Inclusion of SBL markedly increased the proxidation risk as denoted by the higher TBARs in larvae, as well as a higher expression of CAT, GPX and SOD genes. Moreover, SBL tend to produce larvae with a lower number of mineralized vertebrae and a lower expression of OC, OP and BMP4 genes.Finally, increasing dietary MPL or SBL lead to a better assimilation of polyunsaturated fatty acids in the larvae, n-3HUFA (especially 20:5n-3) or n-6 fatty acids (especially 18:2n-6), respectively. In conclusion, MPL had a higher effectiveness in promoting survival, growth and skeletal mineralization of gilthead seabream larvae in comparison to SBL.

Introduction

A main objective in fish larval nutrition is to formulate an effective compound diet able to substitute live preys as early as possible during larval development (Watanabe and Kiron, 1994). Such inert diet must be attractive, adequated to fit larval mouth size and should be able to fulfil the nutritional requirements of the larvae (Kolkovski *et al*., 2009). Among the different nutrients, phospholipids (PL) have been found to play essential roles in larval development (Izquierdo and Koven, 2010), their contents in larval inert diets markedly affecting larval performance (Kanazawa *et al*., 1981, 1983a, b; Sargent *et al*., 2002).Thus, various studies have demonstrated that the inclusion of dietary phospholipids leads to better growth and survival rates, a higher fish resistance to stress, and a reduced incidence of skeletal deformities in larvae (Kanazawa, 1993; Takeuchi *et al*., 1992; Salhi *et al.,* 1999; Izquierdo *et al.*, 2001; Kjørsvik *et al.,* 2009; Izquierdo and Koven, 2010; Saleh *et al*., 2012a, b) and early juveniles (Coutteau *et al*., 1997). Marine fish larvae have a high capacity to utilize phospholipids (Geurden *et al*., 1998b; Salhi *et al*., 1999; Izquierdo *et al*., 2001) and inert diets containing marine phospholipids rather than marine triacylglycerides (TAG) resulted in better larval growth (Izquierdo *et al*., 2001; Cahu *et al*., 2003a; Gisbert *et al*., 2005). PL are the main structural components of cell membranes bilayers (Kagan *et al*., 1984), but also play an important structural role in digestion, promoting the utilization of dietary neutral lipids (Olsen and Ringø, 1997). For instance, elevation of dietary PL levels significantly increases both neutral lipase and phospholipase A2 activity in gilthead seabream larvae (Saleh *et al*., 2012a, b). Several authors had also suggested a significant role of PL in the intestinal absorption of lipids in marine fish larvae and early juveniles (Coutteau *et al.,* 1997; Fontagne *et al*., 1998; Salhi *et al*., 1999). More recently, it has been demonstrated that dietary PL contribute to the assimilation of dietary lipids by enhancing the enteric lipoprotein synthesis required for the extra-cellular transport of lipids (Liu *et al*., 2002; Hadas *et. al*., 2003). Moreover, inclusion of PL in early weaning diets markedly increases alkaline phosphatase activity denoting a better development of fish digestive system (Saleh *et al*., 2012a,b). Besides, they seem to affect inert diets palatability and could contribute to supply essential components such as choline and inositol (Tocher *et al.,* 2008).

Early studies also showed a positive effect of dietary PL inclusion on skeletal development of fish larvae, chicken egg lecithin reducing the incidence of certain bone malformations such as twisted jaw and scoliosis (Kanazawa *et al*., 1981). Inclusion of PL rich in n-3 polyunsaturated fatty acids in early weanning diets for cod induced a faster ossification and a higher number of fin rays in comparison to the control group (Kjørsvik *et al*., 2009). However, little is known on the phisiological mechanisms implied in this effect. Transcription factors, growth factors, cell surface receptors, cell adhesion molecules and extracellular matrix molecules are all involved in the four basic processes of skeletal cell and tissue differentiation, which include: migration of skeletogenic cells to the site of future skeletogenesis, epithelial-mesenchymal interactions, cell condensation and cell differentiation. For instance, a very high correlation between developmental age and gene expression for osteocalcin, a matrix mineralization protein and a marker for late bone cell differentiation, was found in fish fed high PI dietary levels (Sandel *et al*., 2010). Therefore, these effects could be also related to the different PL classes composition of each PL source (Geurden *et al.,* 1998a, b). Indeed certain membrane PL, such as phosphatidyl inositols (PI), are precursors of second messengers regulating calcium entry into the cells and are involved in a signaling system controlling biological processes in the early development of vertebrates (Berridge and Irvine, 1989).

Thus, PL include a large group of compounds and their function in larval development could be related to their specific composition, both in type of lipid classes and fatty acids contents. For instance, inclusion of PL from marine origin (bonito eggs), rich in n-3 highly unsaturated fatty acids, improve growth and survival of larval ayu (*Plecoglossus altivelis*) more effectively than PL from a vegetal source (Kanazawa *et al*., 1981). Similarly, higher length and weight are obtained in gilthead seabream larvae fed marine PL (squid) than soybean lecithin (Salhi *et al*., 1999). Recently, seabream growth, hepatic utilization of dietary lipids and gut health were all improved when 2.5% dietary soybean lecithin was substituted by krill PL (Betancor *et al*., 2012b). However, there are no studies comparing both PL sources at several dietary contents, which are close to the optimum levels for each PL source as determined in previous studies (Saleh *et al*., 2012a, b).

Different fatty acid profiles in the dietary PL could differently affect the susceptibility of larval tissue lipids to peroxidative damage. Lipid peroxidation is recognized as being highly deleterious, resulting in damage to cellular biomembranes (Kanazawa,

1993) and subcellular membranes, such as those of mitochondria, causing several pathological conditions in fish (Sakai *et al*., 1989). The detrimental effects of peroxidative risk include the oxidative damage to molecules of high biological importance – lipids, proteins, carbohydrates and/or DNA causing alterations that can lead to cell death (Halliwell and Gutteridge, 2007). To be protected from this peroxidation risk, fish have an endogenous antioxidant defence system, including radical scavenging enzymes such as catalase (CAT), superoxide dismutase, (SOD) and glutathione peroxidase (GPX) (Halliwell and Gutteridge, 1996). Peroxidative risk seems to be even higher in marine fish larvae than in juveniles, due to the large surface area to volume ratio of microdiets, the very high larval tissue content in polyunsaturated fatty acids and the higher water contents, and, hence, a functional defence against oxidised fat is more important than at later stages of fish life cycle (Betancor, 2012). However, few studies have been aimed to determine the effect of dietary nutrients, and particularly PL on the oxidative status of marine fish larvae. PL requirements for marine fish larvae range between 5 and 10% in microdiets. For instance, the optimum PL dietary levels determined for red seabream (*Pagrus major*) larvae are 5% SBL (Kanazawa *et al.,* 1983a), 7% SBL for Japanese flounder (*Paralichthys olivaceus*) (Kanazawa, 1993) and 12% SBL for European sea bass (*Dicentrarchus labrax*) (Cahu *et al.,* 2003a). For gilthead seabream (*Sparus aurata*) our previous studies feeding 5 different dietary levels have shown an optimum dietary level of 8 % PL when SBL (Saleh *et al*., 2012b) was used as a dietary PL source and a 10% PL when krill PL were used (Saleh *et al*., 2012a). Although a lower amount of SBL seems to be sufficient to fulfil the PL requirements of gilthead seabream larvae, the value of SBL in promoting fish larvae growth and survival could be lower.

Thus, the aim of the present study was to compare the effectiveness of both PL sources, krill phospholipids and SBL, to promote gilthead seabream (*Sparus aurata*) larvae development, when they are included at optimum levels in early weanning diets. For that porpuse, PL from either SBL or krill PL were included at three different dietary levels that were either deficient or optimum, and their effects on survival, stress resistance, growth, skeleton malformation, bone mineralization, biochemical composition, oxidative status and selected genes expression in gilthead seabream larvae were studied.

Materials and methods

Gilthead seabream larvae were obtained from natural spawnings of GIA (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria University (ULPGC), Spain) broodstock. Larvae (initial total length 5.5 mm, dry body weight 125 µg) previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until they reached 16 days old, were randomly distributed in 15 experimental tanks at a density of 2100 larvae $tank^{-1}$ and fed one of the experimental early weanning diets tested in triplicates for 29 days. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 ppm salinity) at an increasing flow rate of 0.4 - 1.0 L/min to assure the good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and maintain high water quality, which was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min-1) attaining 6.5±1 ppm dissolved O2. Average water temperature and pH along the trial were 19.3±2°C and 7.85, respectively. Photoperiod was kept at 12h light: 12h dark, by fluorescent daylights and the light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia).

Five experimental microdiets (pellet size 250-500 µm) with increasing phospholipid contents of krill phospholipids (Qrill, high phospholipids, Aker BioMarine, Fjordalléen, Norway) and soybean lecithin (Agramar S.A., Spain) were formulated containing 20 % total lipids, 60% crude proteins and over 3% n-3 HUFA, which covered the essential fatty acid requirements of gilthead seabream larvae (Izquierdo and Koven, 2010; Hemre et al., in press). Their formulation and proximate analysis are shown in Table 5.1. The fatty acids composition of total and polar lipids of the experimental diets are shown in Tables 5.4 and 5.5. The desired lipid content (about 21% DW) was completed if necessary with a non-essential fatty acid source, oleic acid (Oleic acid vegetable, Merck, Darmstadt, Germany). The microdiets were prepared by mixing squid powder and water-soluble components, then the lipids and fat-soluble vitamins and, finally, gelatine dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany), dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved

(Filtra, Barcelona, Spain) to obtain a particle size between 250 to 500 µm. Diets were prepared and analyzed for proximate and fatty acid composition at GIA laboratories. Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00 for 29 days. Non-enriched rotifers were co-fed during days 16th and 17th (1 rotifer/ml). To assure feed availability, daily feed (pellet size < 250 µm) supplied was maintained at 1.5 and 2.5 g per tank during the first and second week of feeding. The amount of feed added daily was gradually increased to 4-5 g per tank with increasing in pellet size to 250–500µm, where an overlap using a mixture of both pellets sizes was conducted during the third and forth week of feeding. Larvae were observed under the binocular microscope to determine feed acceptance.

Table 5.1 Formulation and proximate composition of the experimental microdiets containing several levels of either krill phospholipids or soybean lecithin

Ingredients (g/100 g diet)	**Control**	**7 MPL**	**9 MPL**	**7 SBL**	**9 SBL**
Squid powder[a]	69	69	59.50	69.5	69.5
Krill PL[b]	0	13	21	0	0
Soybean lecithin[c]	0	0	0	4.5	8.5
Sardine oil[d]	5.5	0	3	0	0
Oleic acid[e]	9	1.5	0	9.5	5.5
Basal premix	16.5	16.5	16.5	16.5	16.5
Proximate analysis % dry weight					
Lipid	19.34	20.13	22.10	21.21	22.23
Protein	61.23	58.89	61.00	60.74	61.48
Ash	7.20	7.5	7.43	7.30	7.39
Moisture	8.7	8.1	9.9	7.8	8.4
Lipid classes composition					
Wax/Sterol esters	0.86	0.90	0.37	0.41	0.39
Triacylglycerols	5.38	7.13	4.42	3.00	4.10
Free fatty acids	5.47	2.53	3.60	6.89	4.90
Cholesterol/Sterols	2.58	2.05	4.28	3.12	2.70
Total neutral lipids	14.30	12.61	12.67	13.42	12.08
Glycolipid	0	0.28	0.55	0.22	0.52
Phosphatidylethanolamine	1.68	1.68	2.57	2.32	2.59
Phosphatidylglycerol/cardiolipin	0.00		0.00	0.00	1.42
Phosphatidylinositol	0.56	0.40	0.81	1.17	1.43
Phosphatidylserine	0.39	0.15	0.60	0.47	0.55
Phosphatidylcholine	1.82	3.98	4.04	2.6	2.46
Sphingomyelin	0.1		0	0.23	0.13
Lysophosphatidylcholine	0.17	0.89	0.76	0.24	0.13
Total polar lipid	4.71	7.38	9.33	7.27	9.40
PC/PI	3.25	9.95	4.99	2.22	1.72

a Rieber and Son, Bergen, Norway.
b Qrill, high phospholipids, Aker BioMarine, Fjordalléen, Norway.
c Agramar S.A., Spain.
d Agramar S.A., Spain.
e Merck KGaA, Darmstadi, Germany.

Before the end of the experiment an activity test was conducted by handling 25 larvae/tank out of the water in a scoop net for 1.5 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 living larvae at the beginning and at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 h) and total length (Profile Projector V-12A Nikon, Tokyo, Japan) of 30 fish tank^{-1} at the beginning, in the middle and at the end of the trial. In addition, at the end of the trial and after 12 h of starvation, the all larvae in each tank were washed with distilled water, sampled and kept at -80 ºC for biochemical composition. Fresh larvae samples (100 mg tank^{-1}) for molecular biology analysis were well washed and conserved into 500 ml RNA Later (SIGMA), a product that rapidly allows tissue to stabilize, protects cellular RNA in situ in unfrozen samples and permits to posterior RNA isolation. The larvae were stored in RNA Later overnight at 4 ºC and after that RNA Later was removed before being stored at – 80 ºC to prevent the formation of salt crystals.

Sixty larvae of 44 dph were randomly chosen per diet and kept in formaldehyde for posterior whole mount staining. Staining procedures with alizarin red were conducted to evaluate the skeletal anomalies and vertebral mineralization (Izquierdo *et al*., 2012).

Moisture (A.O.A.C., 1995), protein (A.O.A.C., 1995) and lipid (Folch, 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC/min and then held at 215 ºC for 10 min. Fatty acid methyl esters were quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) following the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

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. Then, 2.95 ml of freshly prepared 50mM thiobarbituric acid solution were added to the sample 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}.

Molecular biology analysis were carried out at IUSA (Instituto Universitario de Sanidad Animal, ULPGC, Spain). Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30ml of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15,1:20 and 1:25). The product size of the real-time q PCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using β-actin as the house-keeping gene in a final volume of 20ml per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analysed once per gene. The PCR conditions were the following: 95ºC for 3min 30sec followed by 40 cycles of 95ºC for 15sec, 61ºC for 30sec, and 72ºC

for 30sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalised and the Livak method ($2^{-\Delta\Delta Ct}$) used to determine relative mRNA expression levels. Gilthead seabream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in Table 5.2.

All data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation and were treated using one-way ANOVA. Means compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Table 5.2 Sequences of forward and reverse primers (5'-3') for real-time quantitative-PCR of seabream genes

Gene	Primers	Accession n^{o}
Catalase(CAT)	Forward primer: 5´-ATGGTGTGGGACTTCTGGAG-3´ Reverse primer: 3´-AGTGGAACTTGCAGTAGAAAC-5´	FJ860003
Superoxide dismutase (SOD)	Forward primer: 5´- AAGAATCATGGCGGTCCTACTGA-3´ Reverse primer: 3´- TGAGCATCTTGTCCGTGATGTCT -5´	AJ937872
Glutathione peroxidase (GPX)	Forward primer: 5´- TCCATTCCCCAGCGATGATGCC-3´ Reverse primer: 3´- TCGCCATCAGGACCAACAAGGA-5´	DQ524992
Osteocalcin	Forward primer: 5´-AGCCCAAAGCACGTAAGCAAGCTA-3´ Reverse primer: 3´- TTTCATCACGCTACTCTACGGGTT-5´	AF048703
Osteopontin	Forward primer: 5´- AAGATGGCCTACGACATGACAGAC- 3´ Reverse primer: 3´- CCTGAAGAGCCTTGTACACCTGC-5´	AY651247
Osteonectin	Forward primer: 5´- AAAATGATCGAGCCCTGCATGGAC-3´ Reverse primer: 3´- TACAGAGTCACCAGGACGTT-5´	AY239014
[a]RUNX2	Forward primer: 5´- GCCTGTCGCCTTTAAGGTGGTTGC-3´ Reverse primer: 3´- TCGTCGTTGCCCGCCATAGCTG-5´	AJ619023
Alkaline phosphatase	Forward primer: 5´- AGAACGCCCTGACGCTGCAA-3´ Reverse primer: 3´- TTCAGTATACGAGCAGCCGTCAC-5´	AY266359
Matrix Gla protein	Forward primer: 5´- GTGCCCTCCTTCATTCCAC-3´ Reverse primer: 3´- TATGACCACGTTGGATGCCT-5´	AY065652
[b]BMP4	Forward primer: 5´- CCACCAGGGCAGACACGTCC-3´ Reverse primer: 3´- GCGTAGCTGCTCCCAGTCCTC-5´	FJ436409
β-actin	Forward primer: 5´- TCTGTCTGG ATC GGAGGCTC-3´ Reverse primer: 3´- AAGCATTTG CGGTGGACG -5´	X89920

a Runx2 (Runt-related transcription factor 2).
b BMP-4 (Bone morphogenetic protein 4).

Results

All the experimental diets were well accepted by gilthead seabream larvae according to the microscopic observations. Larvae fed diets without PL supplementation (Control diet) showed a very low survival rate at the end of the trial (Figure 5.1). Inclusion of SBL, increasing dietary PL up to 9%, significantly (P>0.05) increased larval survival, but inclusion of MPL even at only 7% dietary PL increased larval survival even further than SBL. Thus, larvae fed 9 MPL and 7 MPL diets significantly showed the highest survival. Larval resistance to stress, determined as the survival rate after handling, increased with increasing of dietary PL levels, being highest for larvae fed any of the MPL diets (P>0.05). On the contrary, larvae fed 0 PL and 4 PLS had a marked drop in survival after stress (Figure 5.2).

The larval growth after thirty days of feeding was significantly lowest in larvae fed the Control diet without PL supplementation and, although the diet 7 SBL was not able to significantly (P>0.05) increase final total length, further elevation of SBL in diet 9 SBL significantly (P>0.05) improved this parameter (Figure 5.3). Besides, inclusion of MPL proportionally increased final total length, being for larvae fed both 7 MPL and 9 MPL significantly (P>0.05) higher than Control or SBL larvae. Final larval total length was also correlated to the dietary n-3 HUFA levels (Figure 5.4), regardless the PL source used ($y = 0.5096x + 7.0074$, $R^2 = 0.92768$). In terms of body weight, larvae fed 9 MPL were significantly (P>0.05) the biggest, followed by 7 MPL, then 9 SBL and, finally, Control and 7 SBL larvae, which were not significantly different between them (Figure 5.5).

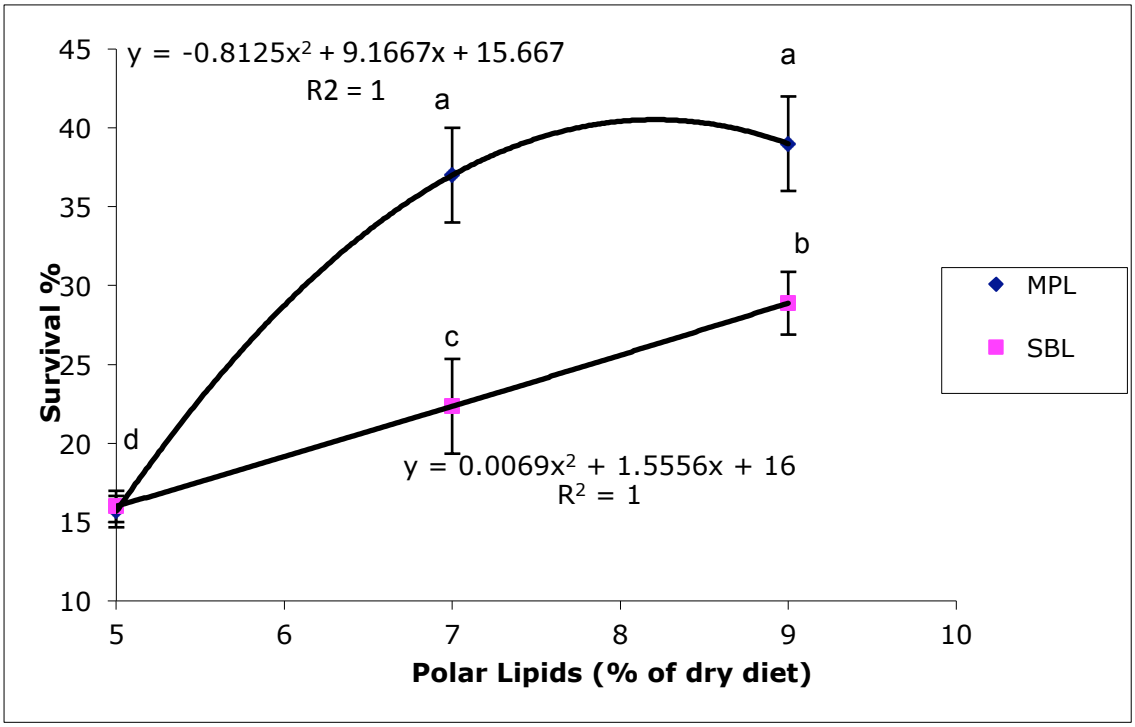

Figure 5.1 Survival rate of larvae (44 dph) fed three dietary PL levels using two different PL sources. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

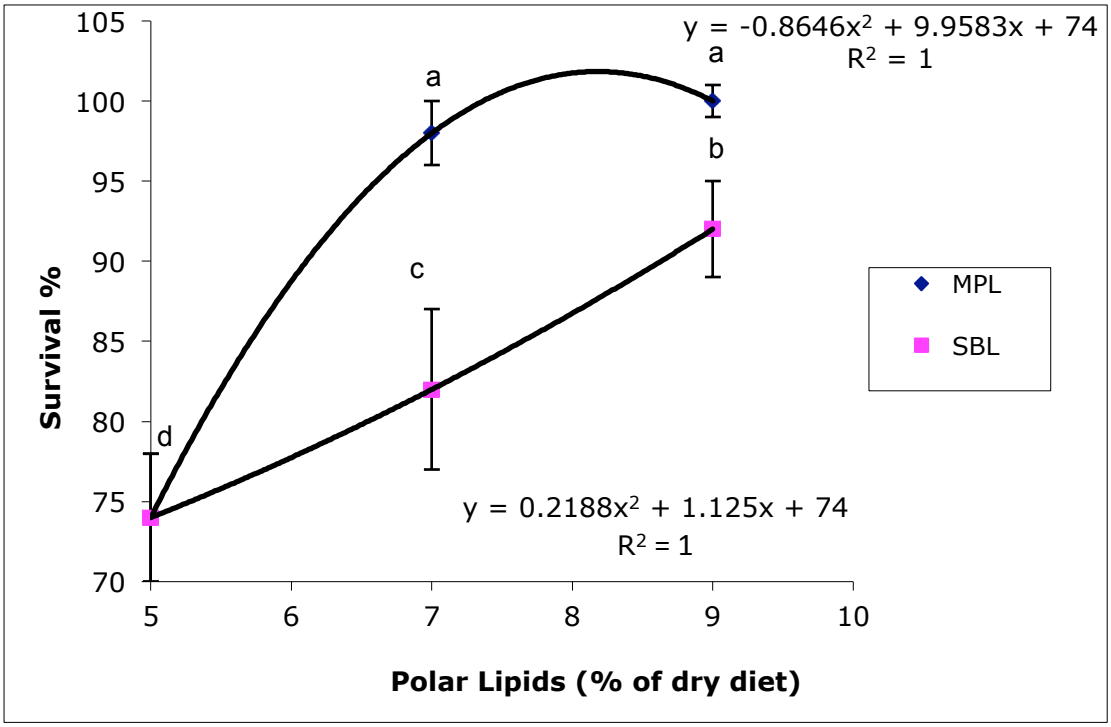

Figure 5.2 Survival rate (24 hours after activity test) of larvae (44 dph) fed three dietary PL levels using two different PL sources. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

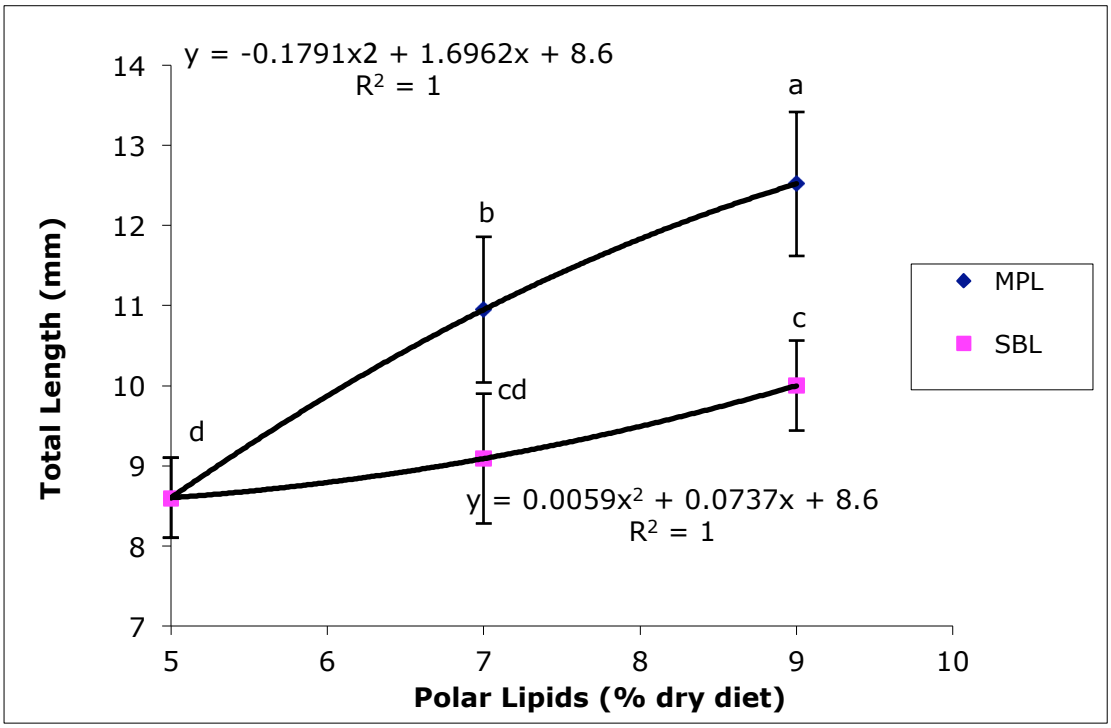

Figure 5.3 Total length of larvae (44 dph) fed three dietary PL levels using two different PL sources. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

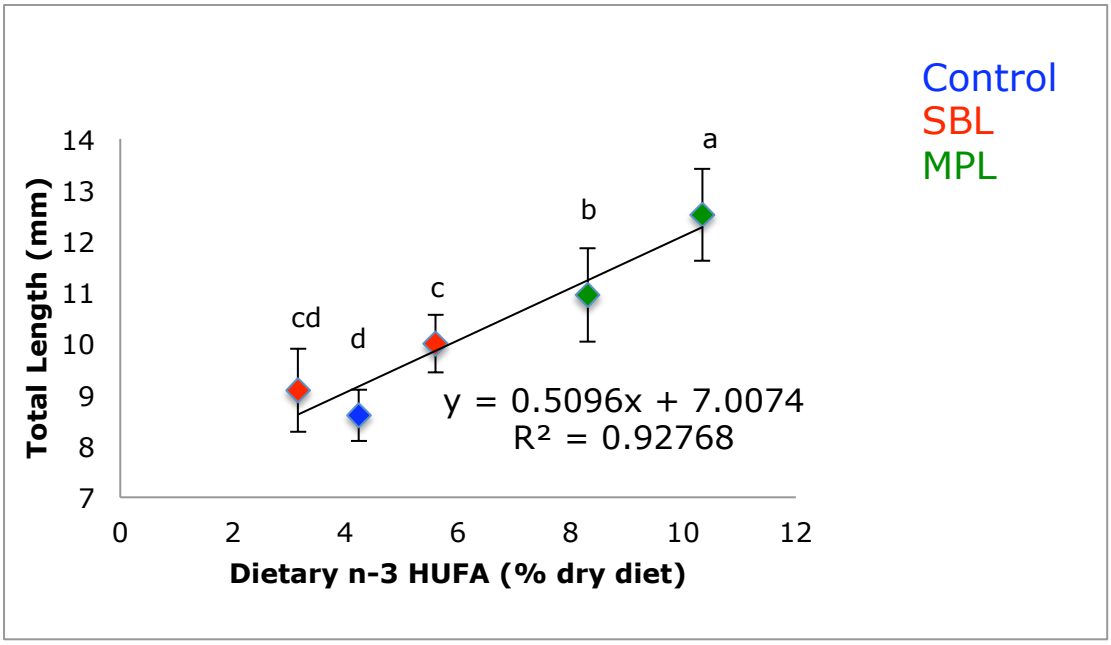

Figure 5.4 Correlation between total length of larvae (44 dph) fed three dietary PL levels using two different PL sources and dietary n-3 HUFA. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

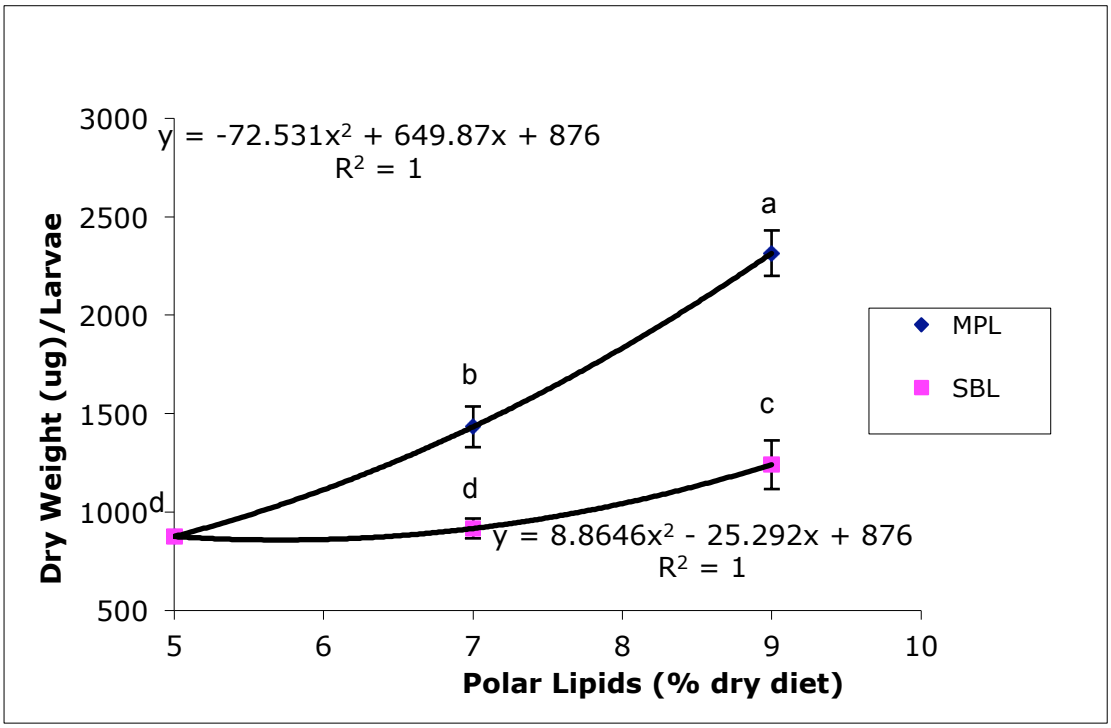

Figure 5.5 Dry whole body weight of larvae (44 dph) fed three dietary PL levels using two different PL sources. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

The effects the different dietary PL levels on skeletal mineralization were analyzed considering the average number of mineralized vertebrae for a given larval size. In general, there was a promoting effect of dietary MPL on the skeletal mineralization. For instance, in the size group 12.40-13.40 mm, when most of the larvae started mineralization of vertebrae, the average number of mineralized vertebrae was 10, 16, 18, 12 and 14 for fish fed diets Control, 7 MPL, 9MPL, 7 SBL and 9 SBL, respectively (Figures 5.6 and 5.7, Table 5.3).

The skeletal anomalies study showed that larvae fed 9 MPL had the lowest percentage of total skeletal anomalies incidence en comparison with the other treatments, the larvae fed Control, 7SBL and 9SBL diet had the highest incidence of lordosis (10 %), and larvae fed 9 SBL had higher incidence of kyphosis (13 %). The larva fed 7MPL diets had lowest mandibula and maxilla anomalies (Figure 5.8).

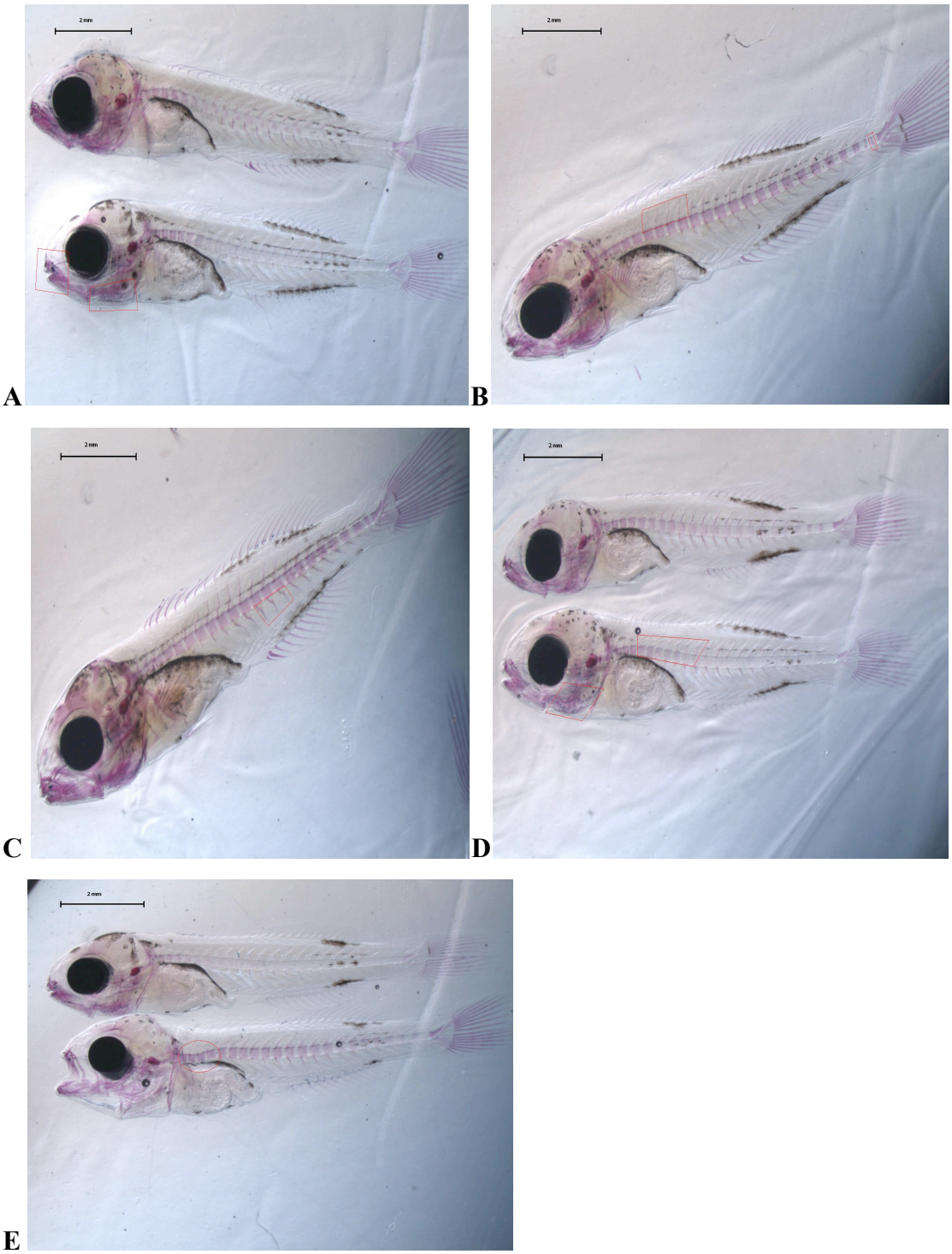

Figure 5.6 Representative pictures of mineralized vertebrae of larvae (44 dah) fed three dietary PL levels using two different PL sources, (A: Control; B: 7 MPL; C: 9 MPL; D: 7 SBL; E: 9 SBL.

Table 5.3 Average numbers of mineralized vertebrae of larvae (44 dah) fed three dietary PL levels using two different PL sources

Average number of mineralized vertebrae						
Size class	**Length (mm)**	**Control**	**7 MPL**	**9 MPL**	**7 SBL**	**9 SBL**
1	8.409-8.909	0	n.a.	n.a.	n.a.	n.a.
2	8.909-9.409	0	n.a.	n.a.	n.a.	n.a.
3	9.409-9.909	0	n.a.	n.a.	n.a.	n.a.
4	9.909-10.409	1	0	n.a.	0	2
5	10.409-10.909	2	2	n.a.	2	0
6	10.909-11.409	3	2	n.a.	1	4
7	11.409-11.909	6	3	n.a.	2	2
8	11.909-12.409	0	5	n.a.	5	10
9	12.409-12.909	8	12	15	9	12
10	12.909-13.409	13	20	21	15	17
11	13.409-13.909	n.a.	n.a.	20	n.a.	n.a.
12	13.909-14.409	n.a.	n.a.	22	n.a.	n.a.
13	14.409-14.909	n.a.	n.a.	23	n.a.	n.a.
% Normal larvae	8.409-14.909	71	84	80	73	71

*n.a. : not available.

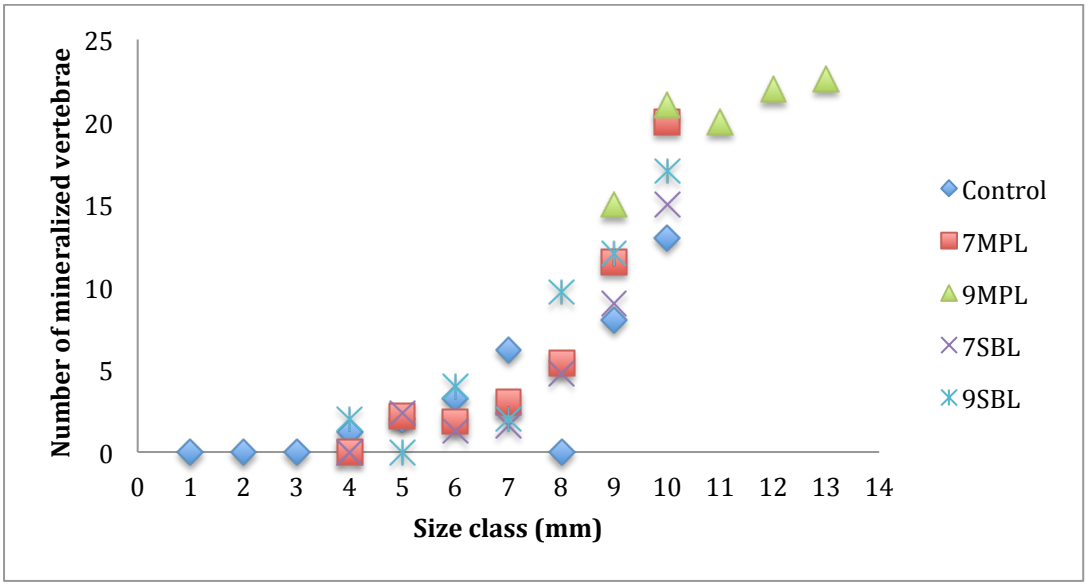

Figure 5.7 Graphical representation of the average number of mineralized vertebrae for each size class of larvae (44 dah) fed three dietary PL levels using two different PL sources.

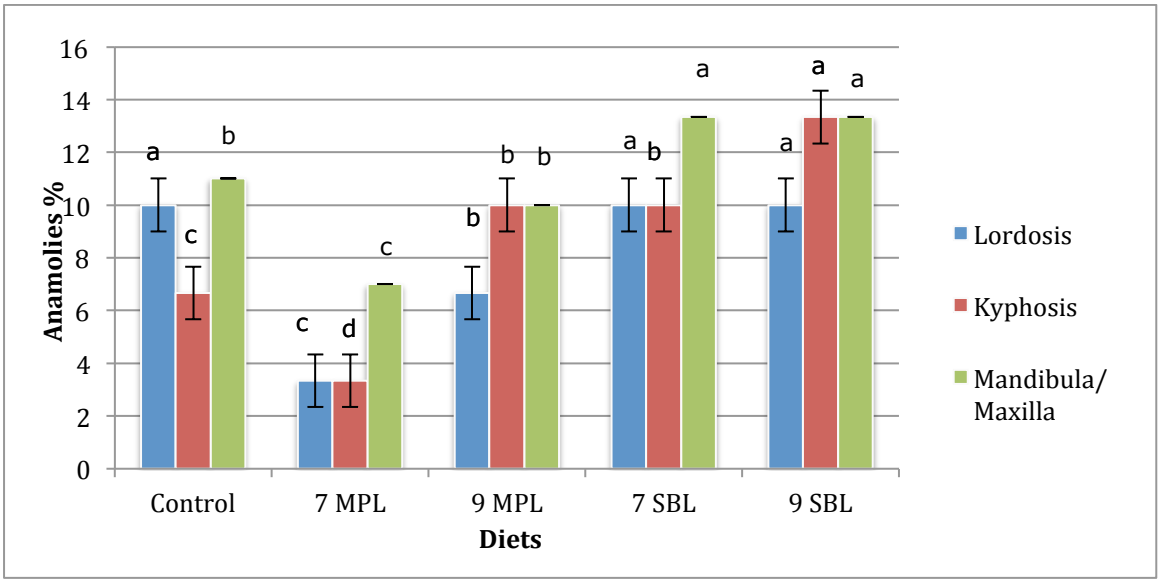

Figure 5.8 Incidence of skeleton anomalies of larvae (44 dah) fed three dietary PL levels using two different PL sources. (P>0.05). Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

Table 5.4 Fatty acids (% dry weight) composition in total lipids of diets three dietary PL levels using two different PL sources

	Control	7 MPL	9 MPL	7 SBL	9 SBL
14:0	0.86	1.69	0.58	0.86	2.29
14:1n-5	0.31	0.05	0.03	0.01	0.04
14:1n-7	0.23	0.04	0.06	0.01	0.03
15:0	0.31	0.09	0.08	0.10	0.09
15:1n-5	0.12	0.01	0.01	0.01	0.02
16:0iso	0.06	0.07	0.07	0.01	0.01
16:0	4.90	4.85	5.39	4.65	4.11
16:1n-7	0.47	1.04	0.92	0.75	0.27
16:1n-5	0.14	0.05	0.04	0.04	0.05
16:2n-6	0.04	0.09	0.04	0.02	0.15
16:2n-4	0.04	0.01	0.01	0.12	0.01
17:0	0.02	0.04	0.04	0.04	0.08
16:3n-4	0.09	0.08	0.21	0.02	0.06
16:3n-3	0.10	0.06	0.03	0.03	0.06
16:3n-1	0.04	0.07	0.06	0.00	0.01
16:4n-3	0.68	0.16	0.05	0.00	0.22
18:0	2.90	0.59	0.73	1.13	0.20
18:1n-9	2.04	3.26	3.57	6.66	2.91
18:1n-7	0.50	1.22	0.76	0.00	0.07
18:1n-5	0.05	0.09	0.10	0.04	0.06
18:2n-9	0.00	0.03	0.07	0.24	0.02
18:2n-6	0.97	0.38	0.68	4.76	6.82
18:2n-4	0.02	0.02	0.07	0.00	0.02
18:3n-6	0.07	0.01	0.01	0.01	0.01

18:3n-4	0.05	0.02	0.05	0.03	0.01
18:3n-3	0.12	0.15	0.24	0.20	0.42
18:4n-3	0.13	0.40	0.39	0.03	0.04
18:4n-1	0.45	0.01	0.04	0.04	0.03
20:0	0.13	0.02	0.03	0.05	0.02
20:1n-9	0.06	0.04	0.06	0.07	0.03
20:1n-7	0.66	0.47	0.48	0.00	0.07
20:1n-5	0.00	0.06	0.03	0.00	0.07
20:2n-6	0.35	0.03	0.09	0.06	0.07
20:3n-6	0.01	0.01	0.02	0.01	0.02
20:4n-6	0.38	0.16	0.14	0.18	0.11
20:3n-3	0.20	0.07	0.06	0.09	0.03
20:4n-3	0.07	0.06	0.07	0.03	0.10
20:5n-3	1.72	3.67	3.63	0.99	2.55
22:1n-11	0.11	0.01	0.04	0.13	0.02
22:1n-9	0.60	0.15	0.05	0.02	0.15
22:4n-6	0.01	0.09	0.05	0.04	0.10
22:5n-6	0.03	0.03	0.03	0.03	0.01
22:5n-3	0.26	0.10	0.26	0.07	0.18
22:6n-3	1.99	4.42	6.33	1.99	2.74
Total Saturated	9.12	7.27	6.85	6.83	6.78
Total n-3	5.28	9.14	11.03	3.42	6.33
Total n-6	1.86	0.81	1.07	5.12	7.29
Total n-9	2.71	3.47	3.77	7.00	3.11
n-3HUFA	4.24	8.33	10.34	3.16	5.60
ARA	0.38	0.16	0.14	0.18	0.11
EPA	1.72	3.67	3.63	0.99	2.55
DHA	1.99	4.42	6.33	1.99	2.74
EPA/ARA	4.57	22.41	26.63	5.41	22.32
DHA/EPA	1.16	1.20	1.74	2.01	1.08
DHA/ARA	5.30	26.98	45.21	10.89	24.05
n-3/n-6	2.84	11.30	10.23	0.64	0.88

Table 5.5 Fatty acids (% dry weight) composition in lipid classes of diets containing three dietary PL levels using two different PL sources

	Control	7 MPL	9 MPL	7 SBL	9 SBL
Phosphatidylcholine					
Saturated	37.07	36.96	32.14	36.14	35.5
Monounsaturated	6.10	11.78	15.95	7.20	11.35
Total n-3	55.58	48.97	48.12	47.77	62.53
ARA	0.38	0.53	0.78	0.35	0.42
EPA	6.76	18.30	29.50	6.08	7.51
DHA	47.86	27.92	14.11	40.14	52.29
Phosphatidylinositol					
Saturated	36.23	45.85	44.78	35.71	47.06
Monounsaturated	19.69	16.24	18.09	19.91	8.85

Total n-3	20.95	34.20	33.50	21.08	11.27
ARA	0.69	1.42	1.77	0.70	0.32
EPA	8.69	17.03	17.61	8.79	3.48
DHA	7.81	15.81	14.46	7.87	3.84
Phosphatidylserine					
Saturated	30.22	34.34	31.01	42.00	34.20
Monounsaturated	18.40	20.72	21.70	17.52	18.53
Total n-3	42.13	41.72	42.69	31.57	32.71
ARA	1.33	1.44	2.21	1.23	1.21
EPA	9.42	12.75	19.61	7.96	7.55
DHA	27.12	26.51	20.73	20.99	22.61
Phosphatidylethanolamine					
Saturated	25.34	23.42	22.23	28.61	24.73
Monounsaturated	13.93	12.89	17.26	11.77	13.84
Total n-3	37.35	56.59	54.71	45.58	38.21
ARA	2.27	3.41	2.28	2.86	2.25
EPA	20.01	30.09	26.08	25.18	19.45
DHA	16.42	24.74	26.99	18.36	15.96

The fatty acid profile of neutral lipids of the larvae (Table 5.6) showed significantly higher contents of saturated and n-3 fatty acids, particularly n-3 HUFA and EPA in fish fed MPL diets in comparison with the control or SBL fed larvae. On the contrary, total n-6 and n-9 fatty acids were higher in larvae fed SBL and control diets than those fed MPL diets. Fatty acid composition of neutral lipids from larvae fed 7 SBL diet was very similar to the Control, being only significantly higher in saturated fatty acids and DHA and lower in ARA. Larvae fed diet 9 SBL were also significantly higher than the control in DHA, n-6 and n-9 fatty acids. Regarding the larval polar lipids, the fatty acids profiles were more similar among larvae fed the different diets than their respective neutral lipids (Table 5.7). In general, the main n-3 polyunsaturated fatty acids EPA and DHA presented a higher concentration in the PL fraction than in the NL, whereas 18:2n-6 content was higher in the NL than in the PL fraction. PL fractions of larvae fed MPL diets were 15% higher in saturated and n-3 fatty acids than fish fed Control or SBL diets. Besides, they were 50% higher in EPA, whereas DHA levels in polar lipids were more similar among the different larvae, being highest in larvae fed 9MPL followed by SBL and, then, 7 MPL and Control larvae. Thus, increasing dietary MPL and SBL lead to better assimilation of n-3HUFA (especially EPA) and n-6 fatty acids (especially 18:2n-6) respectively, denoted by the higher content of these fatty acids in both neutral and polar lipids of

the larvae in comparison to Control fish, regardless their respective dietary fatty acid profiles.

Table 5.6 Fatty acids (% total identified fatty acids) composition of neutral lipids from larvae fed diets containing five phospholipids levels. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	Control	7 MPL	9 MPL	7 SBL	9 SBL
14:0	0.81	4.20	5.08	1.91	1.43
14:1n-5	0.10	0.05	0.03	0.21	0.06
14:1n-7	0.12	0.12	0.12	0.21	0.08
15:0	0.32	0.33	0.41	0.38	0.32
15:1n-5	0.04	0.05	0.04	0.06	0.04
16:0iso	0.10	0.08	0.08	0.10	0.08
16:0	11.10	18.42	20.69	12.53	14.40
16:1n-7	3.89	5.48	4.86	3.28	2.42
16:1n-5	0.19	0.31	0.37	0.26	0.21
16:2n-6	0.38	0.31	0.68	0.37	0.36
16:2n-4	0.48	3.15	2.26	0.58	0.54
17:0	0.97	0.32	0.27	1.04	0.71
16:3n-4	0.03	0.02	0.03	0.00	0.00
16:3n-3	0.09	0.15	0.27	0.09	0.08
16:3n-1	0.00	0.02	0.03	0.00	0.00
16:4n-3	0.09	0.14	0.15	0.06	0.05
16:4n-1	0.04	0.00	0.00	0.00	0.00
18:0	4.48	5.93	6.03	4.82	5.33
18:1n-9	40.19	15.21	11.02	41.20	33.30
18:1n-7	3.47	6.44	5.43	2.31	1.87
18:1n-5	0.81	0.45	0.55	0.65	0.50
18:2n-9	0.15	0.10	0.12	0.09	0.00
18:2n-6	9.87	7.54	2.62	10.29	18.31
18:2n-4	0.16	0.21	0.25	0.15	0.13
18:3n-6	0.24	0.11	0.09	0.09	0.12
18:3n-4	0.10	0.01	0.06	0.06	0.10
18:3n-3	0.99	0.98	0.56	0.91	1.69
18:4n-3	0.19	1.02	0.89	0.19	0.15
18:4n-1	0.02	0.03	0.00	0.00	0.00
20:0	0.19	0.22	0.18	0.15	0.15
20:1n-9	2.88	1.63	2.52	2.06	1.99
20:1n-5	0.18	0.30	0.38	0.14	0.13
20:2n-9	0.00	0.01	0.01	0.01	0.01

20:2n-6	0.28	0.22	0.19	0.21	0.27
20:3n-6	0.05	0.14	0.07	0.03	0.02
20:4n-6	0.90	1.19	0.86	0.73	0.61
20:3n-3	0.19	0.13	0.32	0.18	0.21
20:4n-3	0.17	0.41	0.33	0.05	0.04
20:5n-3	3.20	10.57	10.68	3.00	2.82
22:1n-11	0.75	0.90	0.73	0.50	0.49
22:1n-9	0.43	0.69	0.84	0.03	0.03
22:4n-6	0.04	0.06	0.05	0.03	0.01
22:5n-6	0.12	0.09	0.15	0.10	0.09
22:5n-3	0.63	1.34	1.06	0.31	0.28
22:6n-3	10.55	10.92	18.63	10.64	10.56
Total Saturated	17.86±1.11^{d}	29.41±2.28^{a}	32.66±1.79^{a}	20.83±1.56^{c}	22.33±1.84^{c}
Total n-3	16.12±1.45^{c}	25.66±1.73^{b}	32.89±1.98^{a}	15.43±1.33^{c}	15.88±1.26^{c}
Total n-6	11.87±0.78^{b}	9.67±067^{c}	4.72±0.46^{d}	11.84±0.71^{b}	19.80±0.91^{a}
Total n-9	43.66±1.98^{a}	17.64±1.34^{c}	14.51±1.05^{d}	43.38±2.01^{a}	35.33±1.94^{b}
Total n-3HUFA	14.75±0.67^{c}	23.37±1.34^{b}	31.03±1.58^{a}	14.18±0.78^{c}	13.91±0.87^{c}
ARA	0.90±0.05^{b}	1.19±0.08^{a}	0.86±0.03^{b}	0.73±0.08^{c}	0.61±0.03^{d}
EPA	3.20±0.17^{b}	10.57±0.51^{a}	10.68±0.44^{a}	3.00±0.28^{b}	2.82±0.14^{b}
DHA	10.55±0.79^{b}	10.92±0.95^{b}	18.63±0.96^{a}	10.64±0.88^{b}	10.56±0.79^{b}
EPA/ARA	3.55±0.11^{d}	9.13±0.69^{b}	12.47±0.76^{a}	4.13±0.28^{d}	4.65±0.19^{c}
DHA/EPA	3.25±0.13^{b}	1.03±0. 07^{d}	1.74±0.08^{c}	3.54±0.16^{a}	3.73±0.21^{a}
DHA/ARA	11.65±0.88^{d}	9.43±0.79^{e}	21.76±1.02^{a}	14.62±0.91^{c}	17.36±1.00^{b}
n-3/n-6	1.38±0.04^{c}	2.65±0.09^{b}	7.25±0.54^{a}	1.30±0.03^{c}	0.80±0.01^{d}

Table 5.7 Fatty acids (% total identified fatty acids) composition in polar lipids of larvae fed diets containing five phospholipids levels. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	Control	7 MPL	9 MPL	7 SBL	9 SBL
14:0	0.62	1.12	1.07	1.11	0.38
14:1n-5	0.03	0.02	0.02	0.35	0.02
14:1n-7	0.05	0.04	0.03	0.29	0.03
15:0	0.30	0.23	0.27	0.62	0.23
15:1n-5	0.01	0.01	0.01	0.42	0.00
16:0ISO	0.06	0.04	0.04	0.24	0.04
16:0	22.88	25.63	28.03	22.11	23.12
16:1n-7	0.88	2.25	2.07	1.00	0.72
16:1n-5	0.21	0.24	0.26	0.35	0.17
16:2n-6	0.39	0.15	0.43	0.52	0.36
16:2n-4	0.55	0.97	0.70	0.66	0.48

17:0	0.52	0.21	0.19	0.62	0.34
16:3n-4	0.02	0.00	0.00	0.03	0.00
16:3n-3	0.08	0.18	0.17	0.10	0.07
16:3n-1	0.00	0.80	0.00	0.51	0.54
16:4n-3	0.54	0.00	0.71	0.00	0.00
16:4n-1	0.66	0.36	0.21	0.69	0.59
18:0	7.85	8.72	8.06	6.96	7.42
18:1n-9	19.79	11.33	8.71	17.25	14.05
18:1n-7	2.77	3.95	3.26	2.36	2.06
18:1n-5	0.50	0.26	0.29	0.37	0.38
18:2n-9	0.22	0.14	0.06	0.16	0.20
18:2n-6	7.00	3.63	0.96	7.71	12.84
18:2n-4	0.04	0.06	0.05	0.05	0.06
18:3n-6	0.15	0.09	0.09	0.11	0.08
18:3n-4	0.08	0.00	0.02	0.07	0.08
18:3n-3	0.29	0.20	0.09	0.25	0.45
18:4n-3	0.05	0.11	0.08	0.03	0.03
18:4n-1	0.01	0.02	0.00	0.00	0.01
20:0	0.19	0.16	0.14	0.14	0.13
20:1n-9	1.08	0.47	0.78	0.78	0.69
20:1n-5	0.10	0.14	0.14	0.07	0.03
20:2n-9	0.03	0.01	0.01	0.03	0.01
20:2n-6	0.35	0.16	0.10	0.26	0.34
20:3n-6	0.08	0.13	0.07	0.06	0.04
20:4n-6	1.48	1.42	1.56	1.29	1.19
20:3n-3	0.13	0.06	0.11	0.11	0.12
20:4n-3	0.09	0.21	0.13	0.03	0.02
20:5n-3	4.74	11.18	11.92	5.14	5.23
22:1n-11	0.08	0.11	0.06	0.05	0.04
22:1n-9	0.05	0.09	0.09	0.02	0.02
22:4n-6	0.06	0.06	0.04	0.05	0.04
22:5n-6	0.33	0.14	0.20	0.20	0.27
22:5n-3	0.68	1.46	0.73	0.38	0.36
22:6n-3	23.99	23.42	28.04	26.44	26.73
Total Saturated	32.36 ± 2.3^{b}	36.07 ± 1.38^{a}	37.76 ± 1.4^{a}	31.54 ± 1.6^{b}	31.63 ± 1.2^{b}
Total n-3	30.58 ± 1.41^{c}	36.83 ± 1.36^{b}	41.98 ± 1.89^{a}	32.49 ± 1.09^{c}	33.01 ± 1.13^{c}
Total n-6	9.84 ± 0.78^{b}	5.78 ± 0.20^{c}	3.46 ± 0.81^{d}	10.19 ± 0.45^{b}	15.16 ± 0.84^{a}
Total n-9	21.17 ± 1.63^{a}	12.04 ± 1.10^{bc}	9.65 ± 1.96^{c}	18.25 ± 2.87^{a}	14.97 ± 1.42^{b}
Total n-3HUFA	29.62 ± 2.25^{c}	36.33 ± 2.13^{b}	40.94 ± 2.49^{a}	32.10 ± 1.91^{c}	32.47 ± 1.86^{c}
ARA	1.48 ± 0.10^{a}	$1.42\pm.09^{ab}$	1.56 ± 0.12^{a}	1.29 ± 0.08^{b}	1.19 ± 0.11^{b}
EPA	4.74 ± 0.47^{b}	$11.18\pm.76^{a}$	11.92 ± 0.83^{a}	5.14 ± 0.51^{b}	5.23 ± 0.44^{b}

DHA	23.99±1.12[c]	23.42±1.06[c]	28.04±1.03[a]	26.44±1.01[b]	26.73±1.09[b]
EPA/ARA	3.20±0.12[c]	7.85±0.43[a]	7.62±0.52[a]	3.98±0.18[b]	4.40±0.37[b]
DHA/EPA	5.04±0.33[a]	2.09±0.09[b]	2.35±0.11[b]	5.15±0.19[a]	5.11±0.30[a]
DHA/ARA	16.18±1.12[c]	16.46±1.10[c]	17.91±1.09[c]	20.50±1.01[b]	22.49±1.07[a]
n-3/n-6	3.13±0.32[c]	6.37±0.61[b]	12.15±0.82[a]	3.19±0.12[c]	2.18±0.09[d]

The level of larval lipid peroxides (Figure 5.9), as indicated by the MDA content (nmol/g larval tissues), was significantly higher in the larvae fed 9 MPL, 7 SBL and 9 SBL diets than in control and 7 MPL larvae. Besides, this increase was positively correlated to the sum of dietary n-3, n-6 and n-9 fatty acids ($y = 0.032x + 4.9457$, $R^2 = 0.78$), and also was positively correlated to the dietary PL content for each PL source (Figure 5.10).

The general pattern of antioxidant enzyme genes expression of seabream larvae was characterized by a higher expression in larvae fed SBL diets than larvae fed control and MPL diets (Figures 5.11, 5.12 and 5.13). Thus, the highest CAT and SOD expression was found in larvae fed SBL, followed by that of 9 MPL larvae. There were no significant differences between larvae fed 7 SBL and Control diets. GPX expression level was also significantly highest in the larvae fed SBL, followed by that of 9MPL and then 7MPL, which was significantly higher than the control.

The highest expression of the Bone Morphogenetic Protein 4 (BMP-4) gene, an early marker of osteoblast differentiation, was found in larvae fed MPL, regardless the dietary level tested (Figure 5.14), however this transforming growth factor was not affected by the dietary inclusion of SBL. Runx2, a key regulator of both chondrocyte and osteoblast differentiation, was significantly up-regulated in larvae fed MPL in comparison to the control, whereas in fish fed SBL there were no significant differences with the control or MPL larvae (Figure 5.15). ALP, an early marker of cell mineralization, was significantly up-regulated in larvae fed MPL diets, whereas no significant differences were found for the expression of these gene between SBL fed larvae and any of the other larvae (Figure 5.16). In comparison with the control larvae, the inclusion of dietary PL increased up to 7 times the gene expression of osteocalcin (OC), a skeletal extracellular matrix protein considered an specific marker of late osteoblast differentiation (Figure 5.17). The highest up-regulation was obtained in larvae fed MPL, followed by those fed 9 SBL and, finally, 7 SBL. Similar trends were obtained for other skeletal extracellular matrix proteins such as

osteopontin (OP) and osteonectine (ON), which are indicative of osteogenesis, although not considered specific markers. Thus, expression of both OP and ON was up-regulated up to double the expression of the control by the dietary MPL and up to 1.5 times by SBL (Figures 5.18 and 5.19). Finally, the expression of matrix GLA protein (MGP), a physiological inhibitor of endochondral ossification, was not significantly affected by dietary PL, except in larvae fed diets 7MPL and 9 SBL, where it was slighlty up-regulated (Figure 5.20).

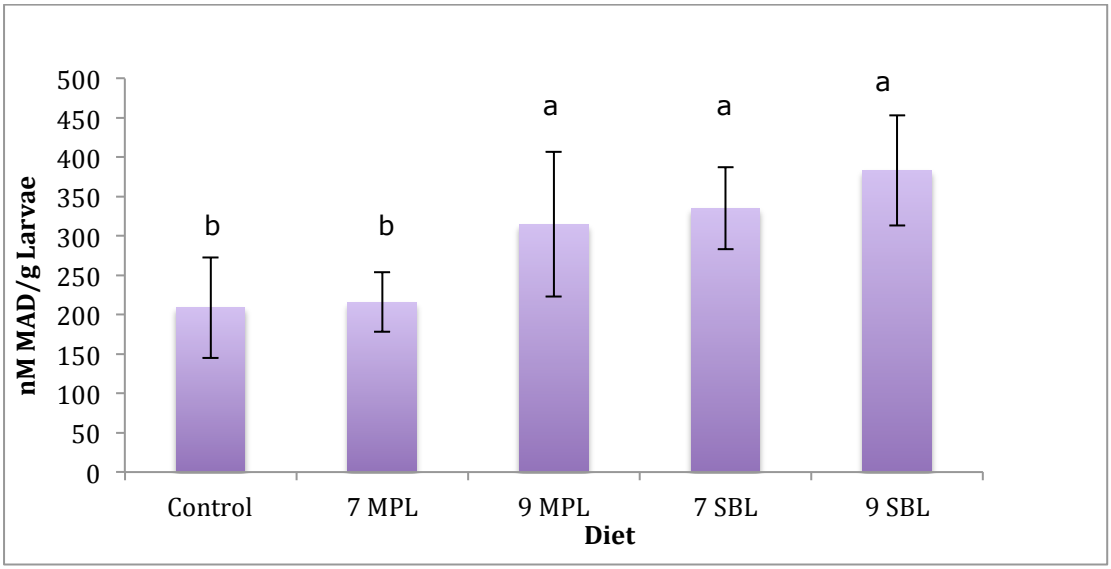

Figure 5.9 The malondialdehyde (MDA) content in larvae (44 dph) fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

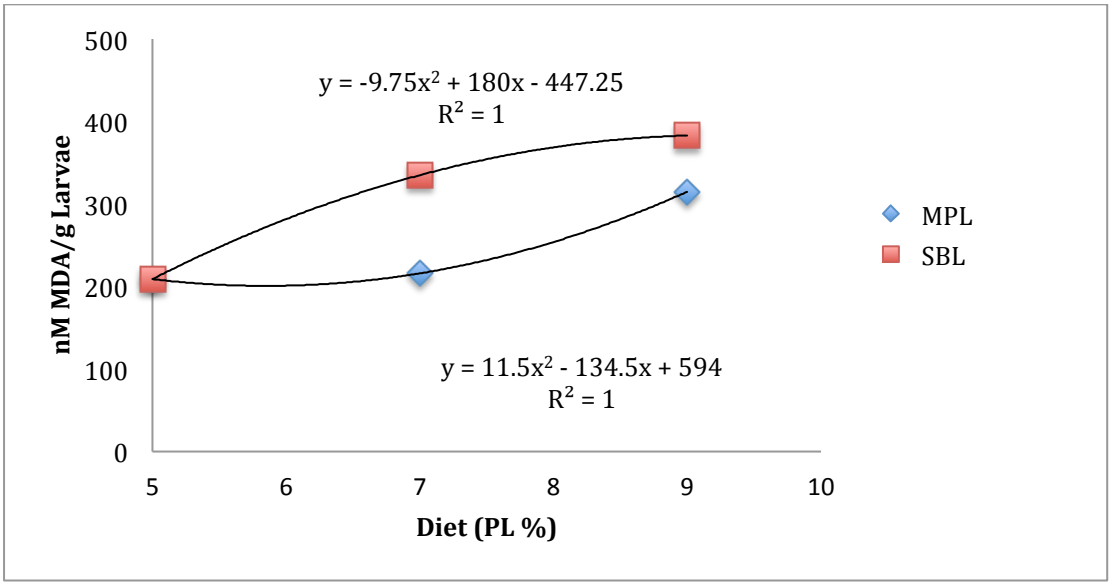

Figure 5.10 The correlation between the malondialdehyde (MDA) content and the dietary phospholipid levels in larvae (44 dph) fed five dietary phospholipid levels.

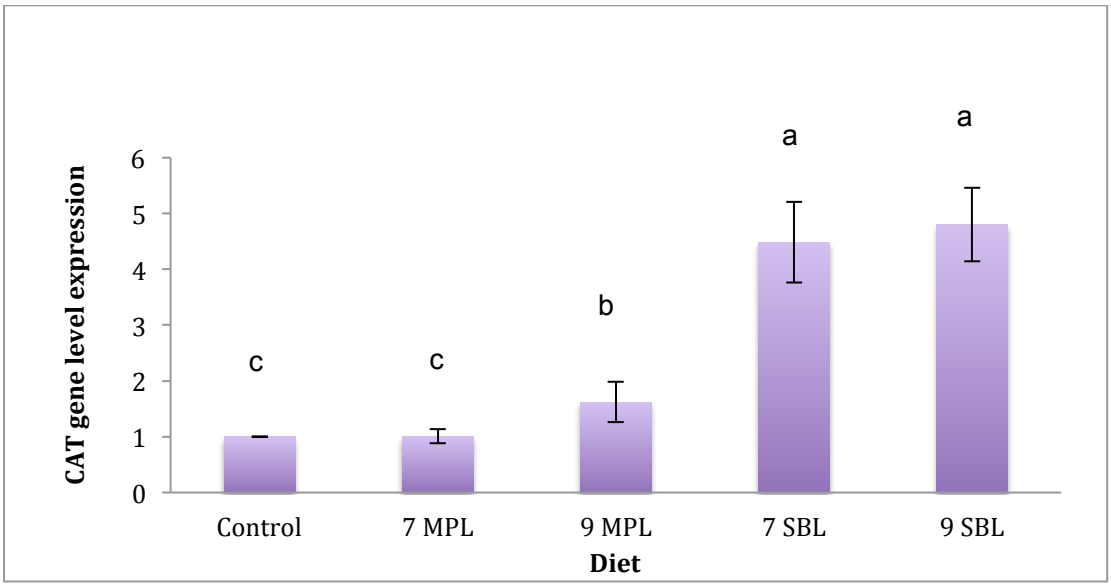

Figure 5.11 Catalase (CAT) gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

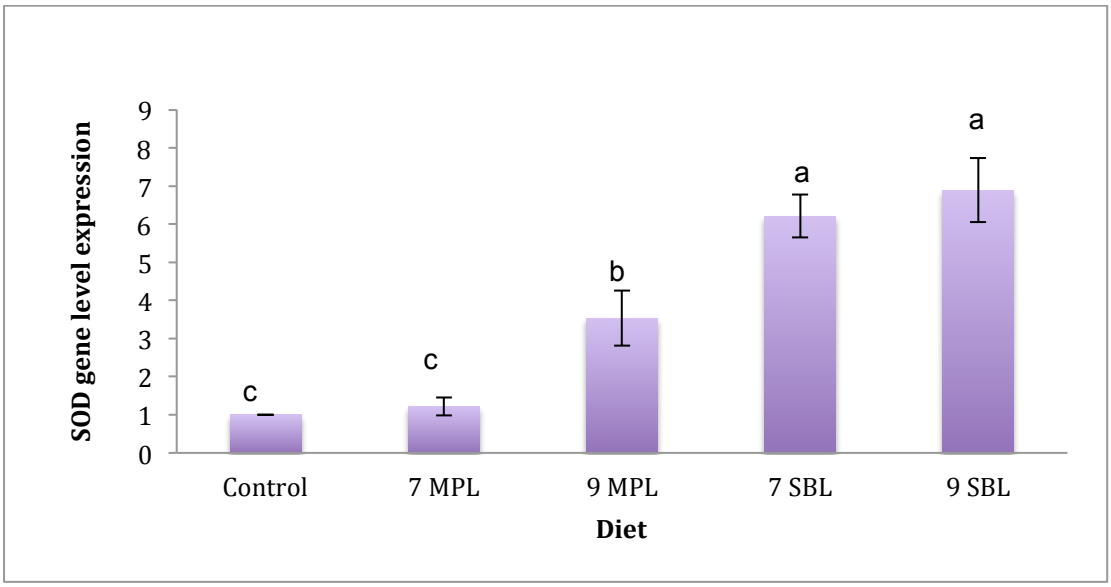

Figure 5.12 Superoxide dismutase (SOD) gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

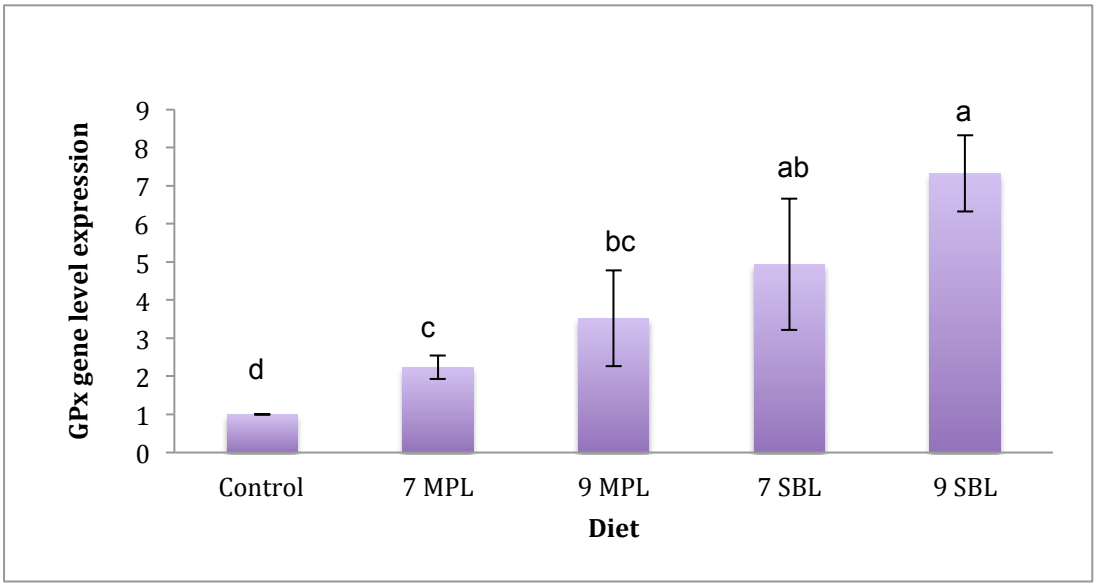

Figure 5.13 Glutathione peroxidase (GPX) gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

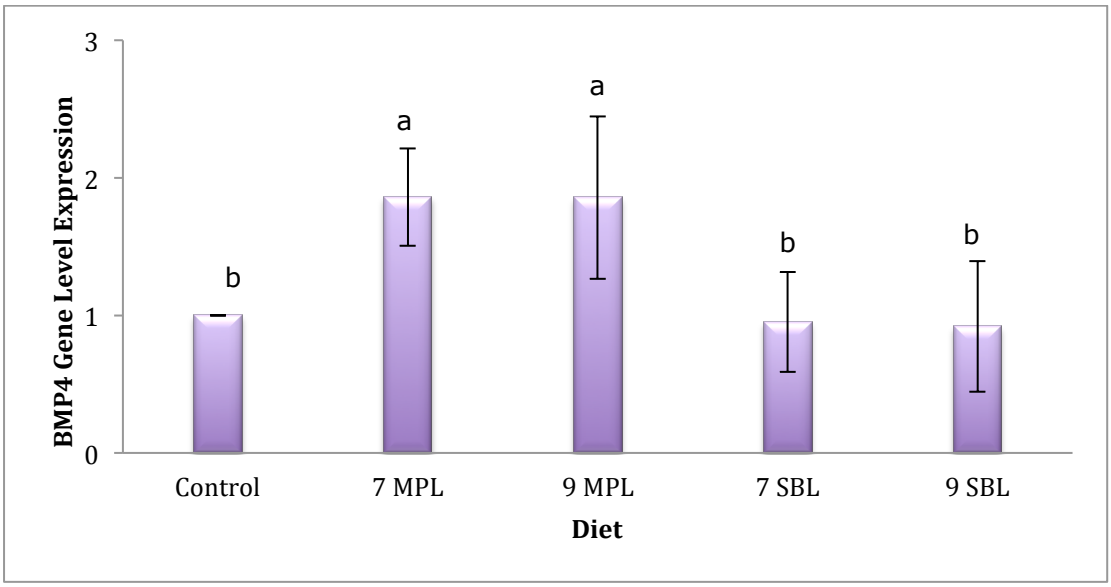

Figure 5.14 BMP4 (Bone Morphogenetic Proteins 2) gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

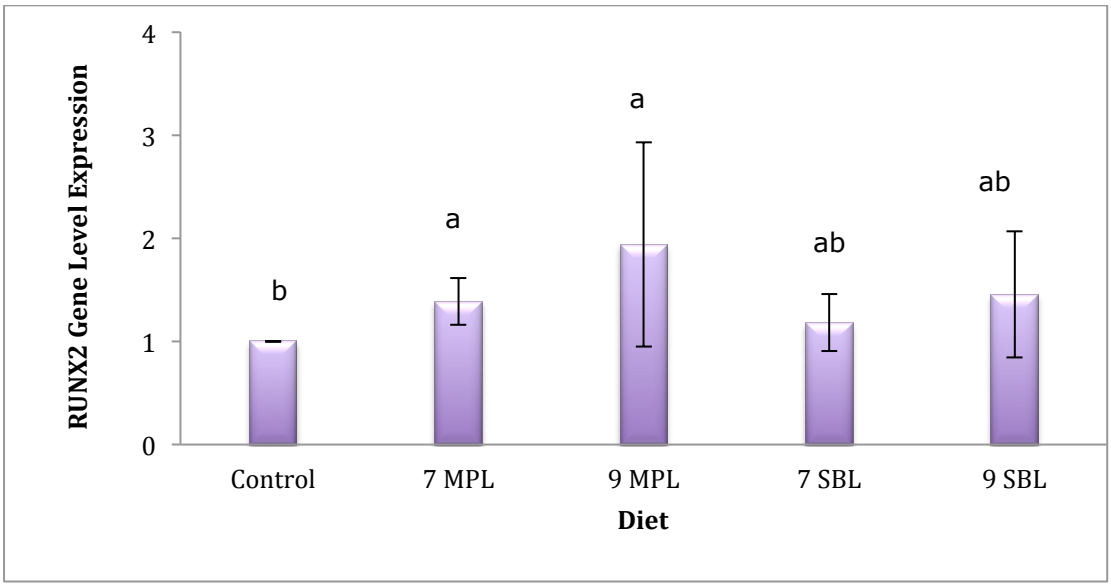

Figure 5.15 Runx2 (Runt-related transcription factor 2) gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

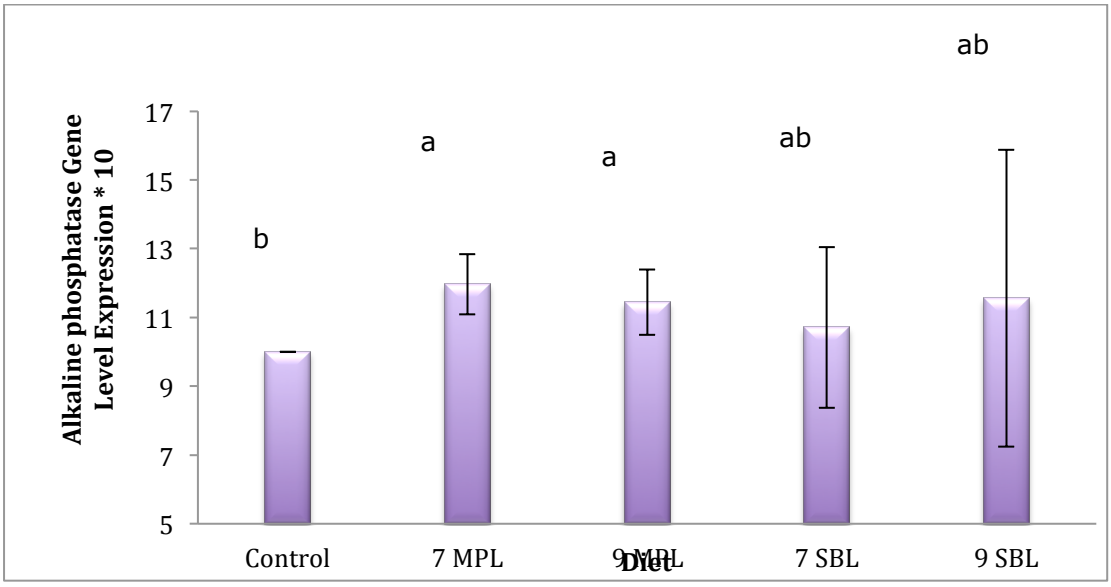

Figure 5.16 Alkaline Phosphatase gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

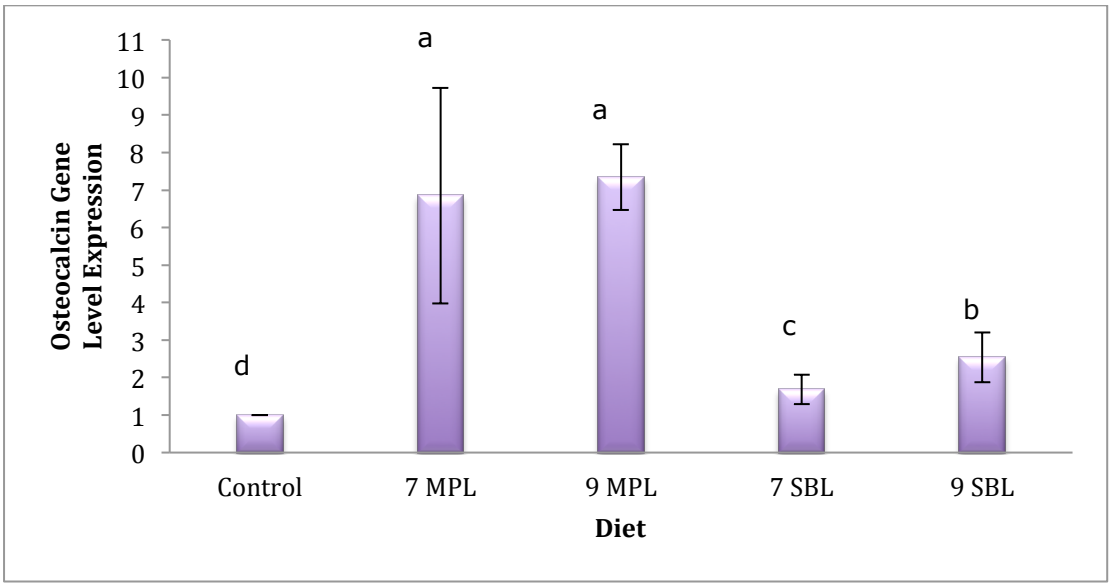

Figure 5.17 Osteocalcin gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

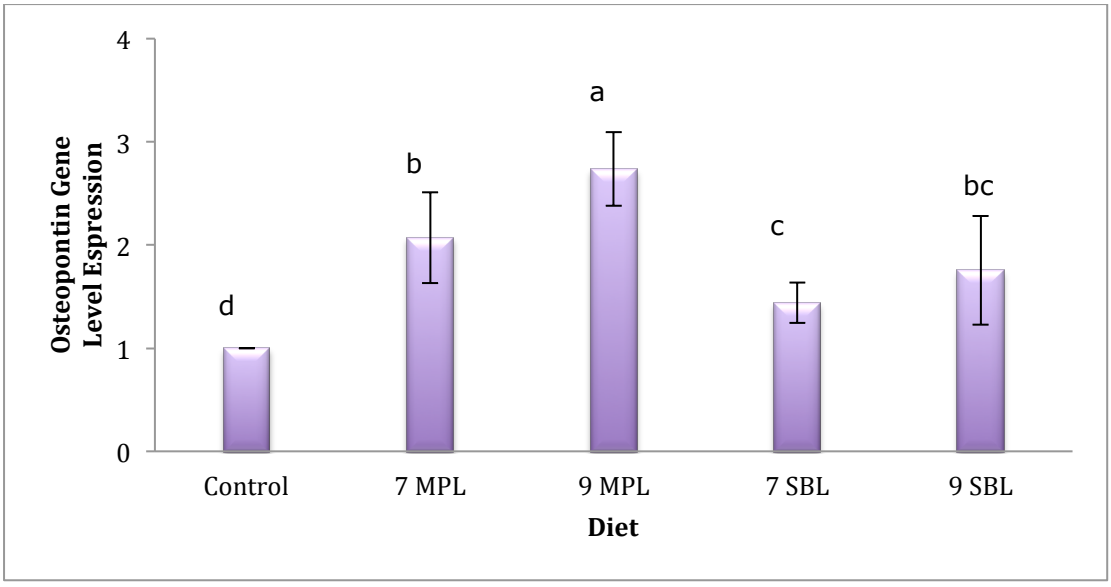

Figure 5.18 Osteopontin gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

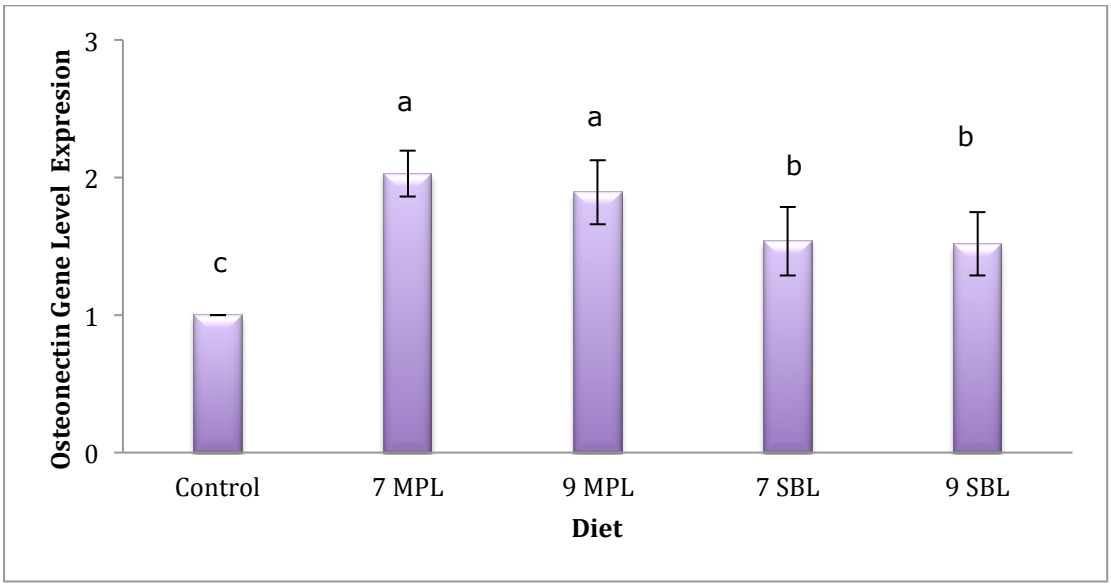

Figure 5.19 Osteonectin gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

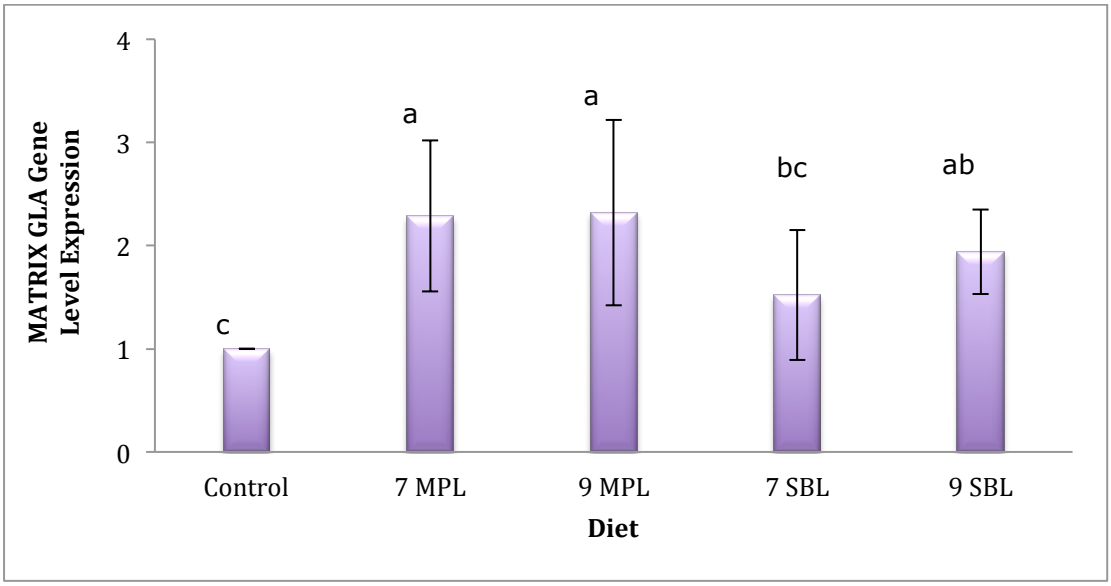

Figure 5.20 Matrix Gla Protein gene expression level measured by real-time PCR in seabream larvae fed five dietary phospholipid levels. Values (mean ± standard deviation) with the same letters were not significantly different (P>0.05).

Discussion

Addition of SBL to the early weanning diets for gilthead seabream raised the dietary contents of PL, particularly PI, PC and PE, and improved seabream survival and stress resistance, promoting the incorporation of dietary DHA into larval polar lipids. These results could be due to the enhancement of digestive enzymes activities, particularly, PLA2 and alkaline phosphatase (Saleh *et al*., 2012b) and gut and liver lipid transport activity (Liu *et al*., 2002) obtained by the inclusion of SBL in previous studies,which would improve the absorption of several nutrients from the lumen (Morais *et al*., 2006). Thus, improved lipid digestion, absorption and transport would be responsible for the high incorporation of n-3 HUFA, particularly DHA into polar lipids of larvae fed increased levels of SBL. Cahu, *et al*. (2003a) found that soybean lecithin incorporation (12% PL dietary dry weight), supported high survival to European sea bass *Dicentrarchus labrax*. However, only the elevation of dietary SBL up to 9% PL improved larval growth. This is in agreement with previous studies, where elevation of dietary PL over 9% with SBL did not further improved seabream larval growth (Saleh *et al*., 2012b).

However, improvement in either larval growth or survival was significantly lower than that obtained by MPL in the present study. Dietary inclusion of SBL, rich in linoleic acid and PI, markedly increased the oxidative risk of gilthead seabream larvae as denoted not only by the higher MAD values obtained, but also by the increase in the gene expression of enzymes involved in the oxidative metabolism such as SOD, CAT and GPX. Despite inclusion of the highest MPL level (9% PL) also raised the MAD values, the expression of CAT, SOD or GPX genes was significantly lower than for SBL fed larvae, and only slightly higher than in the control larvae, suggesting the presence of antioxidant factors in the MPL source used, such as carotenoids. The krill oil is rich in antioxidants factors such as carotenoids (astaxanthin) (Tou *et al*., 2007).

In agreement with the improved growth obtained by increased levels of marine phospholipids in other species such as Atlantic cod (*Gadus morhua*) and European sea bass (*Dicentrarchus labrax*), in the present study, the MPL seemed to be superior to enhance larval growth and development when incorporated into microdiets than SBL. Thus, even the inclusion of only 7 % PL as MPL improved survival, stress

resistance and growth of gilthead seabream in a larger extend than SBL, what could be related to their higher content in PC and LPC rich in DHA and EPA, in comparison to SBL diets. Indeed growth improvement obtained by dietary PL increase has been previously associated to an enhancement in feed intake associated to the estimulation of gustatory response by the trimethyl group of the choline base of PC (Izquierdo and Koven, 2010). Despite it is well known the potent effect of these fatty acids in promoting larval growth and survival, the higher performance of fish fed MPL seems to be due not only to a higher dietary content on these fatty acids. For instance, feeding seabream larvae with two weaning diets containing the same PL (2.5%) and n-3 HUFA (1.6%) levels, the diet containing these fatty acids in the PL fraction caused a significantly higher growth than those fed these fatty acids in the NL (Salhi *et al*., 1999; Izquierdo *et al*., 2001). Moreover, a better utilization of dietary n-3 HUFA is obtained as denoted by the higher incorporation of these fatty acids in the larval PL (Izquierdo *et al*., 2001). These authors found that dietary n-3 HUFA rich PL markedly improves the incorporation of free EPA, but not of free oleic acid, into larval polar lipids (Izquierdo *et al*., 2001), in relation to improved lipid transport, mobilization and deposition in the peripheral tissues by n-3 HUFA rich dietary PL. More specifically, the higher LPC rich in DHA, could specifically promote the higher incorporation of HUFA into larval polar lipids, as it has been found in mammal models. For instance, DHA incorporation into PC of rat brain cells was higher when LPC-DHA was used instead of unesterified DHA and LPC-16:0 (Bernoud *et al*., 1999), suggesting an intact and specific uptake of DHA-PC that was directly re-acilated into PC. The improvement in larval growth could be also related to a faster maturation of the gut suggested by the significant increase in alkaline phosphatase activity in previous studies (Wold *et al*., 2007; Saleh *et al*., 2012a, b) and a better digestive efficiency due to the stimulatory effect of dietary MPL on PLA2 and neutral lipase activity (Saleh *et al*., 2012a). Thus, marine PL rich in HUFA may increase the microdiet intake, promote gut development and nutrient digestion, facilitate direct incorporation of HUFA into polar lipids, and thus, enhance growth, survival and stress resistance in marine fish larvae.

Inclusion of PL in the seabream weaning diets significantly reduced the lordosis incidence, regardless the type of PL or the dietary PI level, despite in carp this PL class was proposed to reduce skeletal deformities (Guerden et al., 1998a). In the present study, the elevation of PI in SBL diets in comparison to control or MPL diets

did not reduced the incidence of deformities. On the contrary, lordosis incidence seemed to be lower in larvae fed higher PE, rich in DHA, and with high DHA incorporation into larval PL.

Lordosis incidence was neither related to the dietary PC/PI ratios, which were either larger in control and MPL diets than the 2.18 reccommended by Cahu *et al*. (2003b) for European sea bass, or lower in diet with 9% SBL in the present study. Regarding kyphosis the lowest incidence was found in larvae fed 9% PL as MPL, the diet with the highest DHA content, and also showed the highest ARA contents in the larval PL fraction. In addition there was not any incidence of cranial deformities in this fish.

The lowest incidence of skeleton deformities found in fish fed 9% PL as MPL could be related to a higher resistance to vertebral deformities by a stronger mineralization as suggested by the highest expression in this fish of the skeletal extracellular matrix proteins, significantly osteopontine, in relation to the higher dietary DHA content.

Indeed, larvae fed MPL showed higher expression of BMP-4 gene, a member of the transforming growth factor b superfamily considered as important regulators of the differentiation of uncommitted mesenchymal cells into osteoblasts during both embryonic development and bone repair. These genes are up-regulated by dietary n-3 HUFA being beneficial for other vertebrates (Kruger *et al*., 2010). In agreement in the present study no maxilar or mandibular deformities were registered in fish fed 9% PL as MPL. Similarly, Runx2, the earliest and most specific marker of osteogenesis, is regulated by dietary DHA and possibly also other n-3 HUFA (Kruger *et al*., 2010). In turn, Runx2 regulates the expression of ALP and OC, both being up-regulated in larvae fed MPL. Thus, n-3 HUFA increase ALP activity in growing male rats as well as insulin-like growth factor I (IGF-1) (Kruger *et al*., 2010). In gilthead seabream, increased in dietary DHA up-regulated IGF-1, enhanced bone mineralization and significantly reduced the incidence of vertebral anomalies such as lordosis and kyphosis (Izquierdo *et al*., 2012).

The n-3 HUFA has a role in promoting bone formation in mammals as well as in fish (Lall and Lewis-McCrea, 2007), but these mechanisms are poorly studied in fish. Feeding dietary n-3 HUFA to growing male rats increased rates of bone formation (Watkins *et al*., 2000). Increasing dietary DHA also increased the ratio of n-3/n-6 in bone compartments, which favored bone formation in rats (Watkins *et al*., 2003, 2006). Larvae fed 9 MPL and 7 MPL diets contained relatively more n-3 HUFA than

the 7 SBL and 9 SBL larvae, thus increasing the ratio of n-3/n-6 to 8.19% and 11.3% respectively.

In summary, despite increased on dietary SBL up to 9% improve larval survival, stress resistance, growth and skeletal development, dietary MPL was more effective in promoting all these parameters. Their higher content in PC, LPC, ARA, EPA and, particularly, DHA, not only promotes digestion, transport and deposition of dietary lipids, but also contributes to reduce skeleton anomalies by up-regulating BMP-4, Runx2, ALP and the skeletal extracellular matrix proteins, inducing early mineralization and resistance of vertebral bodies to reduce anomalies such as lordosis and kyphosis. Inclusion of SBL markedly increased the proxidation risk as denoted by the higher TBARs in larvae, as well as a higher expression of CAT, GPX and SOD genes.

Acknowledgement

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Chapter 6

Biomarkers of bone development and oxidative stress in gilthead seabream larvae fed microdiets with several levels of polar lipids and α–tocopherol

Abstract

Although dietary marine phospholipids are able to improve culture performance of marine fish larvae in a further extend than soybean lecithin, both types of phospholipids (PL) markedly increase oxidative risk. The inclusion of a fat-soluble antioxidant such as the vitamin E α-tocopherol could allow a better control of oxidative stress. The objective of the present study was to determine the combined effect of graded levels of α-tocopherol with different levels and sources of krill phospholipids (KPL) and soybean lecithin (SBL) on growth, survival, resistance to stress, oxidative status, bone metabolism related genes expresion and biochemical composition of seabream larvae. Seabream larvae were completely weanned at 16 dph and fed for 30 days seven microdiets with three different levels of PL (0, 4 and 8%) and two of α-tocopherol 1500 and 3000 mg kg^{-1} diet. Seabream larvae fed diets without PL supplementation showed the lowest survival, growth and stress resistance, whereas increase in PL, particularly KPL, markedly promoted larval survival and growth. However, feeding SBL markedly increased TBARs and GPX gene expression increasing the peroxidation risk in the larvae. Besides, KPL inclusion improved incorporation of n-3 HUFA and, particularly, EPA into larval tissues, these fatty acids being positively correlated to the expression of BMP-4, RUNX 2, ALP, OC and OP genes and to bone mineralization for a given larval size class. The increase in dietary α-tocopherol tend to improve growth in relation to the n-3 HUFA levels in the diet, denoting the protective role of this vitamin against oxidation. Indeed, dietary α-tocopherol decreased the oxidative stress in the larvae as denoted by the reduction in

larval TBARs contents and gene expression of SOD and CAT, but not GPX. Thus, increase in dietary α-tocopherol effectively prevented the formation of free radicals from HUFA, particularly EPA, but did not affected the incidence of bone anomalies or the expression of genes related to osteogenetic processes.

Introduction

Good quality fry is essential for the further development of marine fish culture, but the success of fry high production is greatly affected by the nutritional quality of the initial larval diets (Kolkovski *et al.,* 2009; Izquierdo *et al.,* 2000). Dietary phospholipids are fundamental components of early weaning diets (Izquierdo, 1996; Fontagné *et al*., 2000; Izquierdo and Koven, 2010). Not only they are and essential constituents for cell membranes and tissues and organ development but also play an important role as energy sources (Kanazawa, 1993; Izquierdo *et al.,* 2001). Thus, phospholipid addition to larval diets promote growth and survival and increase fish resistance to stress (Kanazawa 1993; Salhi *et al.,* 1999; Izquierdo *et al.,* 2001; Kjørsvik *et al.,* 2009). In gilthead seabream (*Sparus aurata*), the increase in dietary soybean lecithin up to 8 % improves larval growth, promotes gut development as suggested by higher alkaline phosphatase activity, and improves dietary lipid utilization enhancing lipase and phospholipase activities (Saleh *et al*., 2012b). However, in their natural preys, phospholipids are rich in n-3 highly unsaturated fatty acids (n-3 HUFA) (Sargent *et al*., 1997) and accordingly, inclusion of phospholipids of marine origin, rich in these essential fatty acids, further improves larval performance in comparison to soybean lecithin (Betancor *et al*., 2012b; Saleh *et al*., submitted). Therefore, the dietary inclusion of 7% marine phospholipids improves seabream performance further than soybean lecithin (Saleh *et al*., submitted). Moreover, soybean lecithin increases the peroxidation risk causing higher larval TBARs and expression of antioxidant enzymes genes (Saleh *et al*., submitted). Nevertheless, increase in dietary marine phospholipids up to 9% also increased larval TBARs (Saleh *et al*., submitted).

Lipid peroxidation, is recognized as being highly deleterious, producing toxic compounds such as fatty acid hydroxyperoxides, fatty acid hydroxides, aldehydes and hydrocarbons, resulting in damage to cellular biomembranes (Kanazawa, 1991, 1993)

and subcellular membranes, such as those of mitochondria, causing several pathological conditions (Watanabe *et al*., 1970; Murai and Andrews, 1974; Sakai *et al*., 1989). To counteract this oxidative risk, fish have an endogenous antioxidant defence system (Filho *et al*., 1993), namely radical scavenging enzymes that play an important role in physiological antioxidant protection (Blazer, 1982) such as catalase (CAT) and superoxide dismutase (SOD), acting on hydrogen peroxide and superoxide, respectively, and glutathione peroxidase (GPX), which scavenges lipid hydroperoxides (Winston and Di Giulio, 1991; Halliwell and Gutteridge, 1996). Besides, α-tocopherol acts as well as an antioxidant defence factor, reducing lipid peroxidation products and free radicals (Machlin, 1984). α-tocopherol is one of the lipid soluble vitamins that are absorbed from the digestive tract in association with fat molecules and can be stored in body fat reserves. α-tocopherol is a structural component of cell membranes (Putnam and Comben, 1987), and improves the stability of tissue lipids to oxidation (Stéphan *et al*., 1995). Several studies have demonstrated that α-tocopherol is an essential nutrient for different fish species (Watanabe *et al*., 1970; Murai and Andrews, 1974; González *et al*., 1995). Dietary α-tocopherol and n-3 HUFA had a synergistic effect on the non-specific immune responses and disease resistance (Wang *et al*., 2006, Hamre *et al*., 1994). In marine fish larvae, feeding a high level of docosahexaenoic acid (DHA), an essential polyunsaturated fatty acid that are very susceptible to oxidation, together with a low level of α-tocopherol led to muscular lesions in sea bass larvae (Betancor *et al*., 20111). However, an increase in α-tocopherol or decrease in DHA level reduced the rate of muscular damage (Betancor *et al*., 2011). 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). A dietary requirement of α-tocopherol has been demonstrated as 120 mg/kg diet for Atlantic salmon (Hamre and Lie, 1995), 200 to 300 mg/kg diet for common carp (*Cyprinus carpio)* (Watanabe *et al*., 1977), 1200 mg/kg diet for gilthead seabream and 3000 mg/kg diet for gilthead seabream larvae (Ortuño *et al*., 2000, Atalah *et al*., 2011).

Therefore, high levels of dietary HUFA, specially when the antioxidants compounds are not well balanced, may lead to several changes in fish, including increased intracellular reactive oxygen species (ROS) production that may ultimately damage DNA, proteins and lipids (Halliwell and Gutteridge, 1999). Also, ROS can affect the

transcription of many genes, either acting via various transcription factors or directly as a result of oxidative damage (Di Giulio and Meyer, 2008). 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). Also, dietary HUFA affect the expression of numerous genes involved in cellular growth and differentiation such as Insulin Growth Factor-I (IGF-I), and some genes involved in early development of vertebrates and in osteoblast differentiation such as Bone Morphogenetic Protein (BMP4) (Cahu *et al*., 2009). Thus, to avoid adverse effects and improve high dietary HUFA performance, supplementation of antioxidants, such as α-tocopherol are necessary to larval diets (Betancor *et al*., 2011). 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 is reduced when dietary α-tocopherol was supplemented in the diet.

The aim of the present study was to investigate the combined effect of graded levels of α-tocopherol with marine phospholipids derived from krill (KPL) or soybean lecithin (SBL) in early weaning microdiets on culture performance, resistance to stress, oxidative status and bone formation and mineralization of gilthead seabream larvae.

Materials and methods

Gilthead seabream larvae were obtained from natural spawnings from Instituto Canario de Ciencias Marinas (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain). Larvae, previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 16 dph (5.46±0.31 mm total length, 113±7 µg dry body weight), were randomly distributed in 21 experimental tanks at a density of 2100 larvae tank^{-1} and fed one of the diets tested in triplicate. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure

water renewal and maintain high water quality, which was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min-^{1}) attaining 6.4±1 ppm dissolved O_2. Average water temperature and pH along the trial were 19.8±1.3°C and 7.89, respectively. Photoperiod was kept at 12h light: 12h dark, by fluorescent daylights and the light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia).

Seven experimental microdiets (pellet size 250-500 µm) using krill oil (Qrill, high PL, Aker BioMarine, Fjordalléen, Norway) and soybean lecithin (Acofarma, Barcelona, Spain) as sources of PL, and α-tocopherol (Sigma-Aldrich, Madrid, Spain) as an antioxidant vitamin. Their formulation and proximate analysis are shown in Table 6.1, wheras the fatty acids profile is showed in Table 6.3. All diets were formulated to sufficiently cover the gilthead seabream requierements for essential fatty acids (Izquierdo and Koven, 2010).

Table 6.1 Ingredients and proximate composition of the experimental microdiets

Ingredients (g kg^{-1} Diet)	0KPL/ 1500	0KPL/ 3000	4KPL/ 1500	4KPL/ 3000	8KPL/ 1500	8KPL/ 3000	8SBL/ 3000
Squid Powder[a]	69	69	69	69	59.5*	59.5*	69.5
PLO[b]	0	0	13	13	21	21	0
Soybean Lecithin[c]	0	0	0	0	0	0	8.5
Sardine oil[d]	5.5	5.5	0	0	3	3	0
Oleic acid[e]	9	9	1.5	1.5	0	0	5.5
α-tocopherol[f] (mg/100g Diet)	150	300	150	300	150	300	300
Gelatin	3	3	3	3	3	3	3
Mineral mix	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Vitamin mix	6	6	6	6	6	6	6
Attractants	3	3	3	3	3	3	3
Proximate analysis % dry weight							
Total lipids	22.81	23.29	23.94	24.22	25.55	25.42	25.58
Polar lipids	5.91	6.44	8.92	9.51	10.37	10.81	11.13
Protein	61.06	61.31	60.50	60	58.80	58.92	60.31
Ash	7.04	6.93	7.10	6.98	7.26	6.94	6.80
Humidity	7.93	8.10	8.10	7.93	8.03	7.93	8.13

a Rieber and Son, Bergen, Norway. *Defatted.
b Qrill, high phospholipids, Aker BioMarine, Fjordalléen, Norway.
c Acofarma, Barcelona, Spain.
d Agramar S.A., Spain.
e Merck KGaA, Darmstadi, Germany.
f α-tocopherol acetate, Sigma-Aldrich, Madrid, Spain.

The microdiets were prepared by mixing squid powder and water-soluble components, then the lipids and fat-soluble vitamins and, finally, gelatine dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain a particle size between 250 to 500 µm. Diets were prepared and analyzed for proximate and fatty acid composition at GIA laboratories.

Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00 for 29 days. Non-enriched rotifers were co-fed during days 16th and 17th (1 rotifer/ml). To assure feed availability, daily feed (pellet size < 250 µm) supplied was maintained at 1.5 and 2.5 g per tank during the first and second week of feeding. The amount of feed added daily was gradually increased to 4-5 g per tank with increasing in pellet size to 250–500µm, with an overlap of both pellets sizes during the third and forth weeks of feeding. Larvae were observed under the binocular microscope to determine feed acceptance. Before the end of the experiment an activity test of air exposure was conducted by handling 25 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 living larvae at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 h) and total length (Profile Projector V-12A Nikon, Tokyo, Japan) of 30 fish $tank^{-1}$ at the beginning, in the middle and at the end of the trial. In addition, at the end of the trial and after 12 h of starvation, the all larvae in each tank were washed with distilled water, sampled and kept at -80 ºC for biochemical composition.

Fresh larvae samples (100 mg per tank) for molecular biology analysis were well washed and conserved into 500 ml RNA Later (SIGMA), a product that rapidly allows tissue to stabilize and protect cellular RNA in situ in unfrozen samples and permits to posterior RNA isolation. The larvae were stored in RNA later overnight at 4 ºC and after that RNA Later was removed before storing samples at - 80 ºC to prevent the formation of salt crystals.

Moisture (A.O.A.C. 1995), protein (Kjeldhal) and crude lipid (Folch 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC/min and then held at 215 ºC for 10 min. Fatty acid methyl esters were quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) following the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

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}.

Molecular biology analysis was carried out at GIA laboratories. Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30ml of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using

the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15,1:20 and 1:25). The product size of the real-time q PCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using β-actin as the house-keeping gene in a final volume of 20ml per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analysed once per gene.

Table 6.2 Sequences of forward and reverse primers (5'-3') for real-time quantitative-PCR of seabream

Gene	Primers	Accession n^{o}
Catalase(CAT)	Forward primer: 5´-ATGGTGTGGGACTTCTGGAG-3´ Reverse primer: 3´-AGTGGAACTTGCAGTAGAAAC-5´	FJ860003
Superoxide dismutase (SOD)	Forward primer: 5´- AAGAATCATGGCGGTCCTACTGA-3´ Reverse primer: 3´- TGAGCATCTTGTCCGTGATGTCT -5´	AJ937872
Glutathione peroxidase (GPX)	Forward primer: 5´- TCCATTCCCCAGCGATGATGCC-3´ Reverse primer: 3´- TCGCCATCAGGACCAACAAGGA-5´	DQ524992
Osteocalcin	Forward primer: 5´-AGCCCAAAGCACGTAAGCAAGCTA-3´ Reverse primer: 3´- TTTCATCACGCTACTCTACGGGTT-5´	AF048703
Osteopontin	Forward primer: 5´- AAGATGGCCTACGACATGACAGAC- 3´ Reverse primer: 3´- CCTGAAGAGCCTTGTACACCTGC-5´	AY651247
Osteonectin	Forward primer: 5´- AAAATGATCGAGCCCTGCATGGAC-3´ Reverse primer: 3´- TACAGAGTCACCAGGACGTT-5´	AY239014
[a]RUNX2	Forward primer: 5´- GCCTGTCGCCTTTAAGGTGGTTGC-3´ Reverse primer: 3´- TCGTCGTTGCCCGCCATAGCTG-5´	AJ619023
Alkaline phosphatase	Forward primer: 5´- AGAACGCCCTGACGCTGCAA-3´ Reverse primer: 3´- TTCAGTATACGAGCAGCCGTCAC-5´	AY266359
Matrix Gla protein	Forward primer: 5´- GTGCCCTCCTTCATTCCAC-3´ Reverse primer: 3´- TATGACCACGTTGGATGCCT-5´	AY065652
[b]BMP4	Forward primer: 5´- CCACCAGGGCAGACACGTCC-3´ Reverse primer: 3´- GCGTAGCTGCTCCCAGTCCTC-5´	FJ436409
β-actin	Forward primer: 5´- TCTGTCTGG ATC GGAGGCTC-3´ Reverse primer: 3´- AAGCATTTG CGGTGGACG -5´	X89920

a RUNX2 (Runt-related transcription factor 2).
b BMP-4 (Bone morphogenetic protein 4).

The PCR conditions were the following: 95ºC for 3min 30sec followed by 40 cycles of 95ºC for 15sec, 61ºC for 30sec, and 72ºC for 30sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalised and the Livak method ($2^{-\Delta\Delta Ct}$) used to determine relative mRNA expression levels. Gilthead seabream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular

Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in Table 6.2.

All data were tested for normality and homogeneity of variances with Levene´s test, not requiring any transformation and were treated using one-way ANOVA. Means compared by Duncan's test ($P < 0.05$) using a SPSS software (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA).

Results

All the experimental diets were well accepted by gilthead seabream larvae according to the microscopic observations. Generally, survival was very high for this type of studies and it was significantly correlated to the dietary phospholipids (Figure 6.1). Thus, average survival of larvae fed 4KPL and 8KPL diets (59-65% survival rate) was significantly higher than 8SBL, which in turn had a significantly higher survival than 0KPL larvae. The resistance to stress was significantly higher in larvae fed KPL and SBL diets than in larvae fed 0KPL diets (Figure 6.2).

The larval growth after thirty days of feeding showed a good effectiveness of all experimental dietary PL in terms of total length and body weight (Figures 6.3 and 6.4). Addition of KPL significantly improved total length and body weight in a further extend than SBL. The increase of α-tocopherol from 150 to 300 mg/kg diet did not affected larval growth tended to promote larval growth in fish fed 0KPL diets, whereas it markedly improved growth in 4KPL and 8KPL larvae. Thus, the larvae fed 4KPL/3000 or 8KPL/3000 diets showed a significantly higher body weight than 4KPL/1500 or 8KPL/1500 larvae, respectively.

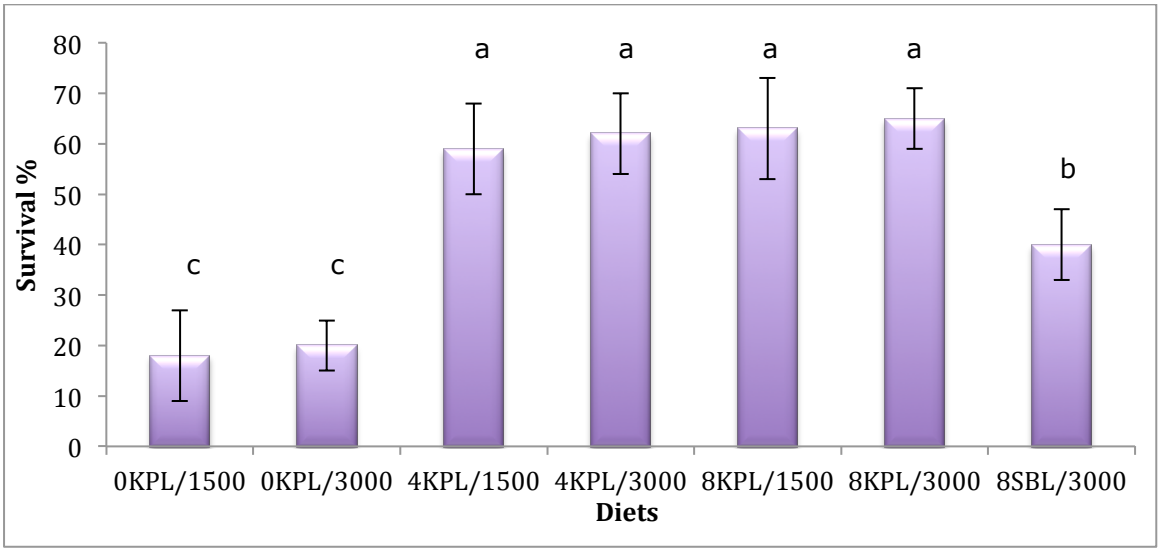

Figure 6.1 Survival rate of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

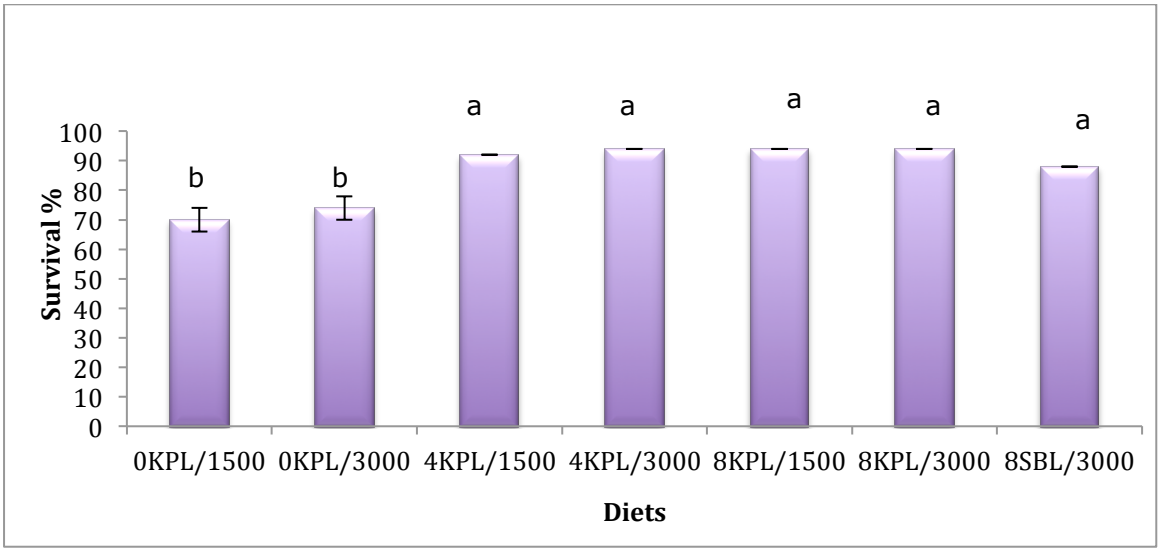

Figure 6.2 Survival rate (24 hours after activity test) of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

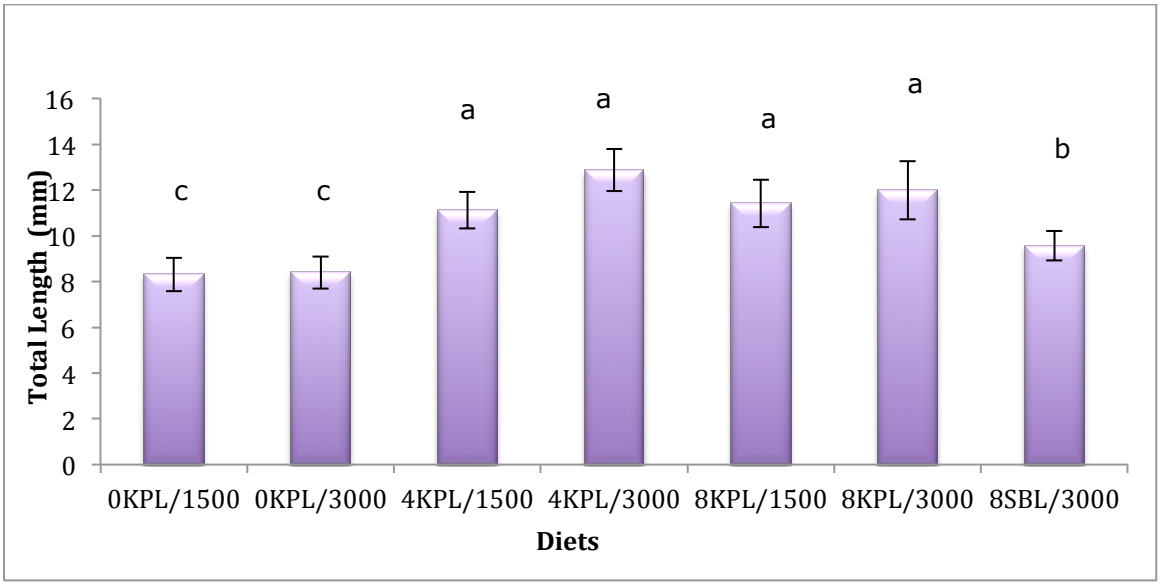

Figure 6.3 Total length of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

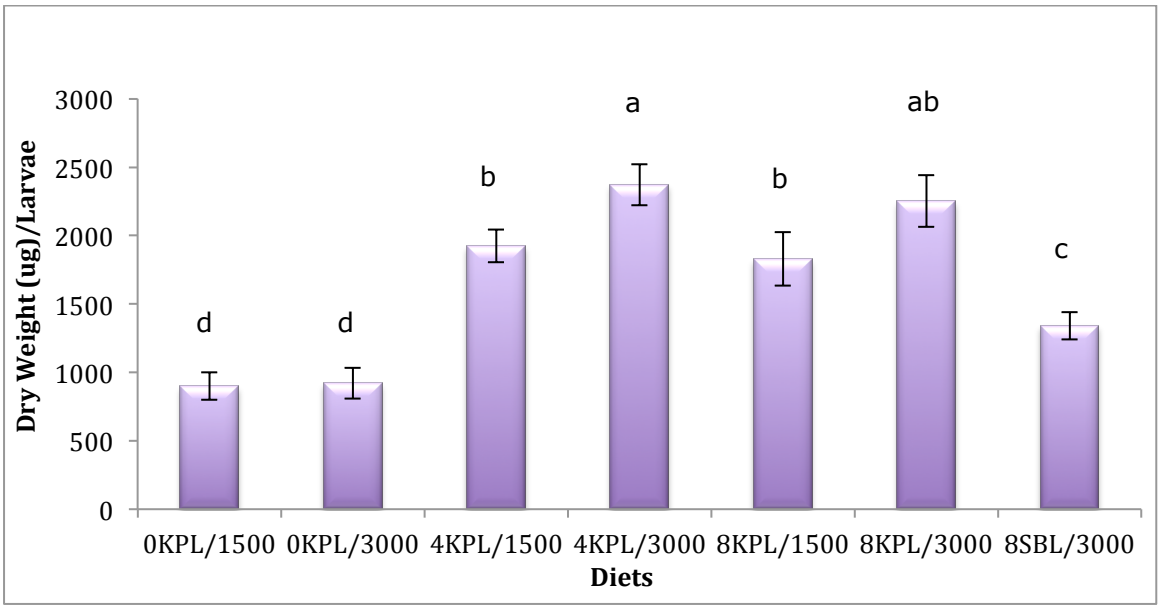

Figure 6.4 Dry weight of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Determination of bone mineralization percentages for the different fish size classes showed that in larvae between 0.8 and 1 cm of total length, those fed 0KPL showed the lowest ossification, whereas increase in either KPL or α-tocopherol markedly enhanced mineralization (Figure 6.5). The highest mineralization percentage was found in larvae fed 4KPL/3000, being significantly higher than in 4KPL/1500 and 0KPL larvae, but not differing from 8KPL larvae. Similarly, in >1 cm larval size, increase in α-tocopherol significantly improved mineralization. Skeleton anomalies could be only determined on mineralized bones, and the results showed in larvae fed 0KPL/1500 a significantly higher incidence of anomalies in caudal fin bones, which were completely mineralized already in all larvae (Figure 6.6). On the contrary, these larvae also showed the lowest incidence of anomalies in vertebral arcs and spines, since in these larvae these bones have not completed mineralization. The increase in KPL or α-tocopherol did not significantly affected vertebral anomalies in the pre-hemal region. Moreover, increase in dietary α-tocopherol tended to reduce the occurrence of chondroid bones anomalies (such as arch/spines), whereas increase in KPL tended to reduce the axial skeletal deformities (such as pre-haemal vertebral bodies anomalies).

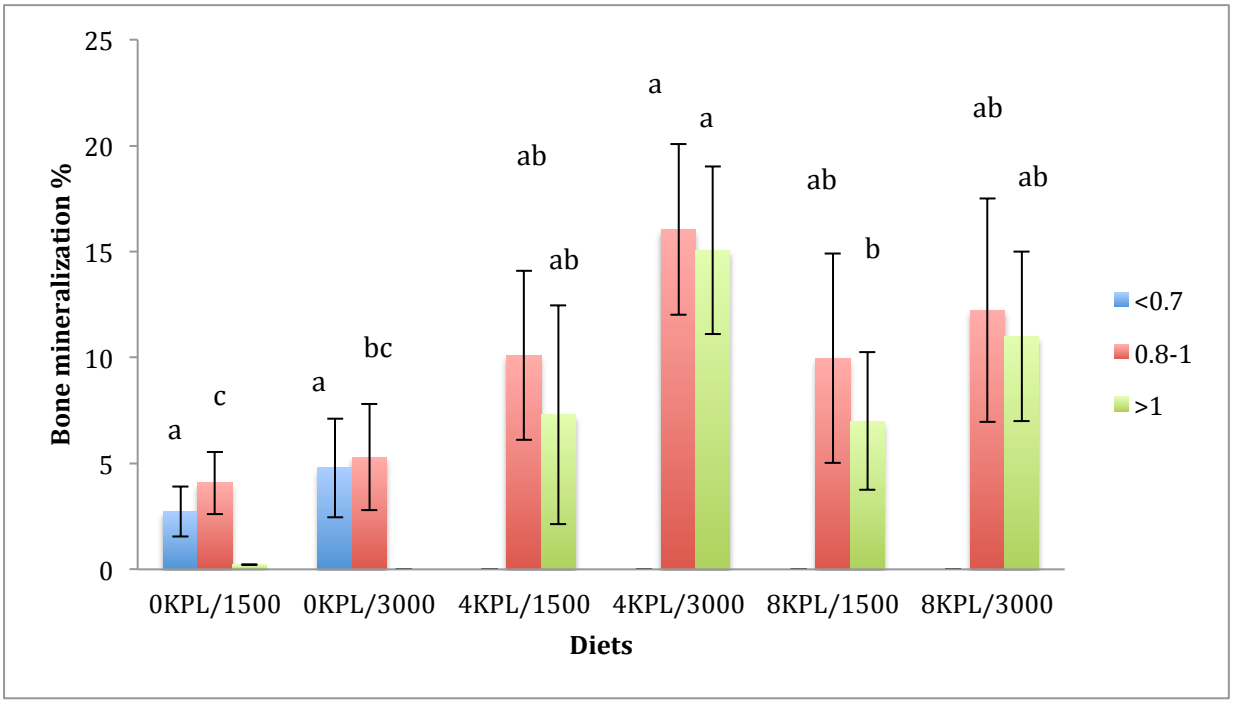

Figure 6.5 Bone mineralization percent for each size class (cm) of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters for a given fish size are not significantly different (P>0.05).

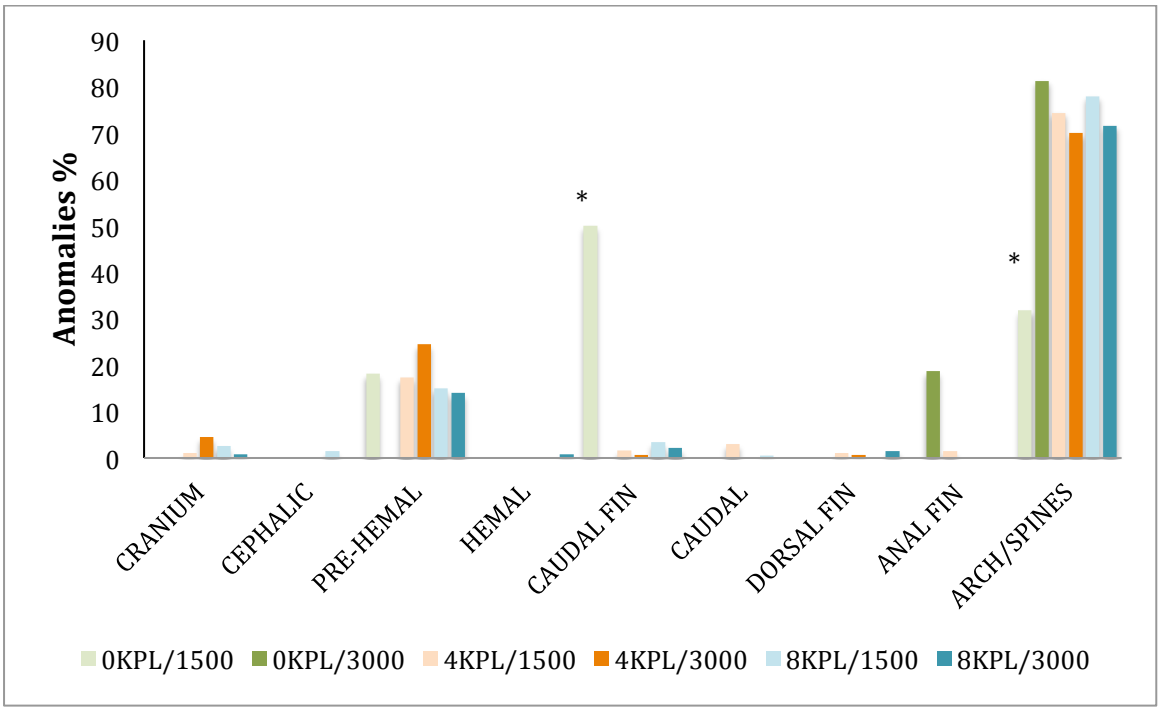

Figure 6.6 Anomalies (craneal deformities; alteration of cephalic, pre-hemal, hemal and caudal vertebral bodies; dorsal, anal and caudal fins and vertebral arch and spines) of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) without letters are not significantly different (P>0.05).

Analysis of the experimental diets showed that all 0KPL diets were higher in saturated fatty acids, slightly lower in monoens and markedly lower in n-3 and n-3 HUFA fatty acids than the other diets (Table 6.3). Besides, this diet also showed higher arachidonic acid (20:4n-6, ARA) than diets 4KPL and 8KPL. Diets 4KPL and 8KPL were very similar in their fatty acid profiles, whereas diet 8SBL was higher in linoleic acid (18:2n-6, LA) and lower in docosahexaenoic acid (22:6n-3, DHA), whereas ARA and eicosapentaenoic acid (EPA) were similar to KPL diets. In general, the fatty acid composition of the larvae (Table 6.4) reflected the dietary fatty acids profiles, although with some clear differences. Thus, larvae fed 0KPL showed significantly lower essential fatty acids such as n-3, n-3 HUFA, EPA and DHA, although the later was closer to the other larvae. Besides, these larvae were higher in LA in concordance with dietary profiles. However, opposite to dietary composition, 0KPL larvae were lower in saturated and higher in monoenoic fatty acids than the other larvae. 4KPL and 8KPL larvae were not significantly different in their fatty acid profiles, except for slightly lower n-6 fatty acids content in 4KPL larvae. These larvae

had significantly higher n-3 and n-3HUFA especially EPA than the other larvae. Finally, in agreement with the dietary profiles, 8SBL larvae had significantly higher n-6 fatty acids, especially 18:2n-6 and lower n-3 HUFA and DHA. However, despite the little differences in the EPA content in the dietary profiles, larvae fed 8SBL showed a markedly lower content in this fatty acid in comparison to larvae fed KPL diets.

Table 6.3 Fatty acids (% dry weight) composition of seven diets containing two different phospholipid sources combined with two α-tocopherol levels

	0KPL/ 1500	0KPL/ 3000	4KPL/ 1500	4KPL /3000	8KPL/ 1500	8KPL/ 3000	8SBL/ 3000
14:0	0.88	0.57	0.59	1.69	1.80	1.83	2.15
14:1n-5	0.31	0.03	0.04	0.05	0.05	0.06	0.05
14:1n-7	0.23	0.06	0.07	0.04	0.04	0.04	0.04
15:0	0.31	0.08	0.08	0.09	0.10	0.11	0.09
15:1n-5	0.12	0.00	0.01	0.01	0.01	0.02	0.01
16:0iso	0.07	0.07	0.07	0.08	0.08	0.02	0.02
16:0	5.02	6.39	5.52	4.94	5.16	5.26	4.58
16:1n-7	0.48	0.92	0.94	1.05	1.10	1.23	1.30
16:1n-5	0.14	0.05	0.04	0.06	0.06	0.06	0.05
16:2n-6	0.04	0.04	0.04	0.09	0.09	0.15	0.13
16:2n-4	0.04	0.01	0.01	0.01	0.01	0.01	0.01
17:0	0.02	0.03	0.04	0.04	0.04	0.07	0.06
16:3n-4	0.09	0.21	0.22	0.08	0.08	0.06	0.05
16:3n-3	0.11	0.03	0.03	0.06	0.06	0.08	0.07
16:3n-1	0.04	0.06	0.06	0.07	0.07	0.02	0.02
16:4n-3	0.70	0.05	0.05	0.16	0.17	0.26	0.23
16:4n-1	0.00	0.01	0.01	0.01	0.01	0.00	0.00
18:0	2.97	2.92	0.75	0.60	0.63	0.51	0.44
18:1n-9	2.08	3.38	3.65	3.29	3.47	3.50	3.04
18:1n-7	0.51	0.79	0.78	1.22	1.30	1.35	1.37
18:1n-5	0.05	0.10	0.10	0.09	0.09	0.08	0.07
18:2n-9	0.00	0.07	0.07	0.03	0.03	0.02	0.02
18:2n-6	0.99	1.71	0.69	0.39	0.41	0.37	3.04
18:2n-4	0.02	0.07	0.07	0.02	0.02	0.02	0.02
18:3n-6	0.07	0.01	0.01	0.01	0.01	0.01	0.01
18:3n-4	0.05	0.05	0.05	0.01	0.02	0.02	0.01
18:3n-3	0.13	0.23	0.24	0.15	0.16	0.11	0.29
18:4n-3	0.14	0.08	0.38	0.44	0.46	0.41	0.56
18:4n-1	0.46	0.04	0.04	0.01	0.01	0.04	0.03
20:0	0.13	0.03	0.03	0.01	0.02	0.02	0.02
20:1n-9	0.06	0.06	0.06	0.04	0.04	0.03	0.03
20:1n-7	0.68	0.47	0.49	0.47	0.50	0.33	0.28
20:1n-5	0.00	0.03	0.03	0.06	0.06	0.09	0.07
20:2n-9	0.01	0.00	0.01	0.00	0.00	0.00	0.00
20:2n-6	0.36	0.09	0.10	0.03	0.03	0.05	0.05
20:3n-9	0.00	0.00	0.00	0.01	0.00	0.01	0.01
20:3n-6	0.01	0.02	0.03	0.01	0.01	0.03	0.02

20:4n-6	0.38	0.14	0.14	0.16	0.17	0.14	0.12
20:3n-3	0.20	0.07	0.06	0.07	0.08	0.04	0.03
20:4n-3	0.07	0.07	0.07	0.06	0.07	0.11	0.10
20:5n-3	1.76	1.45	3.71	3.70	3.91	4.26	3.71
22:1n-11	0.11	0.04	0.04	0.01	0.01	0.03	0.02
22:1n-9	0.62	0.05	0.05	0.15	0.16	0.18	0.16
22:4n-6	0.01	0.05	0.05	0.09	0.10	0.12	0.11
22:5n-6	0.03	0.03	0.03	0.03	0.03	0.02	0.02
22:5n-3	0.27	0.25	0.26	0.10	0.11	0.22	0.19
22:6n-3	2.04	2.37	4.43	4.44	4.71	4.42	2.89
∑ saturated	9.33	10.03	7.00	7.38	7.74	7.80	7.35
∑monoenoics	5.40	5.99	6.31	6.52	6.90	6.99	6.48
∑ n-3	5.40	4.59	8.93	9.19	9.73	9.52	8.07
∑ n-6	1.91	2.09	1.09	0.81	0.86	0.88	3.49
∑ n-9	2.77	3.57	3.85	3.51	3.70	3.75	3.25
∑ n-3HUFA	4.34	4.21	8.53	8.38	8.87	9.06	6.92
ARA	0.38	0.14	0.14	0.16	0.17	0.14	0.12
EPA	1.76	1.45	3.71	3.70	3.91	4.26	3.71
DHA	2.04	2.37	4.43	4.44	4.71	4.42	2.89
EPA/ARA	4.57	10.72	26.63	22.49	22.41	30.86	30.90
DHA/EPA	1.16	1.64	1.19	1.20	1.20	1.04	0.78
DHA/ARA	5.30	17.56	31.73	27.00	26.98	32.03	24.10
n-3/n-6	2.84	2.19	8.19	11.31	11.30	10.80	2.31

Table 6.4 Fatty acids (% total identified fatty acids) composition of gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard error) with the same letters in the same row are not significantly different (P>0.05).

Diet	0KPL/ 1500	0KPL/ 3000	4KPL/ 1500	4KPL /3000	8KPL/ 1500	8KPL/ 3000	8SBL/ 3000
14:0	1.05	1.05	2.66	2.73	2.54	2.48	0.9
14:1n-5	0.11	0.13	0.1	0.1	0.1	0.11	0.06
14:1n-7	0.03	0.03	0.04	0.04	0.03	0.02	0.02
15:0	0.29	0.3	0.31	0.31	0.28	0.26	0.23
15:1n-5	0.03	0.03	0.02	0.01	0.02	0.02	0.02
16:0iso	0.33	0.35	0.44	0.45	0.48	0.51	0.31
16:0	16.3	16.98	21.41	21.52	20.79	21.07	22.29
16:1n-7	1.96	1.98	2.49	2.52	2.5	2.58	1.15
16:1n-5	0.19	0.23	0.19	0.21	0.2	0.2	0.15
16:2n-6	0.08	0.09	0.16	0.16	0.14	0.14	0.03
16:2n-4	0.44	0.44	0.38	0.37	0.26	0.14	0.33
17:0	0.04	0.09	0.08	0.07	0.08	0.1	0.04
16:3n-4	0.6	0.59	0.29	0.29	0.23	0.2	0.34
16:3n-3	0.08	0.09	0.2	0.2	0.22	0.24	0.08
16:3n-1	0.39	0.43	0.38	0.39	0.47	0.54	0.35
16:4n-3	0.48	0.52	0.24	0.24	0.26	0.29	0.32
16:4n-1	0.1	0.12	0.1	0.1	0.13	0.16	0.07
18:0	5.76	6.38	5.87	5.8	5.93	6.17	5.69

18:1n-9	23.67	24.5	12.81	12.44	11.23	10.86	16.97
18:1n-7	3.1	3.32	4.22	4.22	4.35	4.63	2.6
18:1n-5	0.31	0.29	0.34	0.34	0.29	0.25	0.25
18:2n-9	0.1	0.06	0.08	0.08	0.07	0.06	0.07
18:2n-6	4.95	5.03	1.45	1.36	1.97	2.81	13.09
18:2n-4	0.19	0.24	0.11	0.11	0.09	0.08	0.09
18:3n-6	0.1	0.11	0.08	0.08	0.07	0.07	0.06
18:3n-4	0.22	0.23	0??	0.15	0.04	0.02	0.06
18:3n-3	0.38	0.39	0.32	0.32	0.36	0.46	0.74
18:4n-3	0.11	0.12	0.76	0.81	0.82	0.82	0.13
18:4n-1	0.05	0.06	0.04	0.02	0.03	0.05	0
20:0	0.18	0.2	0.15	0.15	0.15	0.16	0.15
20:1n-9	0.12	0.15	0.1	0.09	0.07	0.05	0.09
20:1n-7	1.25	1.33	1.32	1.31	0.97	0.69	1.02
20:1n-5	0.14	0.17	0.23	0.23	0.24	0.25	0.09
20:2n-9	0.01	0.02	0.01	0.01	0.01	0.01	0
20:2n-6	0.35	0.39	0.13	0.12	0.15	0.16	0.27
20:3n-9	0	0.07	0.01	0.01	0.02	0.02	0
20:3n-6	0.11	0.06	0.07	0.06	0.09	0.11	0
20:4n-6	1.4	1.47	1.17	1.16	1.17	1.15	0.89
20:3n-3	0.17	0.18	0.23	0.22	0.14	0.09	0.15
20:4n-3	0.29	0.3	0.3	0.31	0.38	0.44	0.15
20:5n-3	5.53	5.7	12.44	12.69	13.15	13.41	5.11
22:1n-11	0.09	0.08	0.04	0.02	0.03	0.03	0.01
22:1n-9	0.35	0.34	0.58	0.58	0.49	0.43	0.28
22:4n-6	0.13	0.1	0.27	0.28	0.29	0.31	0.08
22:5n-6	0.25	0.29	0.19	0.19	0.17	0.14	0.16
22:5n-3	0.43	1.29	0.94	0.92	1.15	1.44	0.27
22:6n-3	22.75	23.7	26.24	26.24	27.34	25.78	24.83
∑Saturated	23.62±2.34^{b}	25.00±2.06^{b}	30.48±2.14^{a}	30.58±2.88^{a}	29.78±2.56^{a}	30.23±2.76^{a}	29.30±2.63^{a}
∑monoenoics	31.34±2.87^{a}	32.58±2.55^{a}	22.48±1.89^{b}	22.10±2.01^{b}	20.51±1.94^{b}	20.11±2.03^{b}	22.70±1.97^{b}
∑n-3	30.22±2.64^{b}	32.28±1.99^{bc}	41.90±2.85^{a}	41.94±2.92^{a}	43.84±2.69^{a}	42.97±2.73^{a}	31.78±2.14^{c}
∑ n-6	7.39±1.06^{b}	7.54±1.01^{b}	3.52±0.24^{d}	3.41±0.59^{d}	4.05±0.83^{c}	4.89±0.79^{c}	14.59±1.56^{a}
∑n-9	24.24±1.66^{a}	25.14±1.71^{a}	13.91±1.63^{c}	13.20±1.45^{c}	11.88±1.58^{c}	11.43±1.64^{c}	17.41±1.31^{b}
∑ n-3HUFA	29.17±2.26^{b}	31.16±2.70^{b}	40.15±2.91^{a}	40.37±2.88^{a}	42.17±2.69^{a}	41.16±2.72^{a}	30.51±2.24^{b}
ARA	1.40±0.42^{a}	1.47±0.37^{a}	1.17±0.31^{a}	1.16±0.29^{a}	1.17±0.28^{a}	1.15±0.30^{a}	0.89±0.19^{b}
EPA	5.53±1.01^{b}	5.70±1.12^{b}	12.44±1.36^{a}	12.69±1.26^{a}	13.15±1.29^{a}	13.41±1.30^{a}	5.11±1.02^{b}
DHA	22.75±1.10^{c}	23.70±1.33^{bc}	26.24±1.56^{a}	26.24±1.67^{a}	27.34±1.74^{a}	25.78±1.97^{ab}	24.83±1.02^{b}
EPA/ARA	3.95±0.97^{c}	3.88±0.88^{c}	10.59±1.03^{a}	10.95±1.10^{a}	11.25±1.14^{a}	11.65±1.19^{a}	5.71±1.07^{b}
DHA/EPA	4.11±0.92^{a}	4.16±0.83^{a}	2.11±0.24^{b}	2.07±0.27^{b}	2.09±0.30^{b}	1.93±0.22^{b}	4.88±0.91^{a}
DHA/ARA	16.23±1.56^{c}	16.13±1.62^{c}	22.34±1.78^{b}	22.64±1.85^{b}	23.40±1.91^{b}	22.47±1.85^{b}	27.83±1.95^{a}
n-3/n-6	4.09±0.84^{c}	4.29±0.90^{c}	11.90±1.06^{ab}	12.33±1.16^{a}	10.83±0.90^{b}	8.78±1.04^{c}	2.18±0.23^{d}

The degree of larval lipid oxidation, as indicated by larval malondialdehyde (MDA) content was significantly higher in those larvae fed 8SBL/3000 than in any of the other larvae, except those larvae fed 8KPL/1500 diets. The increased in dietary -tocopherol, decreased significantly MDA content in larvae fed free 0KPL diets, and tended to reduce MDA content in larvae fed 4KPL and 8KPL diets (Figure 6.7).

The general pattern of antioxidant enzyme (AOE) genes expression of seabream larvae was characterized by significant up-regulation by the increase in dietary PL contents and a significantly lower expression by the increase in α-tocopherol levels (Figs. 8-10). Thus, there were no significant differences between larvae fed 0KPL/1500 and 0KPL/3000 diets in CAT gene expression, whereas supplementation of 3000 mg -tocopherol kg^{-1} diet in KPL and SBL diets significantly lowered CAT and SOD genes expression in the (Figures 6.8 and 6.9). On the contrary, despite a higher GPX gene expression was found with increased dietary PL, the expression of this enzyme was not affected by increasing α-tocopherol level (Figure 6.10).

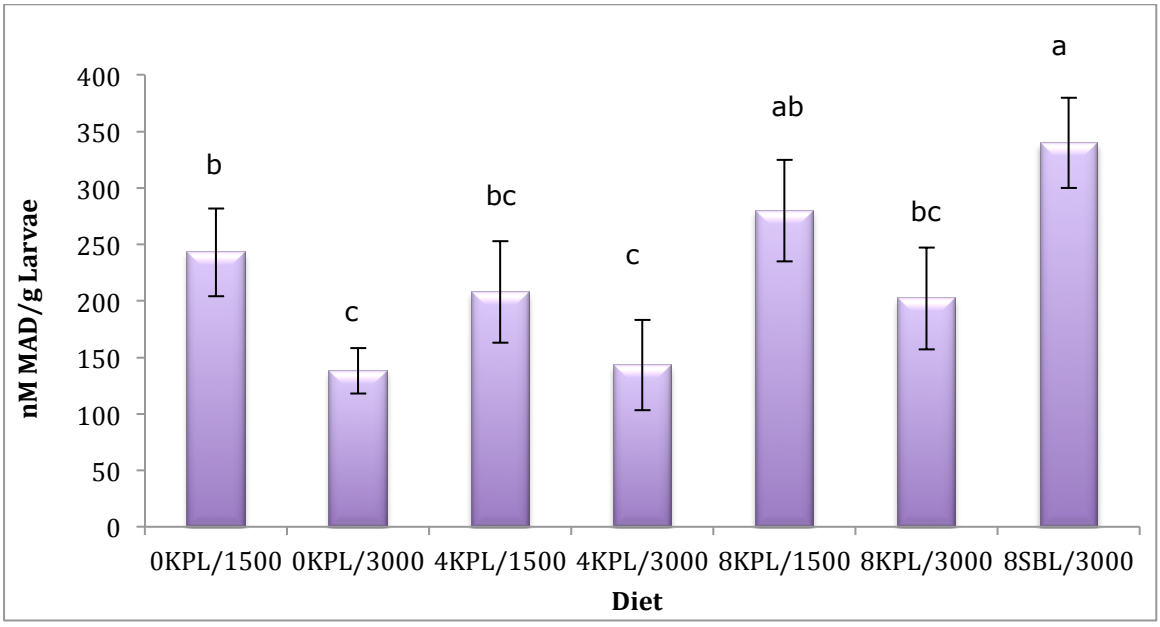

Figure 6.7 The malondialdehyde (MDA) content in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels.. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

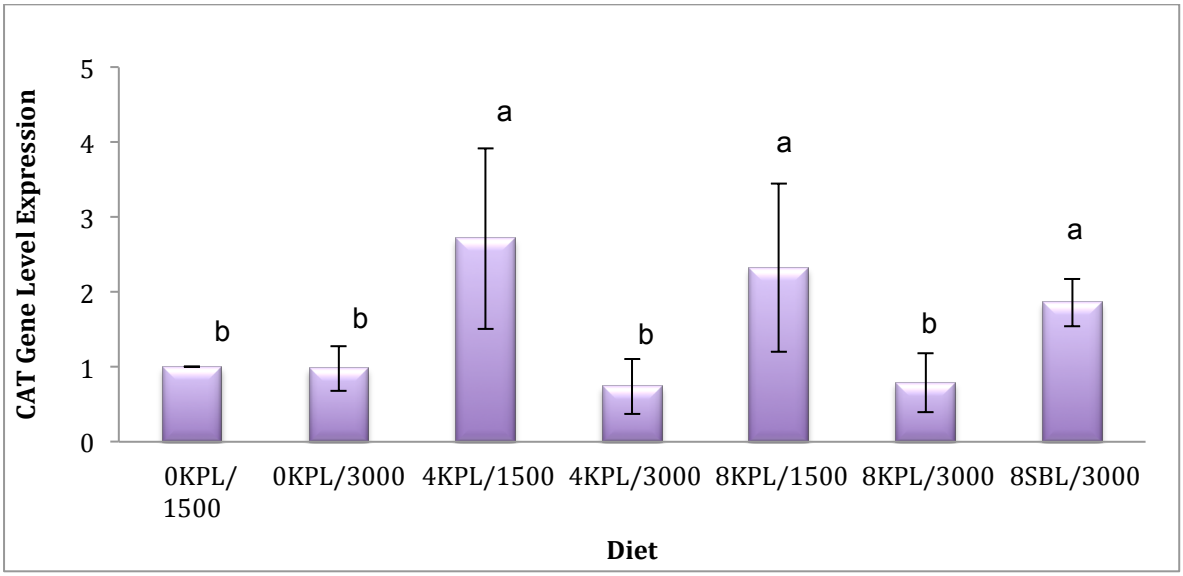

Figure 6.8 Catalase (CAT) gene expression level measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels.Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

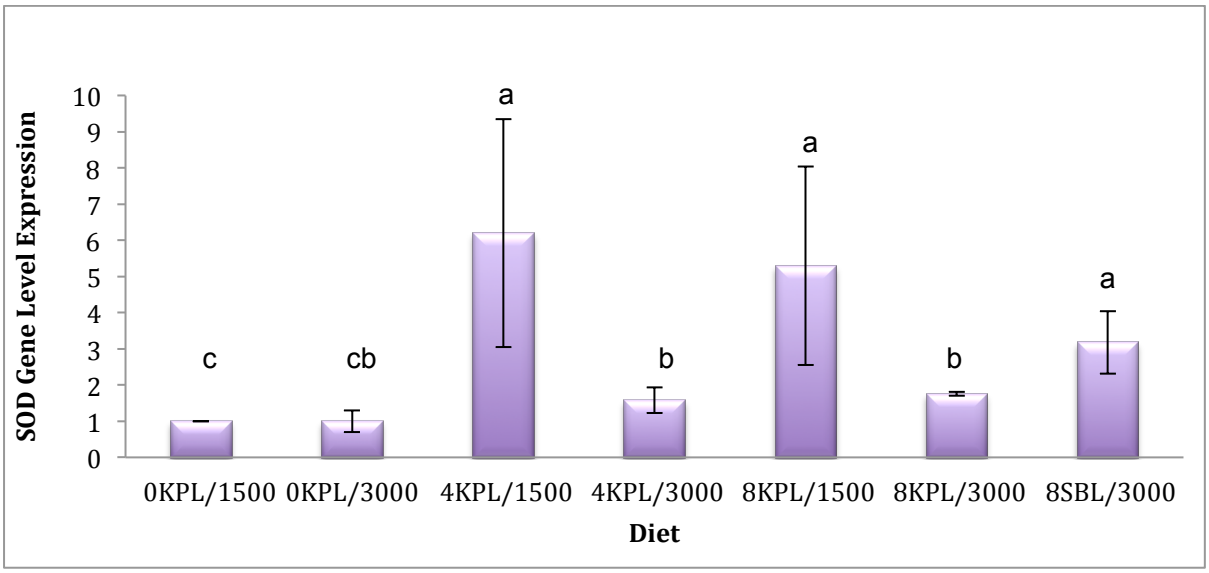

Figure 6.9 Superoxide dismutase (SOD) gene expression level measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

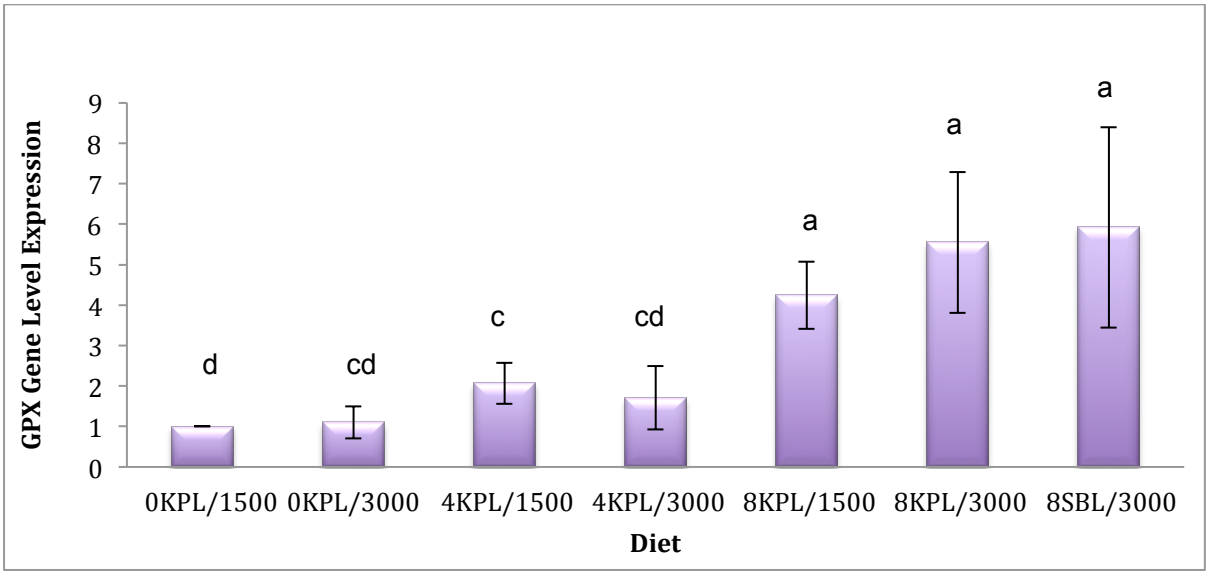

Figure 6.10 Glutathione peroxidase (GPX) gene expression level measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

The gene of BMP4, an early marker of osteoblast differentiation was significantly up-regulated in larvae fed 4KPL and 8KPL in comparison to those fed SBL and free KPL diets (Figure 6.11). Similarly, the expression of RUNX2 was significantly higher in 4KPL and 8KPL larvae than in those fed 0KPL/1500 or 8SBL/3000 (Figure 6.12). Besides, the lowest expression of ALP, an early marker of cell mineralization, was found in larvae fed 0KPL/1500. The genes of osteocalcin (OC), osteopontin (OP) and osteonectine (ON), skeletal extracellular matrix proteins that are markers of osteogenesis, were significantly up-regulated in larvae fed 4KPL and 8KPL in comparison to those fed SBL and free KPL diets (Figures 6.13, 6.14 and 6.15). Finally, the expression of matrix GLA protein (MGP), a physiological marker of endochondral ossification, was significantly up-regulated in larvae fed KPL and SBL diets in comparison to larvae fed free KPL diets (Figure 6.17). However, the expression of these osteogenetic markers was not affected by increasing α-tocopherol levels.

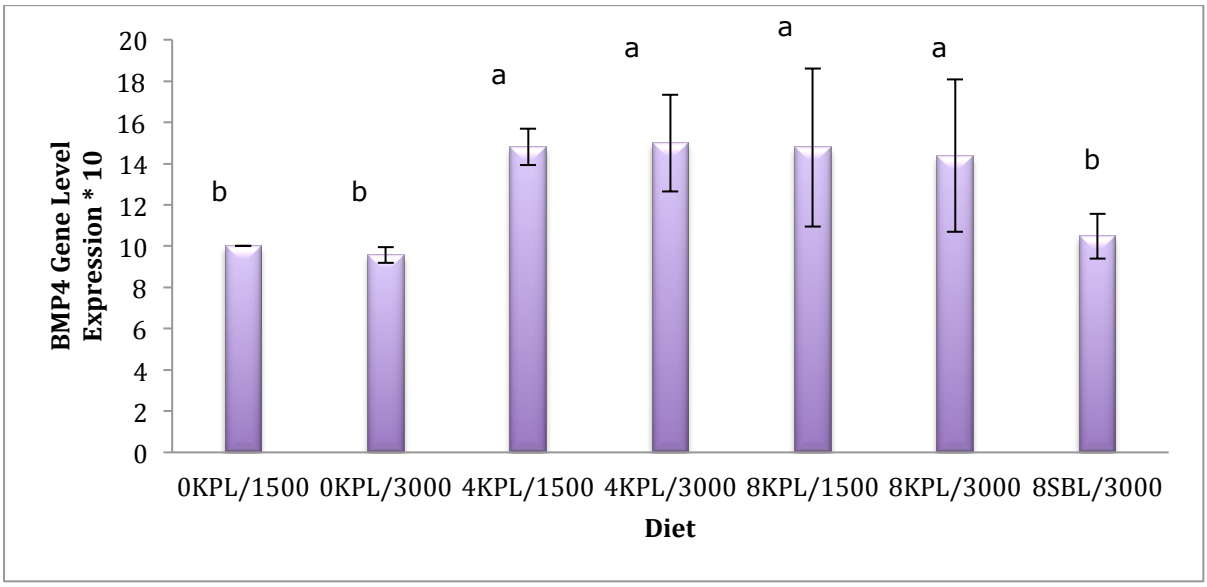

Figure 6.11 BMP4 (Bone morphogenetic protein 4) gene level expression measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

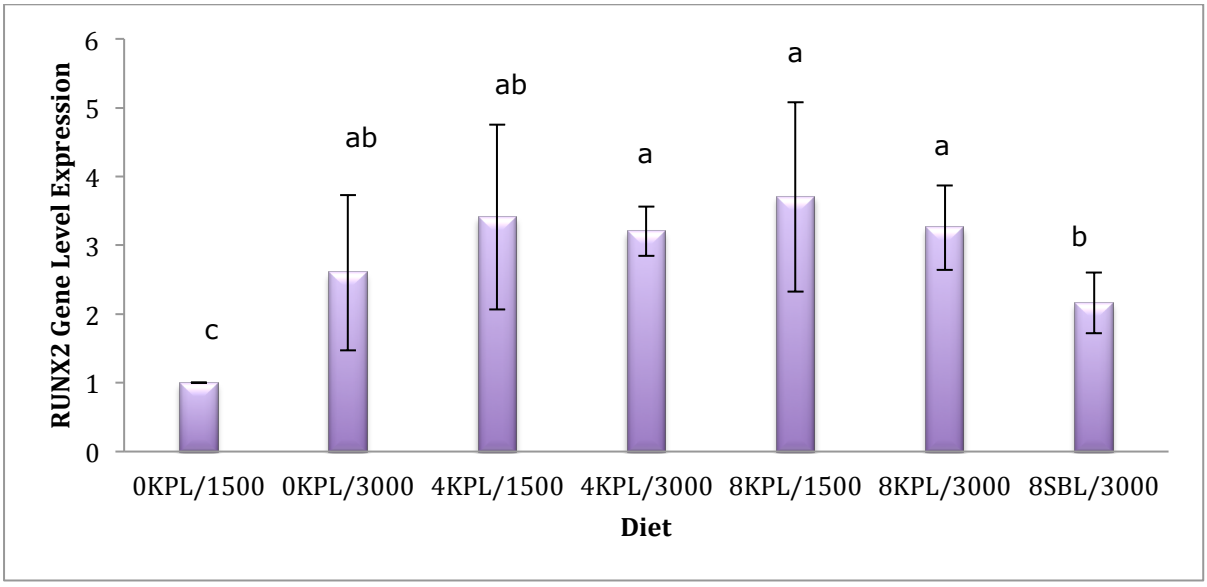

Figure 6.12 RUNX2 (Runt-related transcription factor 2) gene expression level measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

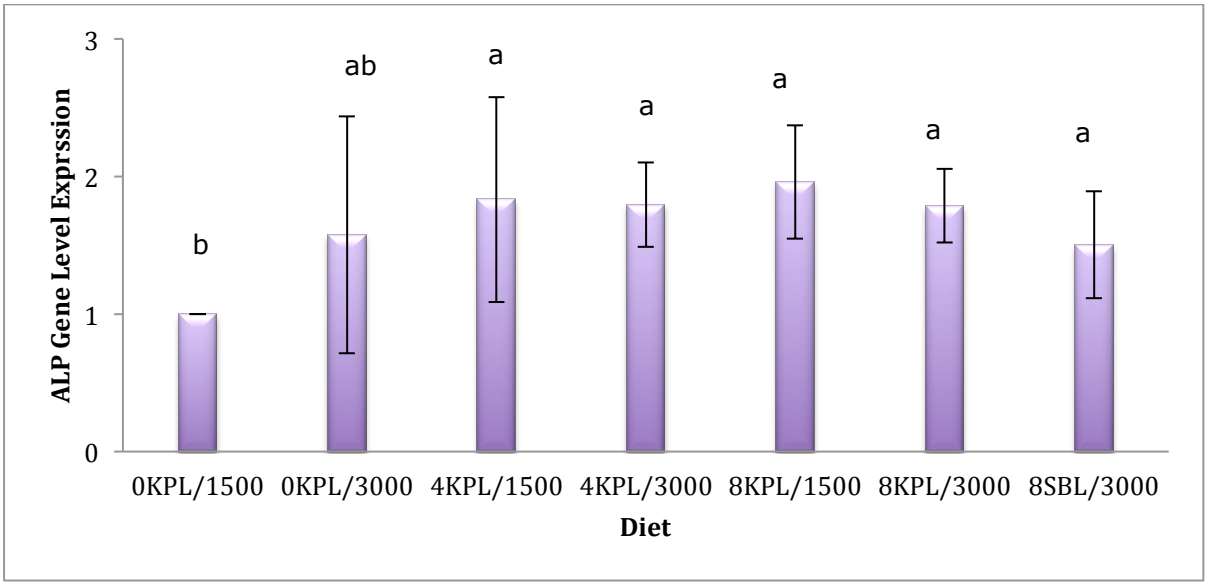

Figure 6.13 Alkaline phosphatase gene level expression measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

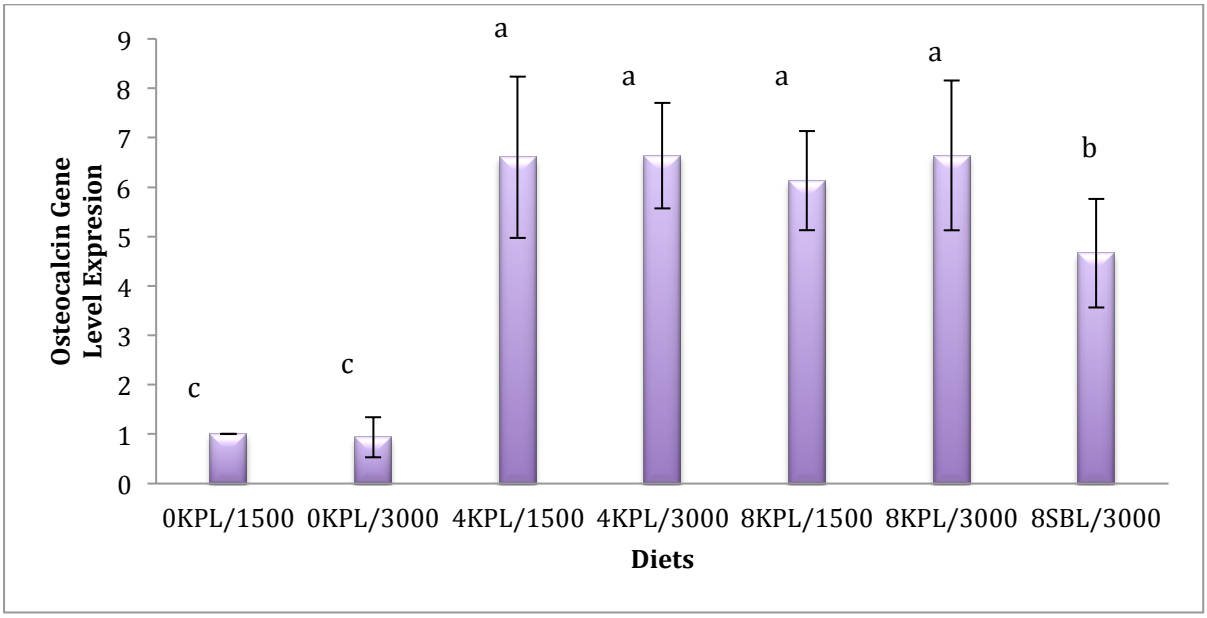

Figure 6.14 Osteocalcin gene expression level measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

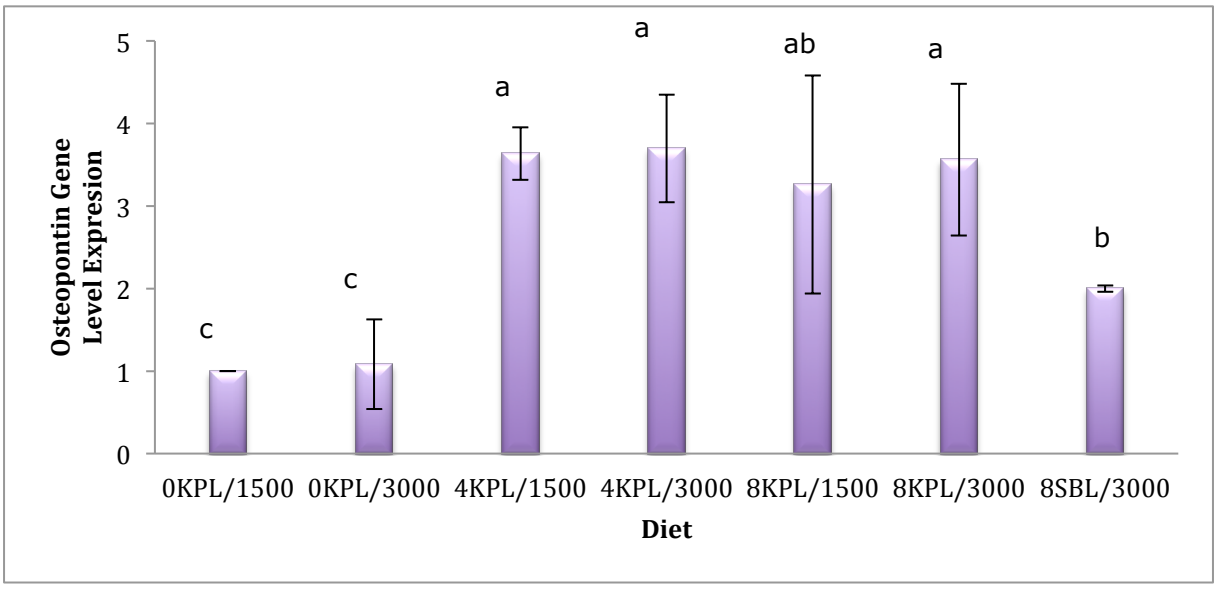

Figure 6.15 Osteopontin gene level expression measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

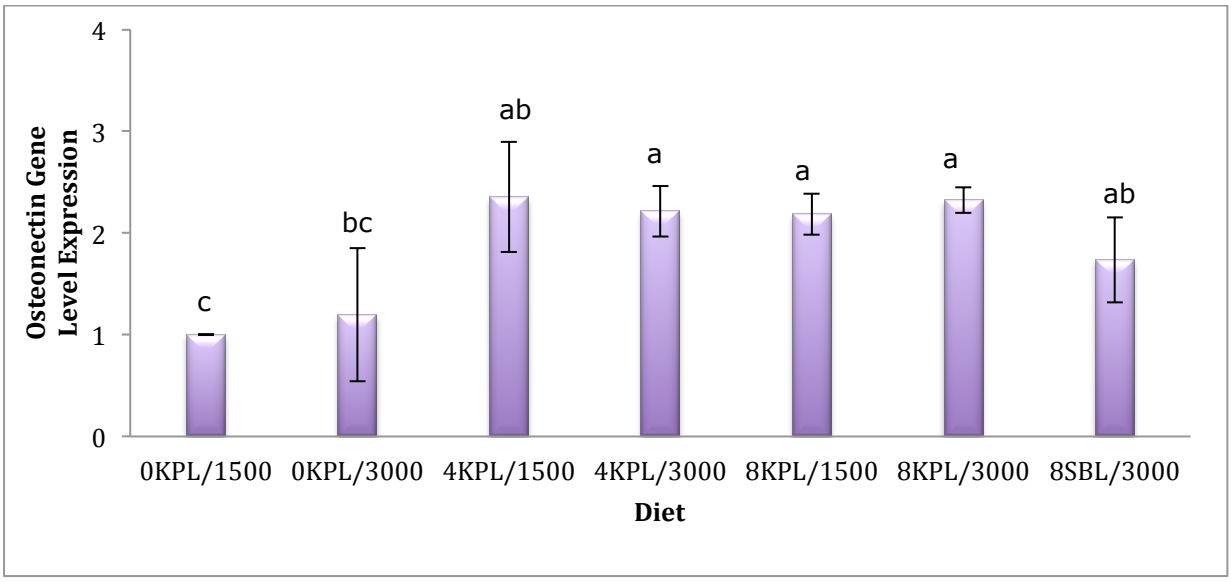

Figure 6.16 Osteonectin gene expression level measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

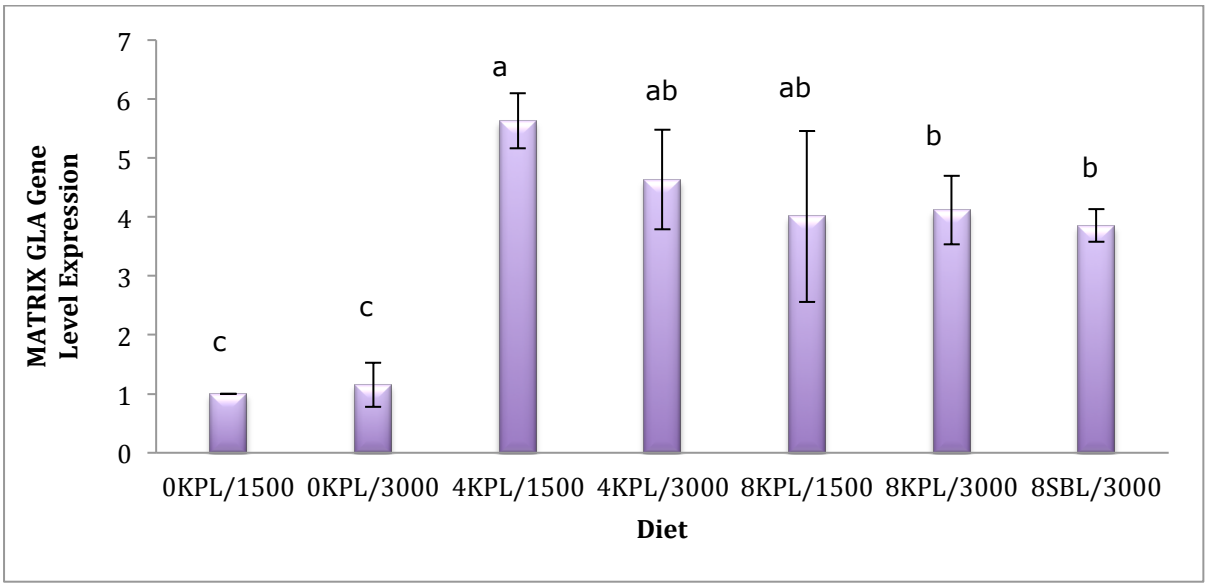

Figure 6.17 Matrix Gla protein gene level expression measured by real-time PCR in gilthead seabream larvae (44 dph) fed different dietary contents of two phospholipid sources combined with two α-tocopherol levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Discussion

Gilthead seabream larvae fed diets without PL supplementation showed the lowest survival, growth and stress resistance. The increase in dietary PL contents markedly improved stress resistance determined as survival after handling, regardless the type of PL, marine or soybean derived. Moreover, increase in PL markedly promoted larval survival ($y = 7.8688x - 24.206$, $R^2 = 0.6284$) and growth what could be related to a faster larval tissues maturation as suggested by the significant increase in alkaline phosphatase activity in previous studies (Wold *et al*., 2007; Saleh *et al*., 2012a, b) and a better dietary nutrients utilization (Saleh *et al*., 2012a, b). Nevertheless, the increase in dietary PL over 9% did not further improved larval survival and growth, in agreement with previous studies that reccomended a 10% marine PL inclusion in early weaning diets for gilthead seabream (Saleh *et al*., 2012a).

The growth promoting efffect of dietary PL was particularly effective when diets included krill derived PL, which contained higher n-3 HUFA and DHA levels than soybean lecithin. Indeed, a significant correlation was found between total length and dietary N-3 HUFA ($y = 0.2819x + 0.2648$ $R^2 = 0.8297$) or DHA ($y =0.9577x - 13.677$, $R^2 = 0.7382$), as well as between final body weight and dietary n-3 HUFA ($y = 94.224x - 1779.9$ $R^2 = 0.81463$) or DHA ($y = 311.98x – 6235$ $R^2 = 0.6886$). Similar

results were also obtained in previous studies (Saleh et al., submitted), where gilthead seabream larvae were fed several levels of marine PL versus soybean PL. Nevertheless, the higher performance of fish fed marine PL seems to be not only due to a higher total content on these fatty acids. For instance, feeding soybean PL markedly increased the peroxidation risk in the larvae as denoted by the higher TBARs and GPX gene expression, as it was also found in other studies (Saleh *et al*., submitted). Besides, comparison of two weaning diets containing the same PL (2.5%) and n-3 HUFA (1.6%) levels for gilthead seabream showed that the diet containing these fatty acids in the PL fraction leads to higher growth rates than those containing n-3 HUFA in the NL (Salhi et al., 1999; Izquierdo *et al*., 2001). Indeed, when larvae are fed marine PL there is a higher incorporation of these fatty acids in the larval PL (Izquierdo et al., 2001). In agreement, in the present study, larvae fed krill PL showed a higher n-3 HUFA content than larvae fed soybean PL. Particularly, EPA contents in marine PL fed larvae were more than double than in soybean PL fed ones, despite the diets had similar EPA levels. In turn, larval n-3 HUFA contents were highly correlated with the expression of BMP-4 gene ($y = 0.4298x-2.9237$, $R^2 = 0.95$). Accordingly, this gene, included in the transforming growth factor b superfamily that regulates mesenchymal cells differentiation into osteoblasts along development, is up-regulated by n-3 HUFA in fish (Lall and Lewis-McCrea, 2007; Saleh *et al*., submitted) and other vertebrates (Kruger *et al*., 2010). For instance, feeding dietary n-3 HUFA to growing male rats markedly increases bone formation (Watkins et al., 2000). The other osteogenetic biomarkers studied such as RUNX 2, ALP, OC, OP were not so strongly correlated to the larval n-3 HUFA ($R^2 = 0.21869$, $R^2 = 0.27996$, $R^2 = 0.42656$, $R^2 = 0.10688$, respectively), what could be suggesting that their up-regulation by n-3 HUFA rather than directly is mediated by BMP-4. Nevertheless, larval EPA contents were highly correlated not only with the expression of RUNX 2, ALP, OC and OP ($y = 0.1959x + 0.8592$ $R^2 = 0.70753$, $y = 0.0643x + 1.012$ $R^2 = 0.64505$, $y = 0.5572x - 0.7555$ $R^2 = 0.73037$, $y = 0.2846x - 0.1597$ $R^2 = 0.8814$, respectively), but also with bone mineralization in larvae from the same larval size (0.8-1.0 cm total length) ($y = 0.7339x + 3.4274$ $R^2 = 0.73408$) denoting the importance of this fatty acid for bone formation, as it also occurs with DHA (Izquierdo *et al*., 2012). Indeed, EPA is a good substrate for both cycloxigenases and lipoxigenases and, in marine fish, it can be an important source of certain eicosanoids.

Actually, PGE3 derived from EPA is the main prostaglandin found in plasma of juvenile gilthead seabream (Ganga *et al*., 2005), prostaglandines being found to regulate mineralization in other vertebrates (Kruger *et al*., 2010).

The increase in α-tocopherol dietary levels did not affected larval survival in agreement with previous studies in this species (Montero *et al*., 1996; Atalah *et al*., 2012). It neither affected survival after the handling stress, despite the importance of this vitamin for stress resistance and immune system regulation (Montero *et al*., 1998), what could suggest that even the lower α-tocopherol levels tested in these diets were enough to cover the basic larval requirements. Nonetheless, increase in dietary α-tocopherol tend to improve growth, particularly in terms of body weight, in relation to the n-3 HUFA levels in the diet denoting the protective role against oxidation of this vitamin. Thus, at lower n-3 HUFA dietary levels there was no effect of α-tocopherol on growth, whereas when dietary n-3 HUFA increase this vitamin significantly increased larval body weight in agreement with previous studies (Atalah *et al*., 2011). Growth may be also improved by increased dietary α-tocopherol in diets for marine fish juveniles (Tocher *et al*., 2002). In the present study, increase in α-tocopherol decreased the oxidative stress in the larvae as denoted by the reduction in larval TBARs contents. These results point out the importance of this vitamin for protection of polyunsaturated fatty acids from peroxidation, being α-tocopherol a structural component of cell membranes (Putnam and Comben, 1987). Several types of compounds are derived from oxidation of polyunsaturated fatty acids, 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 results in damage to cellular and sub-cellular membranes (Puangkaew *et al*., 2005), structural proteins and different fish tissues and organs (Watanabe *et al*., 1970; Sakai *et al*., 1989; Bai and Lee, 1998). Increase in α-tocopherol reduced the gene expression of SOD and CAT, but not GPX. Similarly, increase in α-tocopherol contents in rotifers reduces SOD and CAT gene expression in gilthead seabream larvae but does not affect GPX (Izquierdo *et al*., 2012). SOD prevents the initialization of the radical chain reaction that the superoxide anion produces, by the conversion of superoxide into hydrogen peroxide (Winston and Di Giulio, 1991). In turn, hydrogen peroxide is broken down to molecular oxygen and water by either

CAT or GPX, respectively acting in the peroxisomes or mitochondria principally. N-3HUFA, particularly EPA that indeed was higher in diets containing PL, are mainly catabolized in peroxisomes (Henderson, 1996) and would be responsible for the higher peroxidation risk in these organelles as denoted by the higher CAT gene expression. α-tocopherol intercept peroxyl radicals (ROO-) more rapidly than HUFA and converts it to a hydroperoxide product breaking the chain of reactions involved in lipid auto-oxidation. In the present study, increase in dietary α-tocopherol seems to effectively prevent the fromation of free radicals from HUFA, particularly EPA, in the peroxisomes as denoted by the reduction in CAT gene expression. However, in fish, mitochondrial β-oxidation preferred saturated and monounsaturated fatty acids over HUFA with EPA usually oxidised to a lesser extent (Sargent *et al.* 1989; Tocher 2003), and therefore, protection of EPA by increased α-tocopherol did not affected the oxidative risk in mitochondria as denoted by the lack of effect on GPX. Besides, the positive effect of α-tocopherol in fish growth may be also related to its role in cellular metabolism, signal transduction (Traber and Parker, 1995) or modulation of eicosanoid synthesis (Cornwell and Parganamala, 1993).

The effect of α-tocopherol in skeletal development has been studied extensively in terrestrial mammals but not in fish. α-tocopherol is important for proper skeletal development (Xu *et al*., 1995; Jilka *et al*., 1996) where it associates with the lipid bilayer of bone cells allowing it to be the first line of defense against free radicals (Arjmandi *et al*., 2002). In gilthead seabream larvae, feeding very high DHA rotifers (> 5% in dry basis) without an adequate balance of antioxidant nutrients increases the production of free radicals that damage the cartilaginous anlagen of bones such as skull or vertebral archs and spines and favours the development of skeletal deformities (Izquierdo *et al*., 2012). However, increase in α-tocopherol markedly reduced anomalies incidence in these bones. In the present study, the incidence of cranium anomalies was very low suggesting that the dietary DHA levels used (< 5% in dry basis) were not negatively affecting bone formation. In consecuence, the increased in α-tocopherol from 1500 to 3000 mg kg^{-1} diet did neither affected the incidence of bone anomalies or the genes implicated in the osteogenesis processes.

In summary, feeding soybean PL improved larval seabream growth and survival, but increased TBARs and GPX gene expression increasing peroxidation risks. Krill derived PL improved larval growth and survival more effectively than soybean PL,

enhancing n-3 HUFA and particularly EPA incorporation into larval tissues and up-regulating the gene expression of bone development biomarkers as well as bone mineralization. The increase in dietary α-tocopherol further improved growth in relation to the dietary n-3 HUFA levels denoting its protective role against oxidation and reducing larval TBARs and gene expression of SOD and CAT. Further studies are being conducted to determine the α-tocopherol requirements in gilthead seabream larvae in relation to the dietary contents of other antioxidants.

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Chapter 7

Selenium levels in early weaning diets for gilthead seabream larvae

Abstract

The inclusion of complementary antioxidative factors, such as selenium (Se), could counteract the high oxidation risk in early weanning diets high in polyunsaturated fatty acids (PUFA). The objective of the present study was to investigate the effect of graded levels of Se derived yeast with krill PL (KPL), on skeletal development, survival, resistance to stress, oxidative status and biochemical composition of seabream larvae. Seabream larvae were completely weanned at 16 dph and fed for 30 days five isoenergetic and isoproteic microdiets with different levels of Se: 2SE, 4SE, 6SE, 8SE and 12SE (1.73, 3.91, 6.41, 8.47, 11.65 mg kg^{-1} dietary dry weight, respectively). Increase in Se up to 11.65 mg kg^{-1} dietary dry weight, significantly improved larval survival (54% survival rate) and resistence to stress, whereas did not affected larval growth. Seabream larvae fed diets supplemented with 12SE (11.65 mg kg^{-1}) showed a gradual increase in this mineral according to the dietary Se levels and denoting the progressive absorption of this nutrient. The degree of larval lipid oxidation, as indicated by malondialdehyde (MDA) content and AOE genes expression was significantly lower in those larvae fed diets 8SE and 12SE compared with those of 2SE and 4SE diets. Also, a reactive response as a result of Se inclusion was observed by the increase in osteocalcin, osteonectin osteopontin, alkaline phosphatase and matrix gla protein genes expression in larval tissues, suggesting a well skeletal development. These results denoted the high efficiency of Se as an antioxidant factor and the importance of the inclusion of adequated levels (11.65 mg Se kg^{-1} diet) in early weaning diets.

Introduction

The oxidation of important nutrients like lipids, essential fatty acids and proteins that have critical biological and physiological functions, leads to harmful alterations in fish causing several pathological conditions (Sakai *et al*. 1989) including cellular damages (Halliwell and Gutteridge, 1996), muscles injuries (Betancor *et al*., 2011), negative growth, low feed intake and delayed development in several fish species (Tacon, 1991). Moreover, the oxidation of dietary lipids may also lead to an increased incidence of deformities in marine fish larvae (Lewis-McCr and Lall, 2007). It is well known that dietary lipids constitute a major source of energy and essential fatty acids for fish and play a main role in larval development (Rainuzzo *et al*., 1997; Izquierdo and Koven, 2010). Among them, dietary phospholipids (PL) levels have been found to positively affect survival, growth and resistance to stress and reduce the occurrence of morphological anomalies in several fish and crustaceans (Kanazawa, 1985; Geurden *et al*., 1998a, b; Cahu *et al*., 2003a; Liu *et al*., 2002; Izquierdo and Koven, 2010; Saleh *et al*., 2012a, b). They constitute important sources of essential fatty acids that have a crucial role in maintaining the structure and function of cellular membranes (Tocher, 2003). Besides, they may act as emulsifiers in the gut and improve intestinal absorption of long chain fatty acids (Fontagné *et al*., 2000). Moreover, they stimulate lipoprotein synthesis in intestinal enterocytes (Fontagné *et al*., 1998; Geurden *et al*., 1998b; Liu *et al*., 2002) and play an important role in the transport and assimilation of dietary lipids (Izquierdo *et al*., 2001). However, dietary PL may have high levels of polyunsaturated fatty acids that are very sensitive to peroxidation resulting in production of harmful peroxides and, consecuently, affect their biological and physiological functions. In previous studies, thiobarbituric acid reactive substances (TBARS) contents and antioxidant enzmes genes expression were rised in seabream larvae fed increasing levels of dietary PL rich in n-3 highly unsaturated fatty acids (HUFA) or linoleic acid (Saleh *et al*., submitted). Polyunsaturated fatty acids including those with five and six ethylenic bonds, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are very susceptible to oxidation due to their long chain lengths and the greater number of unsaturated carbon-carbon bonds (Betancor *et al*., 2012c). The mechanism of lipid oxidation begins with auto-oxidation involving the direct reaction of lipids with molecular

oxygen to form hydroperoxides, followed by secondary oxidation reactions yielding diperoxides, which are detrimental toxic compounds. Besides, dietary lipid oxidation is increased by factors such as the presence of lipoxidase, hematin, peroxides, light (involving in the photolysis of peroxides), high temperature, trace metal notably iron, copper, cobalt, and zinc (Dabrowski and Guderly, 2002; Sutton *et al*., 2006). Dietary lipid oxidation is even more exposed in formulated diets for marine fish larvae, which are formed by micro-particles high in polyunsaturated fatty acid contents, with a large surface area to volume ratio and that remain in contact with seawater for long periods until they are reach by the larvae. This type of diets may yield products of secondary oxidation of lipids that contribute to off flavor and include toxic compounds frequently associated with rancidity reducing their nutritional value (Halliwell and Chirico, 1993). Besides, the larval phase is one of the most critical stages in fish life cycle, among other reasons, for the high requirement of n-3 HUFA, as essential fatty acids for normal growth and development, what leads to a high oxidative stress risk in relation to the elevated larval metabolic rate (Evjemo *et al*., 2003). The lipid oxidation in marine fish larvae could be at least partly responsible for the higher disease incidence and subsequent larval mortalities (Tocher *et al*., 2002).

The oxidative stress in fish is an aspect of aerobic life that results of an imbalance between the production of reactive oxygen species (ROS) and antioxidant defences factors in living organisms (Nishida, 2011). ROS at low concentrations may be beneficial or even indispensable in processes such as defence against microorganisms, contributing to phagocitic bactericidal activity. However, the elevated levels of free radicals that are produced by endogenous cellular sources during normal cell metabolism and endogenous ROS that are produced by mitochondrial respiration can cause oxidation of proteins and lipids, alterations in gene expression, and changes in cell redox status giving rise to oxidative stress (Rando, 2002; Livingstone, 2003). In fish there are different effective antioxidant enzymes (AOE) capable of inhibiting the lipid-peroxidation catalytic cycle by catalyzing the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX). Dietary nutrients also constitute antioxidant factors that must be added in feed diets such as selenium, vitamin E and C or carotenoids (Montero *et al*., 2001; Díaz *et al*., 2010; Hamre, 2010; Betancor *et al*., 2011; Betancor *et al*., 2012a, c, d). Selenium is an antioxidant essential trace mineral in nutrition of marine organisms partly obtained

from the surrounding water (Lall and Bishop, 1977), but mostly from the diet (Halver, 2002b). Selenium, as an essential micronutrient, plays an important role in antioxidant defences being a cofactor for antioxidant enzyme GPX (Felton *et al*., 1996), which reduces hydroperoxides by catalyzing the oxidation reaction of glutathione (Arteel and Sies, 2001). Selenium has been shown to prevent dietary hepatic necrosis and exudative diathesis (Schwarz *et al.*, 1957), its deficiency being characterized by cardiomyopathy and muscular weakness (Chen *et al.*, 1980). Despite the fact of being a required micronutrient, Se may be toxic at concentrations only slightly greater than the nutritional requirements (Hilton *et al.,* 1980).

There are very few studies denoting the importance of Se supplementation in fish diets and, despite its particular importance in microdiets for marine fish larvae, none of them has aimed to determine the larval requirements for this micronutrient by feeding at least 5 different dietary levels. Thus, the objective of the present study was to investigate the effect of graded levels of yeast derived Se in early weaning microdiets rich in KPL on gilthead seabream (*Sparus aurata*) larvae performance, resistance to stress, biochemical composition and expression of genes related to oxidative stress and bone metabolism.

Material and methods

Gilthead seabream larvae were obtained from natural spawnings from Instituto Canario de Ciencias Marinas (Grupo de Investigación en Acuicultura (GIA), Las Palmas de Gran Canaria, Spain). Larvae (5.1 mm total length, 100 µg dry body weight) previously fed rotifers (*Brachinous plicatilis*) enriched with DHA Protein Selco® (INVE, Dendermond, Belgium) until 16 dph, were randomly distributed in 15 experimental tanks at a density of 2100 larvae tank^{-1} and fed one of the diets tested in triplicate. All tanks (200 L fibreglass cylinder tanks with conical bottom and painted a light grey colour) were supplied with filtered seawater (37 ppm salinity) at an increasing rate of 0.4 - 1.0 L min^{-1} to assure good water quality during the entire trial. Water entered from the tank bottom and exited from the top to ensure water renewal and to maintain high water quality, which was tested daily and no deterioration was observed. Water was continuously aerated (125 ml min-^{1}) attaining 6.3±1 ppm dissolved O_2. Average water temperature and pH along the trial were 19.8±1.5°C and 7.89, respectively. Photoperiod was kept at 12h light: 12h dark, by fluorescent

daylights and the light intensity was kept at 1700 lux (digital Lux Tester YF-1065, Powertech Rentals, Western Australia, Australia).

Five experimental microdiets (pellet size 250-500 µm) were formulated containing a PL rich krill oil (Qrill, high PL, Aker BioMarine, Fjordalléen, Norway) and five levels of yeast derived Se (Sel-Plex® 2000, 2000 mg Se kg^{-1}, Alltech, Lexington, KY) as source of organic Se. Thus, analysed Se content of the diets was 1.73, 3.91, 6.41, 8.47 and 11.65 mg/kg for diets 2SE, 4SE, 6SE, 8SE and 12 SE, respectively. Diet formulation and proximate analysis are showed in Table 7.1 and their fatty acids profiles in Table 7.4. The microdiets were prepared by mixing squid powder and water-soluble components, then the lipids and fat-soluble vitamins and, finally, the gelatine dissolved in warm water. The paste was compressed pelleted (Severin, Suderm, Germany) and dried in an oven at 38 ºC for 24 h (Ako, Barcelona, Spain). Pellets were ground (Braun, Kronberg, Germany) and sieved (Filtra, Barcelona, Spain) to obtain a particle size between 250 to 500 µm. Diets were prepared and analyzed for proximate and fatty acid composition at GIA laboratories.

Table 7.1 Formulation and proximate composition of the experimental microdiets containing five selenium levels

Ingredients (g/100 g Diet)	2SE	4SE	6SE	8SE	12SE
Sel-Plex® 2000[a]	0	0.1	0.2	0.3	0.5
Squid powder[b]	69	68.9	68.8	68.7	68.5
KPL[c]	13	13	13	13	13
Oleic acid[d]	1.5	1.5	1.5	1.5	1.5
Gelatin	3	3	3	3	3
Min Px	4.5	4.5	4.5	4.5	4.5
Vitamin Px	6	6	6	6	6
Attractant	3	3	3	3	3
Proximate analysis					
% dry weight					
Total lipids	25.54	25.62	25.35	25.42	25.28
Protein	61.5	61.9	60.7	59.5	59.2
Ash	7.11	7.35	7.39	7.41	7.49
Humidity	8.00	7.90	8.10	7.93	8.23
Se mg/kg	1.73	3.91	6.41	8.47	11.65

a Alltech, Lexington, KY.
b Rieber and Son, Bergen, Norway.
c Qrill, high phospholipids, Aker BioMarine, Fjordalléen, Norway.
d Merck KGaA, Darmstadi, Germany.
Diets were manually supplied fourteen times per day each 45 min from 9:00 to 19:00

for 29 days. Non-enriched rotifers were co-fed during days 16th and 17th (1 rotifer/ml). To assure feed availability, daily feed (pellet size < 250 µm) supplied was maintained at 1.5 and 2.5 g per tank during the first and second week of feeding. The amount of feed added daily was gradually increased to 4-5 g per tank with increasing in pellet size to 250–500µm, where an overlap using a mixture of both pellets sizes was conducted during the third and forth week of feeding. Larvae were observed under the binocular microscope to determine feed acceptance. Before the end of the experiment an activity test by thermal shock was conducted allocating 25 larvae tank-^{1} in another tank supplied with seawater at a temperature of 24ºC and aeration and determining their survival after 24 hours. Final survival was calculated by individually counting all the living larvae at the end of the experiment. Growth was determined by measuring dry body weight (105ºC 24 h) and total length (Profile Projector V-12A Nikon, Tokyo, Japan) of 30 fish tank^{-1} at the beginning, in the middle and at the end of the trial. In addition, at the end of the trial and after 12 h of starvation, the all larvae in each tank were washed with distilled water, sampled and kept at -80 ºC for biochemical composition.

Fresh larvae samples (100 mg per tank) for molecular biology analysis were well washed and conserved into 500 ml RNA Later (SIGMA), a product that rapidly allows tissue to stabilize and protects cellular RNA in situ in unfrozen samples and permits posterior RNA isolation. The larvae were stored in RNA later overnight at 4 ºC and after that RNA Later was removed before being stored at - 80 ºC to prevent the formation of salt crystals.

Moisture (A.O.A.C. 1995), protein (A.O.A.C. 1995) and crude lipid (Folch 1957) contents of larvae and diets were analyzed. Fatty acid methyl esters were obtained by transmethylation of crude lipids as described by Christie (1982). Fatty acid methyl esters were separated by GLC (GC -14A, Shimadzu, Tokyo, Japan) in a Supercolvax-10-fused silica capillary column (length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180 ºC for the first 10 min, increasing to 215 ºC at a rate of 2.5 ºC/min and then held at 215 ºC for 10 min. Fatty acid methyl esters were quantified by FID (GC -14A, Shimadzu, Tokyo, Japan) following the conditions described in Izquierdo *et al*. (1990) and identified by comparison to previously characterized standards and GLC-MS.

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 ICP-MS (Thermo Scientific, Cheshire, UK) using argon and hydrogen as carrier gas.

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}.

Molecular biology analysis was carried out at GIA laboratories. Total RNA from larvae samples (average weight per sample 60mg) was extracted using the Rneasy Mini Kit (Qiagen). Total body tissue was homogenised using the TissueLyzer-II (Qiagen, Hilden, Germany) with QIAzol lysis reagent (Qiagen). Samples were centrifuged with chloroform for phase separation (12000g, 15min, 4ºC). The upper aqueous phase containing RNA was mixed with 75% ethanol and transferred into an RNeasy spin column where total RNA bonded to a membrane and contaminants were washed away by RW1 and RPE buffers (Qiagen). Purified RNA was eluted with 30ml of RNase-free water. The quality and quantity of RNA were analysed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Synthesis of cDNA was conducted using the iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's instructions in

an iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). Primer efficiency was tested with serial dilutions of a cDNA pool (1, 1:5, 1:10, 1:15,1:20 and 1:25). The product size of the real-time q PCR amplification was checked by electrophoresis analyses using PB322 cut with HAEIII as a standard. Real-time quantitative PCR was performed in an iQ5 Multicolor Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA) using β-actin as the house-keeping gene in a final volume of 20ml per reaction well, and 100ng of total RNA reverse transcribed to complementary cDNA. Each gene sample was analysed once per gene.

Table 7.2 Sequences of forward and reverse primers (5'-3') for real-time quantitative-PCR of seabream genes

Gene	Primers	Accession n°
Catalase(CAT)	Forward primer: 5´-ATGGTGTGGGACTTCTGGAG-3´ Reverse primer: 3´-AGTGGAACTTGCAGTAGAAAC-5´	FJ860003
Superoxide dismutase (SOD)	Forward primer: 5´- AAGAATCATGGCGGTCCTACTGA-3´ Reverse primer: 3´- TGAGCATCTTGTCCGTGATGTCT -5´	AJ937872
Glutathione peroxidase (GPX)	Forward primer: 5´- TCCATTCCCCAGCGATGATGCC-3´ Reverse primer: 3´- TCGCCATCAGGACCAACAAGGA-5´	DQ524992
Osteocalcin	Forward primer: 5´-AGCCCAAAGCACGTAAGCAAGCTA-3´ Reverse primer: 3´- TTTCATCACGCTACTCTACGGGTT-5´	AF048703
Osteopontin	Forward primer: 5´- AAGATGGCCTACGACATGACAGAC- 3´ Reverse primer: 3´- CCTGAAGAGCCTTGTACACCTGC-5´	AY651247
Osteonectin	Forward primer: 5´- AAAATGATCGAGCCCTGCATGGAC-3´ Reverse primer: 3´- TACAGAGTCACCAGGACGTT-5´	AY239014
[a]RUNX2	Forward primer: 5´- GCCTGTCGCCTTTAAGGTGGTTGC-3´ Reverse primer: 3´- TCGTCGTTGCCCGCCATAGCTG-5´	AJ619023
Alkaline phosphatase	Forward primer: 5´- AGAACGCCCTGACGCTGCAA-3´ Reverse primer: 3´- TTCAGTATACGAGCAGCCGTCAC-5´	AY266359
Matrix Gla protein	Forward primer: 5´- GTGCCCTCCTTCATTCCAC-3´ Reverse primer: 3´- TATGACCACGTTGGATGCCT-5´	AY065652
[b]BMP4	Forward primer: 5´- CCACCAGGGCAGACACGTCC-3´ Reverse primer: 3´- GCGTAGCTGCTCCCAGTCCTC-5´	FJ436409
β-actin	Forward primer: 5´- TCTGTCTGG ATC GGAGGCTC-3´ Reverse primer: 3´- AAGCATTTG CGGTGGACG -5´	X89920

a RUNX2 (Runt-related transcription factor 2).
b BMP4 (Bone morphogenetic protein 4).

The PCR conditions were the following: 95ºC for 3min 30sec followed by 40 cycles of 95ºC for 15sec, 61ºC for 30sec, and 72ºC for 30sec; 95ºC for 1min, and a final denaturing step from 61ºC to 95ºC for 10sec. Data obtained were normalised and the Livak method ($2^{-\Delta\Delta Ct}$) used to determine relative mRNA expression levels. Gilthead seabream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer Analysis software (Molecular Biology Insights, Cascade, CO, USA). Gilthead seabream specific gene primers were designed after searching the NCBI nucleotide database and using the Oligo 7 Primer

Analysis software (Molecular Biology Insights, Cascade, CO, USA). Detailed information on primer sequences and accession numbers is presented in Table 7.2.

Results

All the experimental diets were well accepted by gilthead seabream larvae according to the microscopic observations. Generally, survival was very high for this type of studies and it was significantly correlated to the dietary Se contents (Figure 7.1). Thus, average survival of larvae fed 12SE diet (54% survival rate) was significantly higher to 2SE, 4SE and 6SE treatments, but was not significantly different to 8SE treatment. Generally, there was a very good survival of the termal stress and only survival in larvae fed 12SE diet was significantly different than that of the other larvae. The resistance to thermal shock stress was correlated to the dietary Se contents (Figure 7.2).

The larval growth after thirty days of feeding in terms of total length and body weight was very good regardless the experimental diets used. There was no significant difference in the larval growth, expressed either in total length or body weight, among the larvae fed the five experimental diets (Table 7.3).

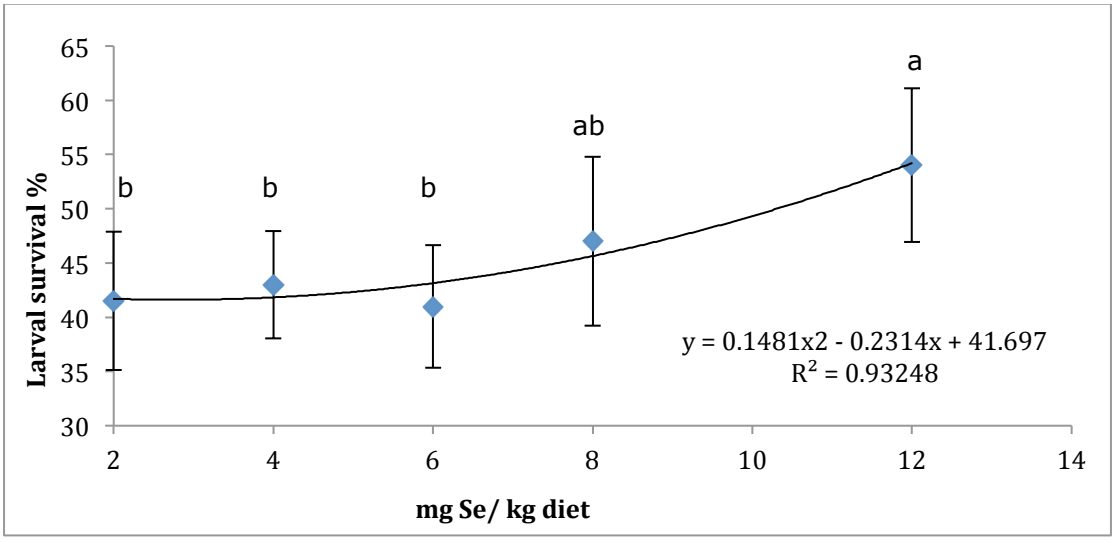

Figure 7.1 Survival rate of larvae (44 dph) fed five dietary selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

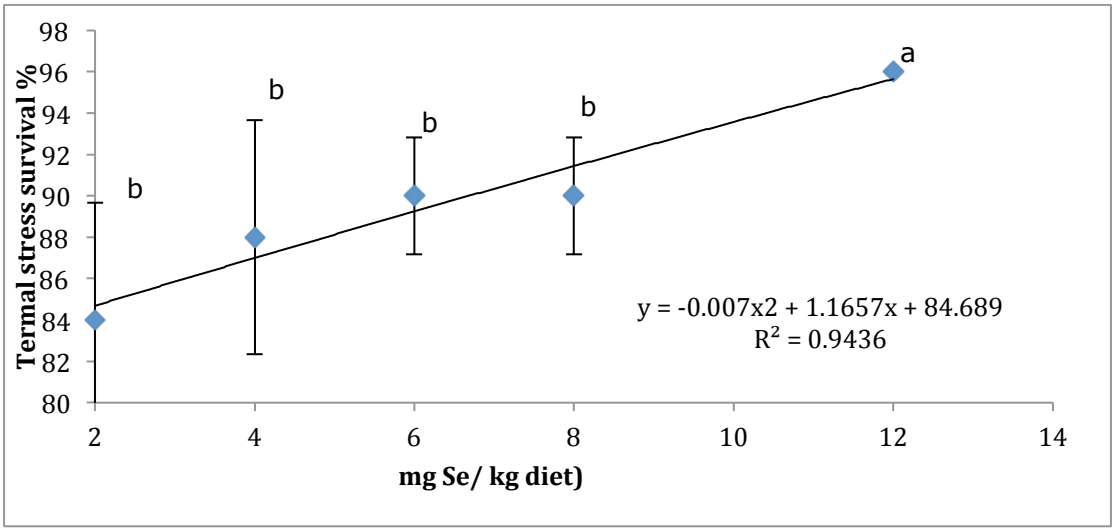

Figure 7.2 Survival rate 24 h after thermal shock of 24ºC of larvae (44 dph) fed five dietary selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Tale 7.3 Growth in total length and body dry weight of larvae (44 dph) fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05)

	2SE	4SE	6SE	8SE	12SE
Total length (mm)	10.68±1.22[a]	10.75±1.11[a]	10.44±1.29[a]	10.72±1.22[a]	10.95±1.00[a]
Dry weight (ug)	1900±119[a]	1920±112[a]	1926±120[a]	1896±130[a]	2015±110[a]

Biochemical composition of the larvae reflected the dietary content. Se content in seabream larvae was correlated to the dietary Se levels. Thus larvae fed diet of 12SE showed the significantly highest content of this mineral, being 6, 3 and 2 times higher than in the larvae fed 2SE, 4SE and 6SE respectively, denoting the progressive absorption of this nutrient (Figure 7.3).

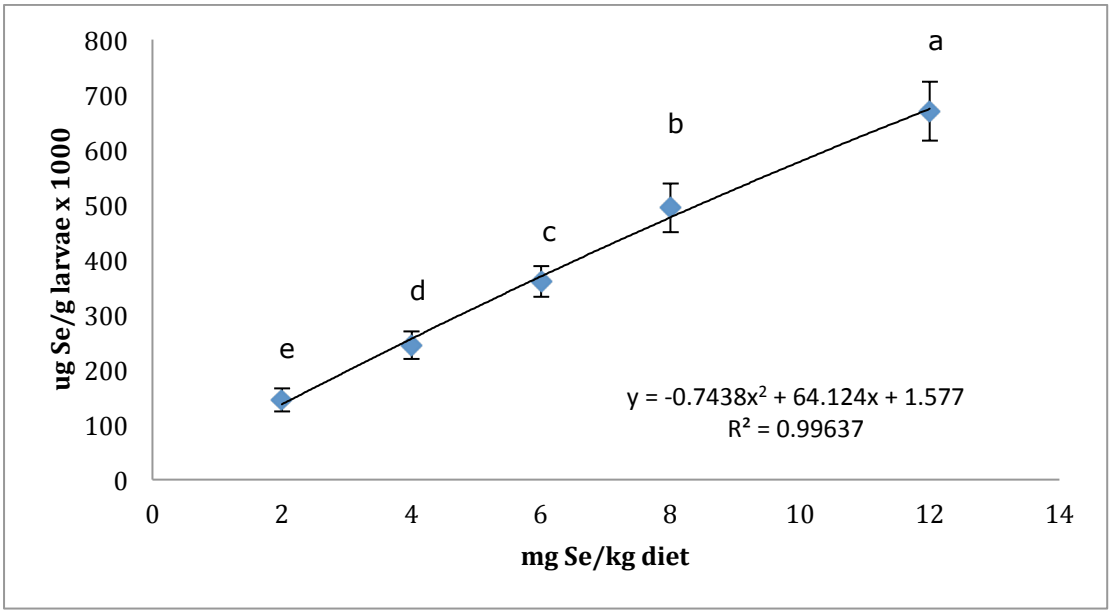

Figure 7.3 The selenium content in larvae (44 dph) fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

The fatty acid composition of the experimental diets was very similar and was not affected by the inclusion of different Se levels (Table 7.4). However, the fatty acid composition of the larvae was significantly affected by the dietary Se inclusion (Table 7.5). Thus, the larvae fed 8SE, and particularly, 12SE diets, were higher in n-3 and n-3 HUFA, mainly due to higher DHA, than 2SE, 4SE and 6SE larvae. Moreover, the larval DHA contents were correlated to the Se contents in larval tissues ($y = 0.0053x + 24.248$, $R^2 = 0.86269$). No significant difference was found in n-6, n-9, EPA and ARA contents among the different larvae.

Table 7.4 Fatty acids (% dry weight) composition in total lipids of diets containing five selenium levels

	0 SE	2 SE	4 SE	6 SE	10 SE
14:0	1.97	1.69	1.83	1.66	1.68
14:1n-5	0.01	0.05	0.05	0.05	0.05
14:1n-7	0.01	0.04	0.04	0.04	0.04
15:0	0.06	0.09	0.09	0.09	0.09
15:1n-5	0.01	0.01	0.01	0.01	0.01
16:0iso	0.08	0.07	0.08	0.07	0.07
16:0	4.55	4.83	4.95	4.61	4.68
16:1n-7	1.09	1.02	1.09	1.03	1.03
16:1n-5	0.04	0.05	0.05	0.05	0.05
16:2n-6	0.01	0.08	0.09	0.09	0.09
16:2n-4	0.09	0.21	0.24	0.23	0.22

17:0	0.01	0.03	0.04	0.04	0.04
16:3n-4	0.09	0.07	0.07	0.07	0.07
16:3n-3	0.02	0.06	0.06	0.06	0.06
16:3n-1	0.09	0.06	0.06	0.01	0.03
16:4n-3	0.09	0.15	0.16	0.16	0.16
16:4n-1	0.02	0.01	0.00	0.01	0.01
18:0	1.51	0.58	0.56	0.56	0.56
18:1n-9	3.56	3.13	3.06	3.10	3.08
18:1n-7	0.65	1.16	1.18	1.18	1.17
18:1n-5	0.07	0.08	0.07	0.08	0.08
18:2n-9	0.02	0.00	0.01	0.03	0.02
18:2n-6	0.37	0.39	0.35	0.39	0.38
18:2n-4	0.03	0.02	0.01	0.02	0.02
18:3n-6	0.01	0.03	0.02	0.03	0.03
18:3n-4	0.02	0.01	0.01	0.01	0.01
18:3n-3	0.20	0.14	0.14	0.15	0.14
18:4n-3	0.03	0.40	0.42	0.43	0.42
18:4n-1	0.00	0.01	0.00	0.01	0.01
20:0	0.04	0.01	0.01	0.01	0.01
20:1n-9	0.02	0.04	0.03	0.04	0.04
20:1n-7	0.26	0.43	0.42	0.44	0.43
20:1n-5	0.03	0.05	0.05	0.06	0.06
20: 2n-9	0.00	0.00	0.00	0.00	0.00
20:2n-6	0.07	0.03	0.03	0.03	0.03
20:3n-9	0.00	0.00	0.00	0.01	0.01
20:3n-6	0.00	0.01	0.01	0.01	0.01
20:4n-6	0.23	0.15	0.14	0.16	0.15
20:3n-3	0.04	0.06	0.05	0.07	0.06
20:4n-3	0.04	0.06	0.06	0.06	0.06
20:5n-3	3.64	3.30	3.43	3.61	3.47
22:1n-11	0.00	0.01	0.01	0.01	0.01
22:1n-9	0.07	0.13	0.12	0.14	0.13
22:4n-6	0.02	0.08	0.08	0.09	0.09
22:5n-6	0.04	0.03	0.00	0.01	0.01
22:5n-3	0.08	0.08	0.07	0.23	0.17
22:6n-3	4.24	4.69	4.09	4.14	4.26
Total Saturated	8.15	7.23	7.49	6.98	7.06
Total Monoenoics	5.82	6.19	6.18	6.23	6.17
Total n-3	8.38	8.94	8.47	8.93	8.80
Total n-6	0.75	0.79	0.73	0.81	0.79
Total n-9	3.68	3.30	3.22	3.31	3.27
Total n-3HUFA	8.04	8.20	7.69	8.11	8.02
ARA	0.23	0.15	0.14	0.16	0.15
EPA	3.64	3.30	3.43	3.61	3.47
DHA	4.24	4.69	4.09	4.14	4.26
EPA/ARA	15.70	22.11	23.94	23.12	22.93
DHA/EPA	1.17	1.42	1.19	1.15	1.23
DHA/ARA	18.30	31.44	28.56	26.54	28.13
n-3/n-6	11.10	11.35	11.67	10.99	11.17

Table 7.5 Fatty acids (% total identified fatty acids) composition in total lipids of larvae (44 dph) fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters in the same row are not significantly different (P>0.05)

	2SE	4SE	6SE	8SE	12SE
14:0	3.16	2.77	3.27	3.25	2.16
14:1n-5	0.11	0.12	0.10	0.12	0.12
14:1n-7	0.05	0.04	0.04	0.05	0.05
15:0	0.32	0.29	0.32	0.34	0.35
15:1n-5	0.03	0.03	0.02	0.02	0.03
16:0iso	0.41	0.47	0.49	0.42	0.45
16:0	21.80	21.78	22.08	17.76	16.32
16:1n-7	2.76	2.59	2.69	2.97	2.94
16:1n-5	0.21	0.22	0.20	0.23	0.24
16:2n-6	0.18	0.17	0.18	0.20	0.20
16:2n-4	0.39	0.86	0.40	0.37	0.42
17:0	0.08	0.08	0.09	0.09	0.09
16:3n-4	0.28	0.27	0.27	0.28	0.29
16:3n-3	0.24	0.23	0.23	0.25	0.26
16:3n-1	0.37	0.43	0.46	0.37	0.41
16:4n-3	0.19	0.20	0.19	0.21	0.21
16:4n-1	0.09	0.12	0.11	0.09	0.10
18:0	5.79	6.30	6.11	5.92	5.97
18:1n-9	12.23	11.76	12.07	12.66	12.74
18:1n-7	4.47	4.29	4.40	4.66	4.75
18:1n-5	0.36	0.34	0.34	0.37	0.38
18:2n-9	0.07	0.08	0.07	0.09	0.09
18:2n-6	1.24	1.19	1.18	1.29	1.29
18:2n-4	0.10	0.08	0.08	0.09	0.10
18:3n-6	0.12	0.10	0.07	0.07	0.12
18:3n-4	0.09	0.09	0.05	0.10	0.12
18:3n-3	0.35	0.32	0.33	0.37	0.37
18:4n-3	0.94	0.83	0.88	1.01	1.02
18:4n-1	0.04	0.03	0.03	0.03	0.03
20:0	0.14	0.15	0.16	0.14	0.15
20:1n-9	0.10	0.08	0.09	0.10	0.10
20:1n-7	1.34	1.19	1.28	1.42	1.41
20:1n-5	0.25	0.24	0.25	0.26	0.27
20: 2n-9	0.01	0.01	0.01	0.01	0.01
20:2n-6	0.11	0.11	0.11	0.12	0.13
20:3n-9	0.01	0.01	0.00	0.00	0.01
20:3n-6	0.06	0.06	0.06	0.06	0.07
20:4n-6	1.14	1.19	1.19	1.17	1.24
20:3n-3	0.22	0.20	0.19	0.23	0.23
20:4n-3	0.23	0.22	0.22	0.24	0.25
20:5n-3	13.14	12.29	12.36	13.87	14.41
22:1n-11	0.03	0.03	0.02	0.03	0.02
22:1n-9	0.59	0.53	0.56	0.62	0.63
22:4n-6	0.29	0.27	0.27	0.31	0.32

22:5n-6	0.19	0.20	0.19	0.16	0.17
22:5n-3	0.84	0.94	1.08	0.92	0.93
22:6n-3	24.85	26.19	25.70	26.63	28.04
Total Saturated	30.97±2.45[a]	31.08±2.03[a]	31.71±2.37[a]	27.17±3.1[b]	24.69±2.91[b]
Total Monoenoics	22.51±1.89[a]	21.48±1.57[a]	22.08±1.65[a]	23.51±1.78[a]	23.67±2.1[a]
Total n-3	41.00±2.91[b]	41.41±2.12[b]	40.68±3.21[b]	43.72±2.96[ab]	45.73±2.25[a]
Total n-6	3.33±0.61[a]	3.29±0.57[a]	3.24±0.49[a]	3.39±0.63[a]	3.54±0.71[a]
Total n-9	13.01±1.13[a]	12.48±1.09[a]	12.80±1.10[a]	13.48±1.21[a]	13.58±1.18[a]
Total n-3HUFA	39.28±2.87[b]	39.84±2.59[b]	39.05±2.64[b]	41.88±3.41[ab]	43.86±2.71[a]
ARA	1.14±0.11[a]	1.19±0.09[a]	1.19±0.12[a]	1.17±0.10[a]	1.24±0.13[a]
EPA	13.14±1.07[a]	12.29±1.10[a]	12.36±1.2[a]	13.87±1.16[a]	14.41±1.4[a]
DHA	24.85±1.62[b]	26.19±1.44[ab]	25.70±1.95[ab]	26.63±1.81[ab]	28.04±1.76[a]
EPA/ARA	11.58±1.18[a]	10.31±1.34[a]	10.38±1.29[a]	11.85±1.19[a]	11.58±1.21[a]
DHA/EPA	1.89±0.21[a]	2.13±0.19[a]	2.04±0.18[a]	1.92±0.17[a]	1.95±0.20[a]
DHA/ARA	21.88±1.98[a]	21.98±1.67[a]	21.16±1.72[a]	22.76±1.95[a]	22.53±1.93[a]
n-3/n-6	12.32±1.2[a]	12.59±1.14[a]	12.54±1.35[a]	12.92±1.62[a]	12.93±1.27[a]

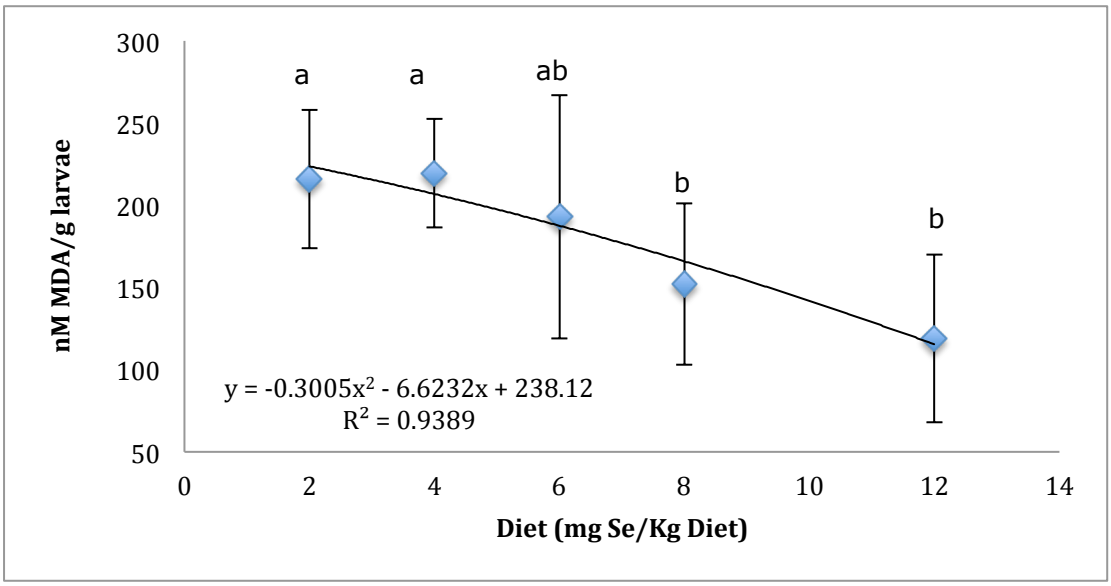

Figure 7.4 The malondialdehyde (MDA) content in larvae (44 dph) fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

The degree of larval lipid oxidation (Figure 7.4), as indicated by larval malondialdehyde (MDA) content, was significantly lower in larvae fed 8SE and 12SE diets (152 and 119 nmol g^{-1} larvae respectively) compared with those of 2SE and 4SE diets (216 and 220 nmol g^{-1} larvae respectively) and were negatively correlated with the dietary Se contents. Besides, the MDA contents in the larvae were also negatively correlated to the larval Se contents ($y = -0.2041x + 257.94$, $R^2 = 0.94804$).

The general pattern of antioxidant enzyme genes expression of seabream larvae was a decreased expression with increasing dietary Se levels (Figures 7.5, 7.6 and 7.7). Thus, larvae fed 12SE diets showed significantly lower CAT gene expression than larvae fed 2SE, 4SE and 6SE diets, being CAT gene expression in larvae negatively correlated to the Se content in the larvae ($y = -0.0136x + 10.83$, $R^2 = 0.90955$). Larvae fed 12SE diet also showed significantly lower GPX gene expression than larvae fed 2SE and lower SOD gene expression than 2SE and 4SE treatments. There was no significant difference between larvae fed 4SE, 6SE and 8SE diets in all antioxidant enzyme gene expression.

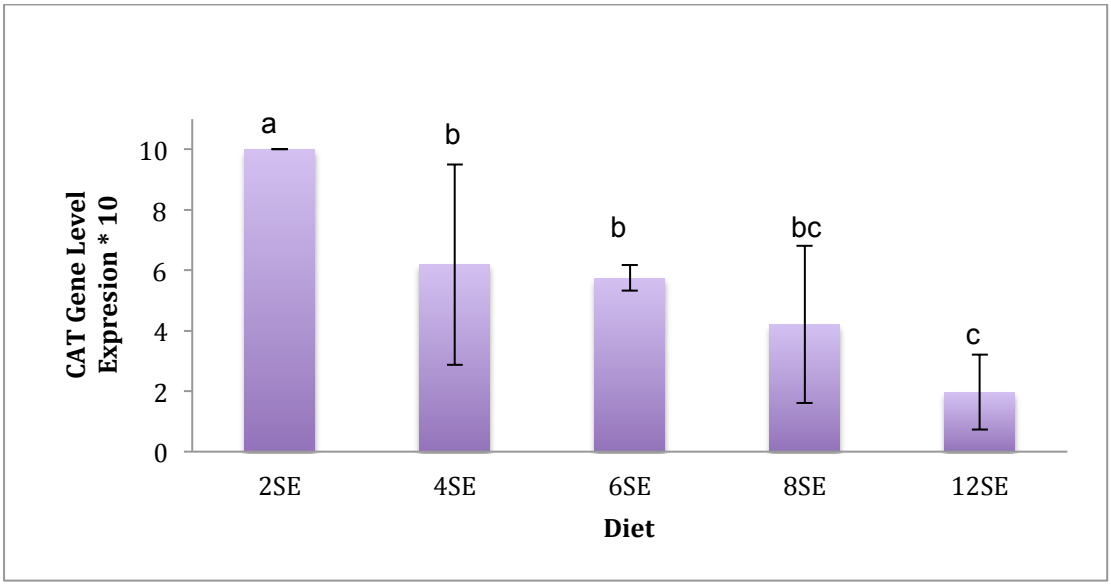

Figure 7.5 Catalase (CAT) gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

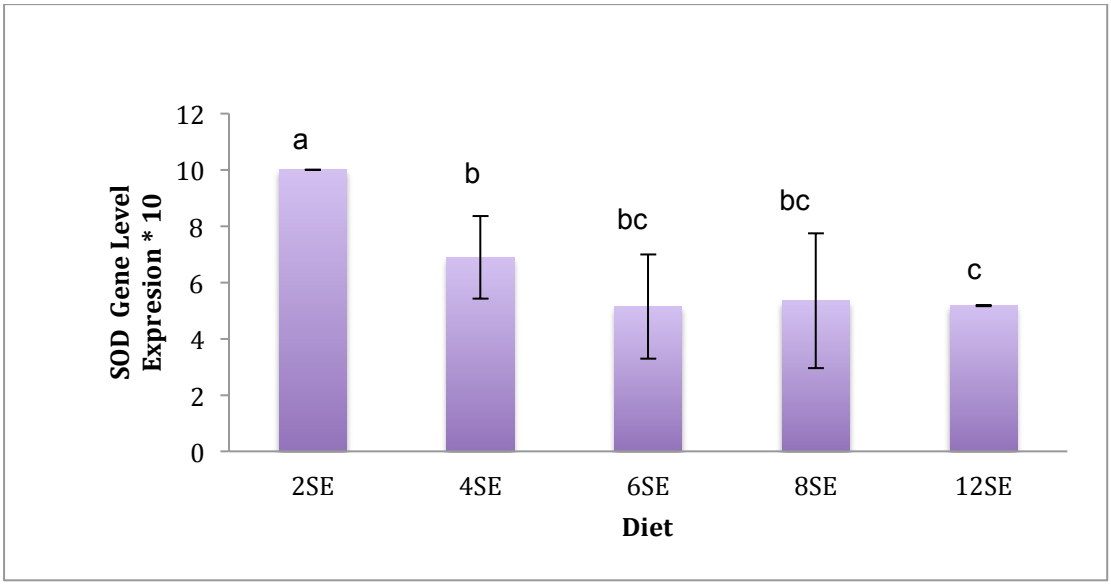

Figure 7.6 Superoxide dismutase (SOD) gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

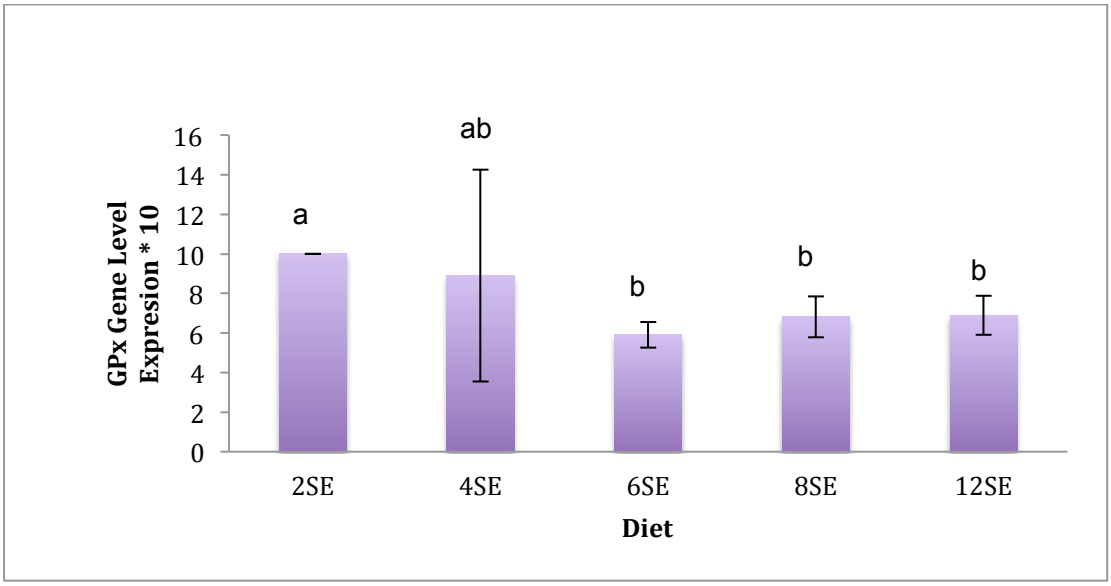

Figure 7.7 Glutathione peroxidase (GPX) gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

The osteological genes that have role in bone-building, formation of cartilage and bone remodeling and mineralization were expressed in slightly higher levels in larvae fed higher dietary SE contents (8SE and 12SE). The osteocalcin, osteonectin osteopontin, alkaline phosphatase and matrix gla protein genes showed a significantly higher expression in larvae fed 8SE and 12SE diets than larvae fed 2SE, 4SE and 6SE diets, while there were no significant differences among the last treatments (Figures 7.8, 7.9, 7.10, 7.12 and 7.13). A significant correlation was found between ALP gene expression and larval Se contents ($y = 0.0179x + 6.7818$, $R^2 = 0.97113$) or larval n-3 HUFA contents ($y = 1.7433x - 57.472$, $R^2 = 0.9068$). There were no significant differences among all treatments in Runx2 (Runt-related transcription factor 2) gene expression (Figure 7.11). The larvae fed 8SE diet have higher BMP4 gene expression than the all treatments, while there was no significant difference between the 4SE, 6SE and 12SE treatments (Figure 7.14).

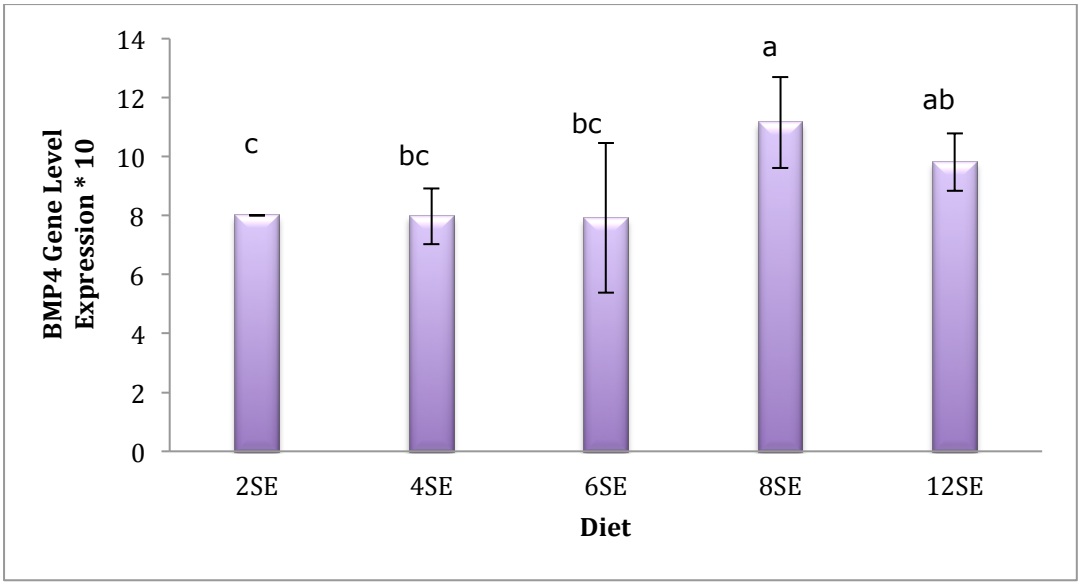

Figure 7.8 BMP4 (Bone morphogenetic protein 4) gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

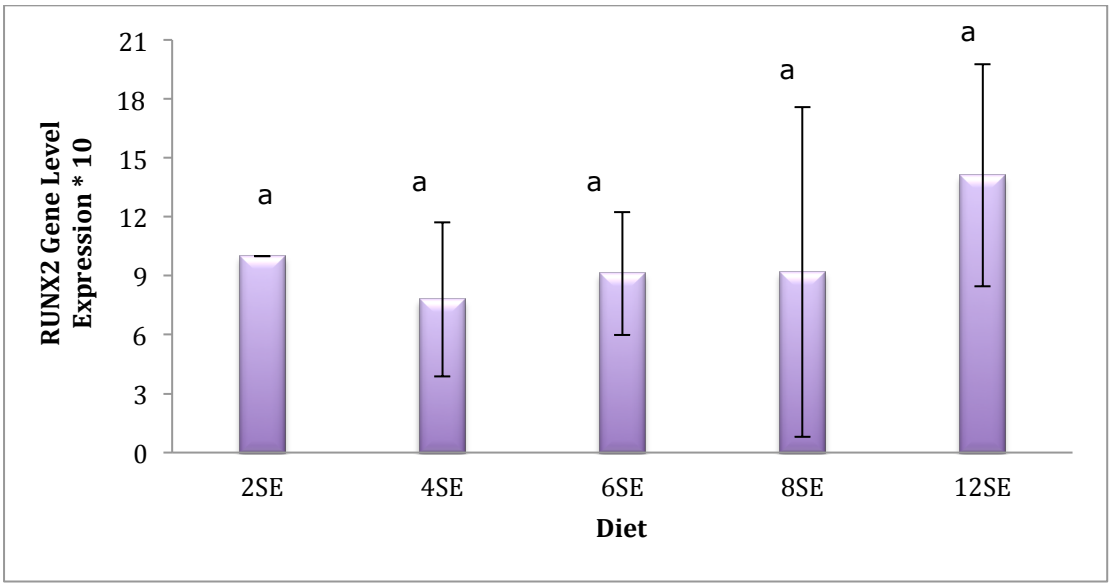

Figure 7.9 Runx2 (Runt-related transcription factor 2) gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

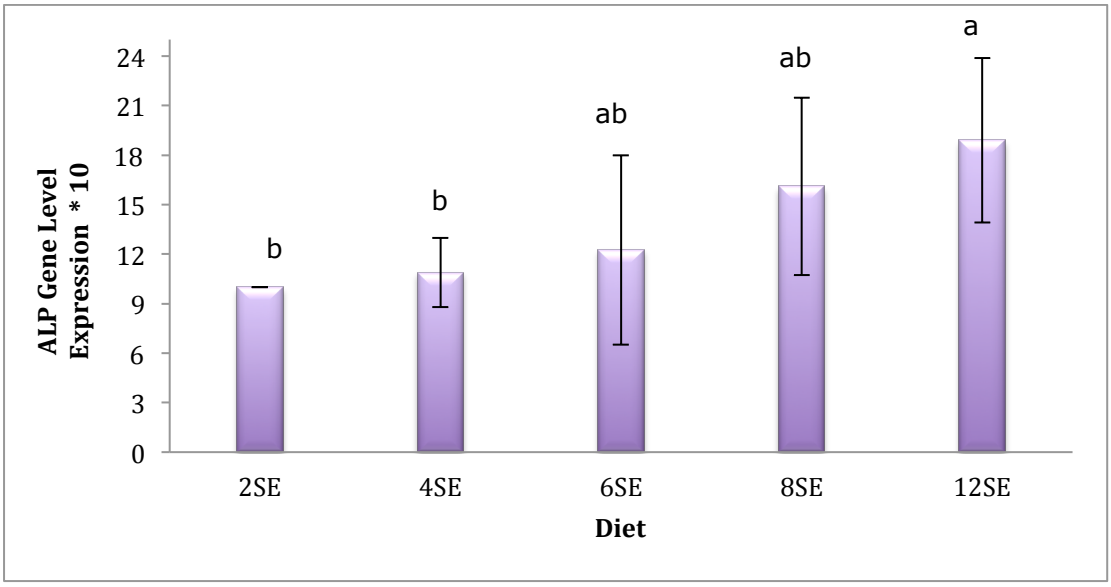

Figure 7.10 Alkaline phosphatase gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

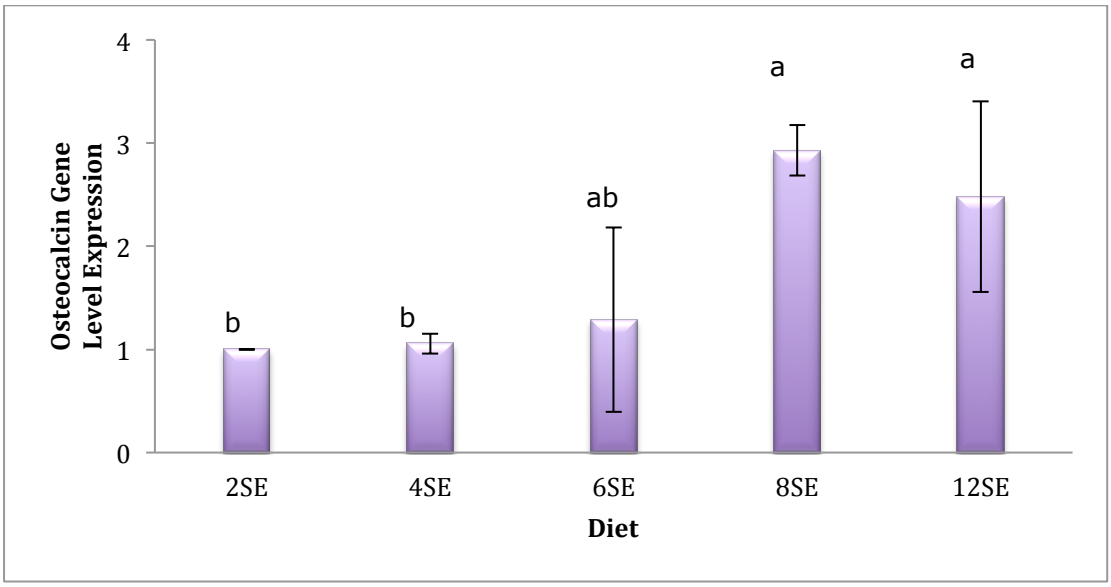

Figure 7.11 Osteocalcin gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

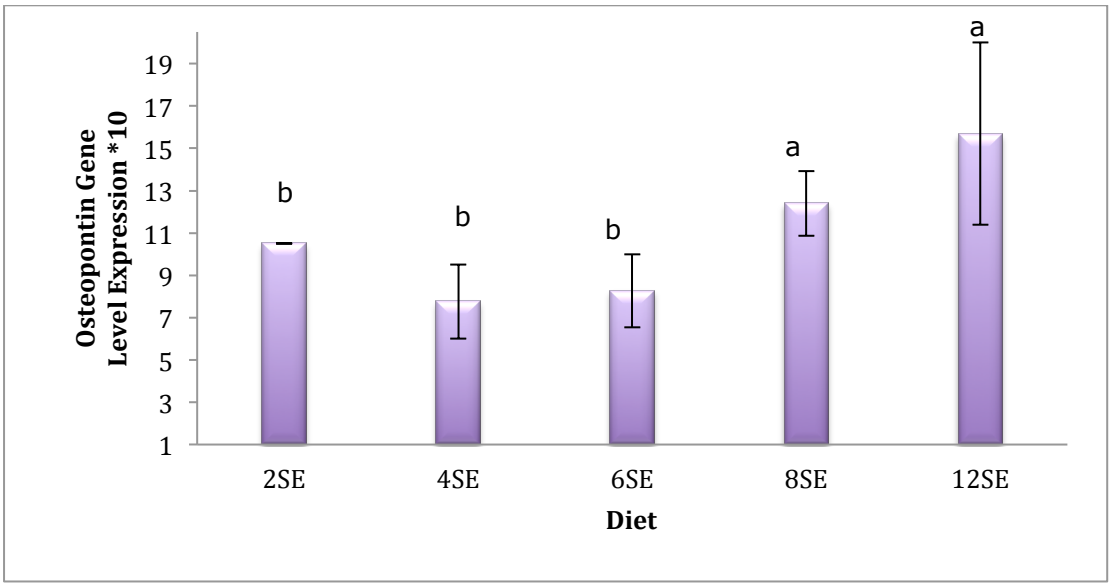

Figure 7.12 Osteopontin gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

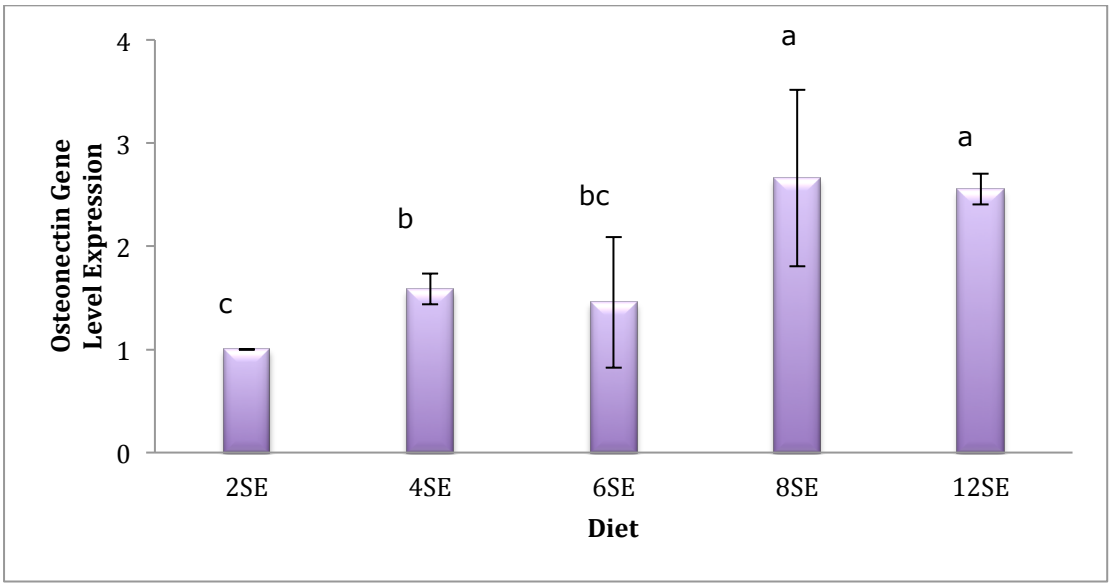

Figure 7.13 Osteonectin gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

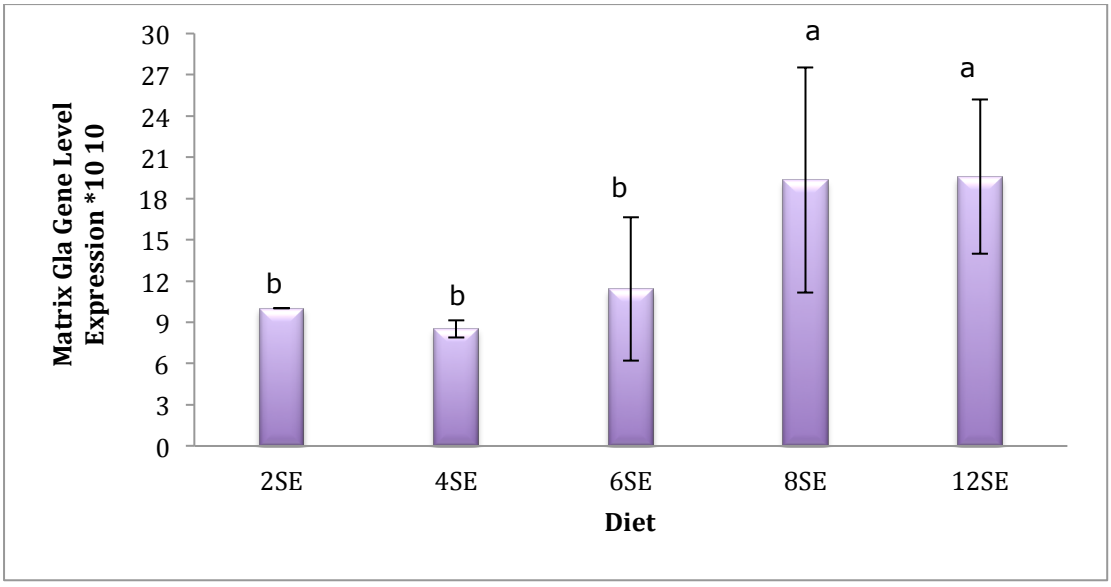

Figure 7.14 Matrix Gla gene expression level measured by real-time PCR in seabream larvae fed diets containing five selenium levels. Values (mean ± standard deviation) with the same letters are not significantly different (P>0.05).

Discussion

The aim of the present study was to evaluate the effect of graded levels of Se derived yeast in diets containing KPL rich in n-3HUFA on larval production performance, osteological genes expression, oxidative status and biochemical composition of seabream larvae. Although the increased Se levels did not affected larval growth, the larvae highest dietary Se content (11.65 mg/kg diet) promoted larval survival rate and resistance to stress. This possitive effect in larval survival or stress resistance could be related in one hand to the n-3 HUFA increase on larval tissues caused by a higher protection of these fatty acids against oxidation by the increased Se content in the larvae. Indeed, n-3 HUFA are known to play a crucial role in larval survival (Izquierdo and Koven, 2010). On the other hand, the higher survival and stress resistance could be also related to the reduction in the presence of oxidation products, which can be severely harmful for fish fish causing several pathological damages and larval mortalities (Halliwell and Gutteridge, 1996; Betancor *et al*., 2011). For instance, the inclusion of Se in diets for European seabass proved to be efficient in controlling the damage caused by the reactive oxigen species, reducing the incidence of muscle injury to almost half as compared with the diet without Se supplementation (Betancor *et al*., 2012c).

Indeed, in the present study, increased dietary Se levels resulted in higher Se contents in larval tissues and an improved protection against peroxidation denoted by the decreasing MDA concentrations and showing the essential role of Se as an anti-oxidant factor that reduces hydroperoxides (Arteel and Sies, 2001; Betancor *et al*., 2012c). Moreover, the increase in Se tissue contents was negatively correlated to the CAT gene expression and were also related to lower SOD and GPX, suggesting as well a reduced peroxidation risk and a better oxidative stress status in larvae fed the higher dietary Se levels. These results are in agreement with those found in Manchurian trout (*Brachymystax lenok*) and sea bass (*Dicentrarchus labrax*) larvae, where feeding high dietary lipid levels increased MDA production and induced an antioxidant response noticeable by an increase in the activity of antioxidant enzimes genes (Zhang *et al.*, 2009; Betancor *et al*., 2012a, c, d). These results agree well with other studies that demonstrated that Se supplementation has a protective effect against oxidative stress caused by high dietary DHA in sea bass or methyl parathion in *Brycon cephalus*, as denoted by the decrease in CAT and SOD activity (Monteiro *et*

al., 2009) or gene expression (Betancor *et al*., 2012a). Thus, the inclusion of antioxidative factors, such as Se, could counteract the high oxidation risk in early weanning diets high in PUFA that are more susceptible to oxidation not only in the inert diets but also in larval tissues.

The impact of the inclusion of Se on microdiets with high dietary PL on the expression of genes considered as biomarkers of bone development and mineralization is still limited. Antioxidants may interact with cellular receptors and transcriptional factors that may further lead to changes in mRNA and protein levels or directly interact with enzymes through protein–protein-binding properties (Olsvik *et al*., 2011). Elevated intracellular ROS production may ultimately damage DNA, proteins and lipids (Halliwell and Gutteridge, 1999). Also, ROS can affect the transcription of many genes, either acting via various transcription factors or directly as a result of oxidative damage (Di Giulio and Meyer, 2008). As observed in our study the higher dietary Se levels led to promoted expression of bone formation and mineralization genes, where the BMP4, alkaline phosphatase, osteocalcin, osteonectin osteopontin, and matrix gla protein genes, all important for skeletal development, bone formation and mineralization, were up-regulated by the dietary inclusion at 8.47 and 11.65 µg Se/mg diet. This up-regulation of several bone metabolism related genes could be due to the higher larval tissue content in n-3 HUFA, and particularly DHA, in agreement with the higher expression of these genes when DHA raised in gilthead seabream larval tissues (Saleh *et al*., submitted). In other vertebrates, these genes, particularly BMP-4, are up-regulated by dietary n-3 HUFA exerting a beneficial effect (Kruger *et al*., 2010). Amongst the important mechanisms of PUFA action are their effects on gene transcription that can be mediated by fatty acid binding to the peroxisome proliferator activator receptor (PPAR) transcription factors that have important effects on bone physiology (Kruger *et al*. 2010). The present study demonstrated improved osteological genes expression with higher larval DHA content, where DHA enhance expression of key transcription factors such as specific transcription factors such as Runx2 (also known as core binding factor-1, Cbf1) and osterix that enhance differentiation of pre-osteoblasts into mature osteoblasts that elevate bone mass (Kruger *et al*., 2010). The present study had shown a coincidence between the lower larval MDA content and lower AOE genes expression and the higher larval n-3 HUFA content in the larvae fed higher dietary Se levels that resulted in improving the regulation of the genes involved in skeletal development and

mineralization as BMP4 osteocalcin, osteonectin osteopontin, alkaline phosphatase and matrix gla protein genes.

Betancor *et al*., (2012c) found increased in CAT, SOD and GPX expression in sea bass larvae fed a high-DHA and Se-free diet but similarly, exposure to high-DHA diets caused a significant increase in CAT and GPX in the larvae fed the Se-supplemented diets, that can interpreted by the higher requirements of sea bass larvae of Se (more than 6.27 µg/mg) to be more effective. This is similar to the present study, where the increased of se levels up to 6.41 µg/mg did not affect significantly larval performance, MDA content and AOE genes expression, but the increased up to 8.47 µg/mg and 11.65 µg/mg resulted in better larval survival and resistance to stress, and also resulted in lower MDA content and AOE expression levels. It can be concluded that, supranutritional levels of Se (more than 8.47 µg/mg) are required for marine fish larvae to reduce significantly the larval MDA content and consequently AOE genes expression. Also, another study showed that supranutritional levels of Se are required to reduce the incidence of human and animal diseases (Brown *et al*., 2001).

Finally, the Se levels used did not seem to be excessive, as the larvae fed the highest Se dietary contents did not show reduced growth or any other detrimental effects. Excessive levels of dietary Se have been associated with growth reduced. Chronic Se toxicity for rainbow trout juveniles (1.3 g) occurred at feed levels of around 13 mg Se kg−1 supplied as sodium selenite that resulted in growth reducing (Hilton *et al*., 1980), but Rider *et al*. (2010) demonstrated that there was no toxic effects at 8 mg Se kg−1 supplied mainly as Se via selenoyeast for rainbow trout juveniles. The lower lengthwise growth of cod larvae occurred at 7-17 dph, may indicate excessive dietary Se in the early larval period when fed rotifers with 3 mg Se kg−1 DW (Penglase *et al*., 2010). In agreement with our previous results (Betancor *et al*., 2012c), the Se organic source used derived from yeast, could had a lower toxicity than inorganic Se (Hilton et al., 1980; Rider et al., 2010), since Se toxicity is highly dependent on its speciation (Tinggi, 2003).

Hamre *et al*. (2008) found 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), Atlantic cod and *Epinephelus malabaricus* Juveniles (0.25 and 0.7 mg Se kg−1 DW, respectively; NRC, 1993; Lin and Shiau, 2005) and copepod levels (3-5 mg kg-1 DW)

and may contain insufficient Se to meet larvae requirements. Enrichment of rotifers with sodium selenite and sodium iodide promoted survival in Atlantic cod larvae, but no differences were observed in growth compared to the control group (Hamre *et al*., 2008). 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 oxidative derivatives, which can be abundant in feeds enriched with n-3 LC-PUFA.

In the present study, the improved larval survival and stress resistance by increased organic Se dietary levels could be related in one hand to the reduction of toxic free radicals and in another hand to the improved utilization of dietary lipids. The low levels of MDA and AOE content observed in the larvae fed 11.65 µg Se/mg diet, demonstrate an adaptive response in attempting to neutralize the generated ROS. Moreover, a reactive response was observed by the increase in BMP4, alkaline phosphatase, osteocalcin, osteonectin osteopontin, and matrix gla protein genes expression in larval tissues, suggesting a well skeletal development. Inclusion of 11.65 mg Se kg^{-1} diet in microdiets is recommended to enhance the larval antioxidant capacity and regulation of the genes involved in skeletal development.

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Chapter 8: General Conclusions

1- PL deficiency reduced fish larvae performance, delayed gut development and bone mineralization, inhibited digestive enzymes activity and lowered dietary lipid utilization in seabream larvae.
2- Optimum inclusion levels of krill PL in microdiets that completely substitute live preys from 16 dph are around 12% krill PL, providing about 10% total PL.
3- Optimum inclusion levels of SBL in microdiets that completely substitute live preys from 16 dph are around 8% krill PL, providing about 9% total PL. However, inclusion of SBL markedly increased the proxidation risk as denoted by the higher TBARs in larvae, as well as a higher expression of CAT, GPX and SOD genes.
4- Dietary krill PL were an excellent source of lipids for gilthead seabream larvae, being markedly better than SBL.
5- The dietary PL requirements of gilthead seabream from 16-45 dph are about 9-10% dietary in diets to completely substitute live preys during this period. These values seem to promote digestion, transport and deposition of dietary lipids and contribute to reduce skeleton anomalies by up-regulating BMP-4, Runx2, ALP and the skeletal extracellular matrix proteins, inducing early mineralization and resistance of vertebral bodies to reduce anomalies such as lordosis and kyphosis.
6- The increase in dietary α-tocopherol further promoted the beneficial effects of dietary PL, promoting growth, denoting its protective role against oxidation and reducing larval TBARs and gene expression of SOD and CAT.
7- Inclusion of 11.65 mg Se kg^{-1} diet in microdiets enhanced the larval antioxidant capacity and regulation of the genes involved in skeletal development. The improved larval survival and stress resistance by increased organic Se dietary levels seems related in one hand to the reduction of toxic free radicals as indicated by low levels of MDA and AOE content, and in another hand to the improved utilization of dietary lipids.

Chapter 9: Resumen en español

9.1 Introducción

La acuicultura ha sido el sistema de producción de alimentos que más ha crecido y el sector de producción animal con el mayor desarrollo a nivel mundial. En la última década ha crecido a una tasa superior al 5% anual (Izquierdo, 2005), con una producción mundial que ha crecido desde 0,6 millones Tm en 1950 a 7,3 millones en 1980 y 55,1 millones Tm en 2009 (APROMAR, 2009; FAO, 2011).

Este rápido incremento en producción fue promovido por la mejora en el control de la reproducción, condiciones de cultivo, formulación de alimentos y otras innovaciones tecnológicas en el cultivo de peces, permitiendo con esto un rápido desarrollo de la acuicultura en tierra y mar (UNEP/MAP/MED POL, 2004). El futuro éxito del sector acuícola está íntimamente relacionado con el incremento de la demanda de alimentos de origen marino, lo que ha permitido que esta actividad sea muy atractiva para los inversionistas, así como una alternativa a otras actividades como la pesca de ejemplares silvestres. Sin embargo, su éxito en peces marinos está limitado por la cantidad y calidad de la producción de alevines (Izquierdo and Fernández-Palacios, 1997; Watanabe *et al*., 1983; Yúfera and Pascual, 1984; Sargent *et al*., 1997; Izquierdo *et al*., 2000).

A pesar de que se ha incrementado la producción de varias especies de peces marinos en el Mediterraneo, aún la dorada (*Sparus aurata)* y la lubina (*Dicentrarchus labrax*) son los peces marinos más cultivados y los que han caracterizado el desarrollo de la acuicultura marina europea en las últimas tres décadas (FAO, 1999; Izquierdo, 2005).

La demanda de alevines de buena calidad se ha incrementado a una tasa de 10% anual, alcanzando en 2008 una producción de alevines de dorada y lubina de 497,7 y 457,7 millones, respectivamente (FEAP, 2008).

La mejora en el desarrollo de la producción de alevines depende esencialmente de la efectividad de los protocolos de alimentación en sus primeras fases del cultivo y por la calidad de las raciones de destete utilizadas (Kolkovski *et al*., 1993; Sargent *et al*., 1997; Izquierdo *et al*., 2000). El cultivo de larvas de peces marinos conlleva la administración de nutrientes externos a través de la alimentación usando presas vivas por algunos días, seguida por microdietas para un destete precoz. Sin embargo, ambos alimentos, presas vivas y microdietas, deben satisfacer los requerimientos

nutricionales de cada especie, asegurando un mayor crecimiento y una alta tasa de sobrevivencia (Kolkovski *et al*., 1993; Sargent *et al*., 1997).

Debido a su adecuado tamaño (90-130 µm) algunas cepas del rotifero *Brachionus plicatilis* constituyen la primera presa que consumen las larvas de dorada. Posteriormente, es utilizada Artemia y un alimento complementario, utilizando raciones de destete de acuerdo al tamaño de la boca de la larva. Sin embargo, los costes extras en términos de mano de obra, estructura, tiempo y energía necesarias para la producción de rotíferos y Artemia son significativos (Kolkovski, 2009). Adicionalmente, el valor nutricional de la presas vivas varía notablemente de acuerdo a su alimentación y condiciones ambientales tales como régimen e intensidad de luz, temperatura y salinidad del agua, etc., poniendo en riesgo la calidad nutricional de las larvas (Jones *et al*., 1993; Kolkovski *et al*., 1993; Barnabé and Guissi, 1994; Rosenlund *et al*., 1997). Además, desde un punto de vista experimental, el enriquecimiento efectivo de las larvas vivas está limitado por algunos nutrientes como fosfolípidos (PL) y proteínas (Rosenlund *et al*., 1997; Koven *et al*., 2001). Por todas estas razones, la sustitución de las presas vivas por microdietas es crucial para una producción eficiente y sostenible en términos de costes, además de una alta y constante producción de juveniles de buena calidad.

Durante las últimas tres decadas se ha hecho grandes esfuerzos para desarrollar microdietas que permitan una sustitución parcial o total de rotíferos y/o Artemia. La mayoría de los trabajos han sido enfocados a los requerimientos nutricionales de las larvas.

A pesar de que se han conseguido grandes logros, aún no es posible la sustitución completa del alimento vivo como primer alimento en la mayoría de las especies marinas. Hay estudios que muestran un pobre rendimiento en el cultivo larvario cuando se utilizan dietas inertes como primer alimento exógeno. Esto puede ser debido a la composición, palatabilidad y/o a las características físicas del alimento seco (Person Le Ruyet *et al*., 1993). Hay que considerar que, además de la calidad nutricional del alimento, también hay características físicas de la dieta que pueden afectar su desempeño, tales como su tasa de sedimentación, estabilidad nutricional y perfil de lixiviación, siendo factores que pueden contribuir a que la microdieta sea atractiva y afectando así su tasa de ingestión, digestión y el crecimiento larvario. Sorprendentemente, estos factores han recibido muy poca atención. La producción de microdietas es una alternativa conveniente y económica en lugar del alimento vivo, a

pesar de los problemas de estabilidad en el agua y lixiviación de nutrientes que se encuentran normalmente en la producción de micropartículas (Baskerville-Bridges and Kling, 2000; Onald and Landgon, 2000; Poussão-Ferreira *et al.,* 2003). Por todo esto es importante considerar la fisiología y la ontogenia del sistema digestivo de las larvas alimentadas, el protocolo y técnicas de alimentación a utilizar, así como los requerimientos nutricionales de cada especie, en el proceso de preparación de microdietas destinadas a larvas de peces marinos.

En los últimos años, se han realizado varios estudios para determinar la composición lipídica óptima en las dietas utilizadas para el destete de larvas de peces marinos, estudiándose con especial atención los ácidos grasos poliinsaturados de cadena larga (LCPUFA), así como los requerimientos de fosfolípidos y su papel en el desarrollo larvario (Watanabe, 1982; Takeuchi, 1997; Sargent *et al.,* 1999a; Izquierdo *et al.,* 2003).

9.1.1 Larvas de peces marinos y la utilización de microdietas

Actualmente, la dependencia de la disponibilidad de alevines es uno de los factores críticos para el éxito comercial de la producción industrial de peces marinos (FAO 1998). Existen ciertas fases que son señaladas como puntos críticos durante las primeras etapas del desarrollo larvario:

a) El cambio de la alimentación endógena a la exógena, que comienza con el suministro de rotíferos entre los días 3 y 4 post-eclosión.
b) El momento del destete o la transición de la ingestión de alimento vivo a un alimento inerte utilizado para realizar un destete precoz.

El desarrollo del cultivo de larvas de peces marinos ha sido posible gracias al uso de presas vivas, principalmente rotíferos y nauplios y metanauplios de *Artemia*. Puesto que el cultivo de rotíferos fue desarrollado tempranamente (Ito, 1960), este organismo ha sido ampliamente utilizado en el cultivo larvario de varias especies de peces marinos. Según Verreth (1994), los cuellos de botella en el desarrollo de técnicas para el cultivo larvario son:

I. La falta de conocimiento sobre las condiciones ambientales adecuadas y los requerimientos alimentarios de las especies durante los primeros estadíos de vida.
II. El desconocimiento de los requerimientos nutricionales óptimos, difíciles de evaluar en ausencia de una tecnología de cultivo adecuada.
III. Los cambios en el cultivo y de las prácticas de alimentación específicas de una especie a otra debido a los cambios ontogenéticos y sus fases de crecimiento.
IV. El pequeño tamaño de las larvas y la necesidad de partículas de alimento pequeñas, lo que impone problemas específicos en la tecnología de alimentación desarrollada.

Las larvas de peces marinos son muy vulnerables durante las primeras etapas de desarrollo y tienen estrictos requerimientos bióticos y abióticos para sobrevivir, desarrollarse y crecer. La mayoría de las investigaciones sobre la nutrición de larvas ha contribuido con los conocimientos necesarios para estimar los requerimientos nutricionales de las larvas de peces. Sin embargo, no es fácil cuantificar este tipo de requerimientos. Obviamente, un buen conocimiento de los requerimientos nutricionales de las larvas durante todo el desarrollo larvario contribuiría a optimizar dietas y protocolos de alimentación.

Debido a que las especies de peces marinos experimentan grandes cambios morfológicos y fisiológicos durante la ontogenia, sus requerimientos nutricionales pueden diferir cualitativamente y cuantitativamente en su fase larvaria, estadios juveniles y fase adulta. Por otra parte, las larvas de peces crecen muy rápidamente, por lo que su frecuencia alimenticía y por lo tanto, la ingestión total de nutrientes debe ser alta. El requerimiento nutricional de un nutriente en particular puede ser definido desde el punto de vista fisiológico como la ingesta de nutrientes necesarios para cumplir un papel fisiológico, pero con frecuencia se define como el " requerimiento para el máximo crecimiento y/o supervivencia", donde la relación alimentación-dieta-pez tiene un efecto importante en la determinación de sus necesidades cuantitativas (Izquierdo y Lall, 2004). Se han realizado grandes esfuerzos para mejorar la eficiencia de microdietas con el objetivo de sustituir total o parcialmente el alimento vivo (rotíferos y *Artemia)* en el cultivo larvario de peces

marinos (Koven *et al*., 2001; Kolkovski, 2004; Kolkovski *et al*., 2009.).

Ha habido logros sustanciales en el destete temprano de larvas usando microdietas y una sustitución parcial del alimento vivo, sin embargo, en la mayoría de las especies, tales microdietas todavía no pueden reemplazar por completo el alimento vivo. La sustitución parcial del alimento vivo ha demostrado ser exitosa. Las microdietas formuladas dadas en conjunto con el alimento vivo ("co-feeding") ha permitido obtener destetes precoces, por ejemplo, se ha logrado una sustitución de hasta un 90% de rotíferos por dietas artificiales en la larvicultura de besugo (*Pagrus major*), platija japonesa (*Paralichthys olivaceus*) (Kanazawa *et al*., 1989) y dorada (Liu *et al*., 2001). Aunque el destete de larvas sustituyendo *Artemia* por una microdieta se ha logrado durante la metamorfosis de muchas especies (Foscarini, 1988; Hardy, 1989; Koven *et al*., 2001; Curnow *et al*., 2006a, b), la introducción temprana de dietas formuladas para una total sustitución del alimento vivo ha tenido un éxito limitado (Kanazawa *et al*., 1989; Walford *et al*., 1991; Fernández Díaz-y Yúfera, 1997; Rosenlund *et al*., 1997; Kolkovski *et al*., 2009).

La adición de nauplios de *Artemia* combinada con un microdieta aumenta el movimiento peristáltico del tracto digestivo de las larvas, mejorando su ingestión y la producción de enzimas digestivas (Kolkovski *et al.,* 1995). El diseño y la formulación de estas dietas de destete precoz requiere conocer los requerimientos nutricionales ideales para satisfacer las necesidades fisiológicas de las larvas (Kolkovski *et al.,* 2009). La eficiencia en la utilización de partículas en la alimentación de larvas de peces marinos se ve afectada por muchos factores externos e internos (Figura 9.1) (Kolkovski, 2001, 2004; Koven *et al*., 2001). Sin embargo, son escasos los estudios de requerimientos nutricionales que usen métodos directos debido principalmente a lo difícil que es diseñar dietas experimentales controlando totalmente su composición nutricional y los factores ambientales en todas las unidades experimentales (tanques) utilizadas. Las dietas formuladas tienen problemas de estabilidad y lixiviación variable lo que impide conocer con exactitud la composición nutricional entre la dieta formulada y la que realmente es ingerida.

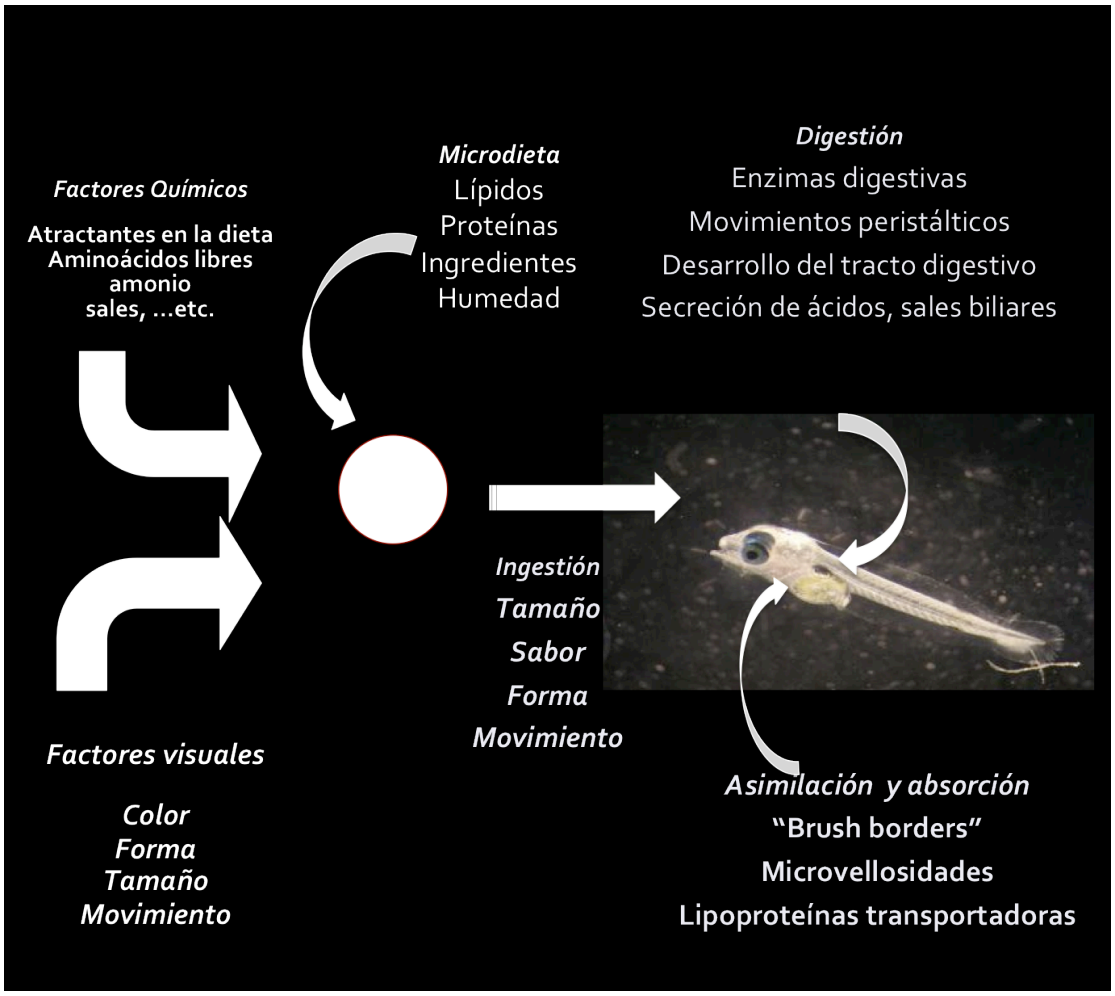

Figura 9.1 Factores que afectan la utilización de las partículas de alimento (Adaptado de Kolkovski *et al.,* 2009).

De acuerdo a Kolkovski (2009), las dietas microparticuladas pueden ser clasificadas en tres tipos generales:

I. Dietas aglomeradas: es la microdieta más simple. Los ingredientes en polvo se microenlazan con una matriz estable en agua tal como agar o carragenina (López -Alvarado *et al*., 1994).

II. Dietas microcubiertas: su proceso de fabricación está basado en recubrir los ingredientes en polvo con una capa de glúcidos (carragenina, alginato), proteínica (gelatina, caseína) o un aglutinante lípidico para reducir la lixiviación (Onal y Langdon, 2004).

III. Dietas microencapsuladas: son hechas encapsulando una solución, coloide o suspension de los ingredients de la dieta con una membrane, usando un agente aglutinante (Yúfera *et al*., 1999; Kolkovski, 2009).

9.1.2 Importancia de los fosfolípidos en la nutrición larvaria de peces marinos

Así como en todos los vertebrados, en peces los fosfoglicéridos son muy importantes, particularmente en la producción de larvas debido a su función esencial como componentes celulares indispensables en la formación de biomembranas y orgánelos (Izquierdo y Koven, 2010). Adicionalmente, los fosfoglicéridos también pueden servir como fuente de energía durante el desarrollo de los huevos y la alimentación endógena en las etapas pre-larvarias de peces marinos (Izquierdo y Koven, 2010). Además pueden actuar como emulsionantes en la formación de micelas mixtas en el tracto digestivo (Olsen y Ringø, 1997). De hecho, los juveniles de peces contienen abundantes fosfolípidos durante su desarrollo embrionario y larvario, que pueden provenir de fuentes endógenas (saco vitelino) o exógenas (lípidos del alimento vivo) (Rainuzzo *et al.,* 1997; Van der Meeren *et al.*, 2008). Los estudios sobre fosfolípidos en la dieta de peces fueron iniciados por Kanazawa *et al.* (1981, 1983), quienes demostraron que a pesar de que juegan un papel importante en su metabolismo, los peces poseen una capacidad limitada para sintetizarlos durante su desarrollo (Kanazawa *et al.,* 1985) y, en consecuencia, deben ser incluidos en dietas para juveniles. Por otra parte, digestión, absorción y transporte de los lípidos son muy limitados en las larvas que inician la alimentación con un sistema digestivo poco desarrollado. Los fosfolípidos tienen diferentes funciones muy importantes en todos estos procesos. Sin embargo, las larvas de peces pueden ser incapaces de sintetizar eficientemente los fosfolípidos en una cantidad suficiente para cubrir esta alta demanda y, por lo tanto, deben ser incluidos en la dieta (Izquierdo y Koven, 2010). De hecho, las larvas presentan enterocitos poco desarrollados y una escasa síntesis de fosfolípidos (Deplano *et al.,* 1991). Caballero *et al.,* (2003), aislando microsomas a partir de enterocitos, demostraron que en dorada la síntesis de fosfolípidos se produce principalmente a través de la vía del glicerol-3-fosfato, cuya actividad es modulada por los lípidos de la dieta. Adicionalmente, las restricciones en la tasa de síntesis de fosfolípidos puede limitar la síntesis de lipoproteínas (Liu *et al.,* 2002), lo que es crucial para el transporte de lípidos (Salhi *et al.,* 1999; Caballero *et al.,* 2006). Se ha observado que la adición de 20 g de lecitina de soja kg^{-1} de peso seco de la dieta (dw) en microdietas de dorada utilizadas el día 15 post-eclosión contenía 220 g lípidos kg^{-1} dw, lo que aumentaba significativamente la aparición de partículas de lipoproteínas en

la lámina propia y promovía el transporte y la utilización de los lípidos de la dieta así como el crecimiento larvario (Liu *et al.,* 2002). Los fosfolípidos de la dieta no sólo aumentan la síntesis y liberación de lipoproteínas en la lámina propia por los enterocitos, sino que también reducen significativamente el tamaño de las lipoproteínas mediante la promoción de la síntesis de lipoproteínas de muy baja densidad (VLDL, con más fosfolípidos) en lugar de la producción de quilomicrones (con más lípidos neutros) (Liu *et al.,* 2002; Caballero *et al.,* 2003, 2006b). La inclusión de fosfolípidos en las dietas de juveniles ha mostrado que mejora el rendimiento de cultivo de varias especies de peces marinos y dulceacuícolas (Izquierdo y Koven 2011), aumentando también el crecimiento y la supervivencia (Kanazawa, 1993; Salhi *et al.,* 1999; Izquierdo *et al.,* 2001; Kjørsvik, 2009). Además de su importancia en el crecimiento larvario y su función en la utilización de lípidos de la dieta, se ha observado que los fosfolípidos también son necesarios para mejorar la resistencia al estrés, el desarrollo esquelético e, incluso, la metamorfosis y pigmentación de peces planos (Kanazawa *et al*., 1981; Kanazawa, 1993; Fontagné *et al*., 2000; Koven, 2003; Zambonino Infante and Cahu, 2007; Hamza *et al.*, 2008).

9.1.2.1 Los fosfolípidos como fuente de ácidos grasos esenciales

Los fosfolípidos pueden ser una fuente importante de energía (ácidos grasos) en los peces, en particular durante el desarrollo embrionario y el larvario inicial en especies que producen huevos ricos en fosfolípidos (Tocher, 1995). En su primera alimentación, las larvas de peces pueden estar predispuestas a la digestión y metabolismo de los fosfolípidos y al uso como fuente de energía de los ácidos grasos de tales fosfolípidos (Sargent *et al.,* 1997). Las principales funciones de los ácidos grasos en las larvas de peces concuerdan con las que se presentan en juveniles y adultos. Funcionan como una fuente de energía metabólica, como componentes estructurales en los fosfolípidos de las membranas celulares y como precursores de moléculas bioactivas (Sargent *et al*., 1999, Izquierdo *et al*., 2000; Tocher 2003). Los fosfolípidos de la dieta tienden a ser una fuente más rica de ácidos grasos esenciales (EFA) que los lípidos neutros como son los triglicéridos (Tocher, 1995). Además, debido a su mejor digestibilidad, los fosfolípidos pueden ser muy superiores a los lípidos neutros como fuente de EFA en las larvas de peces (Sargent *et al.,* 1997, 1999). Recientemente, se ha demostrado que los fosfolípidos son el modo más

eficiente de suministrar EPA y DHA en la dieta de larvas de lubina que si se utilizan triglicéridos (Gisbert *et al.,* 2005, Tocher *et al.,* 2008). En cualquier caso, la mayoría de los estudios para determinar los requisitos de fosfolípidos se han realizado utilizando lecitina de soja o de huevo, que son ricos en 18:2 n-6, pero también deficientes en los ácidos esenciales de peces marinos: n-3 HUFA, EPA y DHA (Watanabe *et al.,* 1984; Coutteau *et al.,* 1997; Sargent *et al.,* 2002; Tocher *et al.,* 2008).

9.1.3 Requerimientos de fosfolípidos

Estudios realizados con un gran número de especies marinas y de agua dulce, han confirmado que los PL son un requerimiento esencial en la dieta de los peces. Se ha observado que el nivel ideal de suplementación de PL en la dieta depende de la especie, la fuente y clase de PL, así como de los criterios utilizados para evaluar tal efecto: crecimiento, supervivencia, resistencia al estrés, malformaciones en las larvas, etc. Tocher *et al.* (2008) han registrado los requerimientos alimentarias de diferentes especies en sus etapas larvarias y juvenil (Tabla 9.1). Estos autores observaron una tendencia que sugiere que los requerimientos cuantitativas de PL disminuyen desde los estadios larvarios a los primeros juveniles. No fueron observados requerimientos en peces de más de 5 g. Se ha visto que el requerimiento de PL está relacionada con la edad de las larvas y el grado de desarrollo de su sistema digestivo (Kanazawa, 1993; Izquierdo y Koven, 2010). Sin embargo, tanto el nivel ideal y composición de PL, así como su rol en la dieta siguen sin estar claros en la mayoría de las especies. Sargent *et al.* (1999) consideraron que la composición de lípidos del huevo es una aproximación de la composición lipídica ideal en una dieta larvaria. Los requerimientos cuantitativos de fosfolípidos para larvas de peces varia de 2 al 12% de la dieta, siendo menor en las larvas de carpa común (*Cyprinus carpio*) (2% de huevo de gallina) (Geurden *et al.,* 1995), seguida por el besugo (*Pagrus major*) (5% SBL: lecitina de soja), la dorada japonesa (*Oplegnathus fasciatus*) (7,4% SBL) (Kanazawa *et al.,* 1983) y las larvas de platija japonesa (*Paralichthys olivaceus*) (7% SBL) (Kanazawa, 1993), y los mayores valores observados en dorada (*Sparus aurata*) (15% SBL) (Seiliez *et al.,* 2006). Sin embargo, en estudios más recientes con otras especies (Capítulo 3) se ha encontrado una inclusión óptima de aproximadamente 10% usando el aceite de krill como fuente de fosfolípidos (Saleh *et al.,* 2012a).

Tabla 9.1 Requerimientos cuantitativos y cualitativos de fosfolípidos de peces teleósteos (Adaptado de Tocher *et al.,* 2008)

Especie	Estadío de desarrollo	Suplemento de fosfolípidos[a] y niveles estudiados[b]	Requerimiento ideal y criterios utilizados[c]	Periodo de alimentación	Referencia
Ayu (*Plecoglossus altivelis*)	Larva	0 y 3% SBL o EL	3% (G,S,M)	20 días	Kanazawa *et al.* (1981)
	Larva	0, 1, 3 y 5% SL	3% (M), 5% (G,S)	50 días	Kanazawa *et al.* (1983a)
	Larva	0 y 3% EL or BPL	3% (G,S,M)	50 días	Kanazawa *et al.* (1983a)
	Juvenil	0 y 3% SBL o BPL	3% (G)	33 días	Kanazawa *et al.* (1981)
	Juvenil	0, 1, 3 y 5% EL	3% (G)	33 días	Kanazawa *et al.* (1981)
Platija japonesa (*Paralichthys olivaceus*)	Larva	0, 3, 5 y 7% SBL	7% (G,S)	30 días	Kanazawa (1993)
	Juvenil	0, 3, 5 y 7% SBL	7% (G)	30 días	Kanazawa (1993)
Dorada japonesa (*Oplegnathus fasciatus*)	Larva	0, 2.5, 5 y 7.4% SBL	7.4% (G,S)	22 días	Kanazawa *et al.* (1983b)
	Larva	0, 3, 5 y 7% SBL	5% (G,S,R)	28 días	Kanazawa (1993)
	Juvenil	0, 3, 5 y 7% SBL	3% (G)	60 días	Kanazawa (1993)
Lubina (*Dicentrarchus labrax*)	Larva	3, 6, 9 y 12% SBL	12% (G,S,M)	40 días	Cahu *et al.* (2003b)
	Juvenil	0 y 3% SBL	3% (G)	40 días	Geurden *et al.* (1995a)
	Juvenil	0 y 2% EPC or SPC	2% (G)	40 días	Geurden *et al.* (1995a)
Besugo (*Pagrus major*)	Larva	0 y 5% SBL	5% (G,S)	20 días	Kanazawa *et al.* (1983b)
Carpa común (*Cyprinus carpio*)	Larva	0 y 2% EL	2% (G,S)	25 días	Geurden *et al.* (1995b)
	Larva	0 y 2% PL	2% (G,S)	21 días	Geurden *et al.* (1995b)
	Larva	0 y 2% SPC, SPI or EL	2% (G,S, M except El)	25 días	Geurden *et al.* (1997a)
Lucioperca (*Sander lucioperca*)	Larva	1, 5 y 9% SBL	9% (G)	24 días	Hamza *et al.* (2008)
Dorada (*Sparus aurata*)	Larva	9, 11 y 15% SL	>9% (G,S)	23 días	Seiliez *et al.* (2006)
	Larva	6, 7, 9, 10 y 11 KPL	10% (G,S,R)	30 días	Saleh *et al.* (2012a)
	Larva	5, 6, 7, 9 y 10 SBL	9% (G,S,R)	30 días	Saleh *et al.* (2012b)
Palometa (*Pseudocaranx dentex*)	Juvenil	0, 0,5, 1, 1,5 y 2% SPC	1.5% (G,S,R)	6 semanas	Takeuchi *et al.* (1992)

	Juvenil	0 y 1,5% SPE	1.5% (G)	6 semanas	Takeuchi *et al.* (1992)
Trucha arco iris (*Oncorhynchus mykiss*)	Juvenil	0, 2, 4 y 8% SL	4% (G)	20 semanas	Poston (1990a)
	Juvenil	0 y 14%	14% (G)	8 semanas	Rinchard *et al.* (2007)
Rodaballo (*Psetta maximus*)	Juvenil	0 y 2% EL	2% (G)		Geurden *et al.* (1997b)
Salmón Atlántico (*Salmo salar*)	Juvenil (180 mg)	0, 2, 4, 6 y 8% SBL/CPL	6% (G)	14 semanas	Poston (1991)
	Juvenil (180 mg)	0 y 4% SBL	4% (G)	16 semanas	Poston (1990b)
	Juvenil (1,0 g)	0 y 4% SBL	4% (G)	12 semanas	Poston (1990b)
	Juvenil (1,7 g)	0 y 4% SBL	4% (G)	12 semanas	Poston (1990b)
	Juvenil (7,5 g)	0 y 4% SBL	0% (no tiene requerimientos)	12 semanas	Poston (1990b)
Esturión blanco (*Acipenser transmontanus*)	Juvenil (5-10 g)	0 y 8% SBL	0% (no tiene requerimientos)	6 semanas	Hung y Lutes (1988)

[a]BPL, lípidos polares de huevos de bonito; CPL, lípidos polares de maíz; EL, lecitina de huevo de gallina; EPC, fosfatidilcolina de huevo purificada; KPL, fosfolípidos de krill; PL, varias fuentes de suplementación de fosfolípidos representando el 2% de fosfolípidos en la dieta, incluyendo EL, SL, girasol, colza y fosfolípidos marinos: SBL, lecitina de soja; SPC, fosfatidilcolina de soja purificada; SPE, fosfatidiletanolamina purificada de soja; SPI, fosfatidilinositol purificado de soja; [b]Porcentaje de peso de la dieta; [c]G, Crecimiento; S, Supervivencia; M, Malformaciones; R, Resistencia al estrés.

9.1.4 Digestión, absorción y transporte de fosfolípidos

9.1.4.1 Digestión de fosfolípidos

La digestión y la eficiencia de asimilación puede ser inferior en larvas de peces marinos que en ejemplares adultos debido a que las larvas tienen características digestivas diferentes a la de los juveniles (Cahu y Zambonino Infante, 1995). Se ha planteado la hipótesis de que las larvas de peces marinos tienen una capacidad digestiva insuficiente para aprovechar una dieta inerte (Lauff y Hofer, 1984). El cambio que se produce de un modo primario de digestión a uno adulto es característico en animales en desarrollo y ha sido descrito extensamente en vertebrados superiores (Henning, 1987). La actividad de las enzimas digestivas en

larvas de peces es baja en la primera alimentación y aumenta junto con el desarrollo (Govoni *et al.,* 1986). La digestión de los alimentos ingeridos se produce en el intestino larvario, donde el pH se mantiene alcalino y la actividad proteolítica es ejercida por enzimas "tipo-tripsina" (Walford y Lam, 1993). En la primera alimentación, la actividad de las enzimas pancreáticas e intestinales es generalmente baja (Cousin et al., 1987). La actividad de las enzimas digestivas aumenta durante los primeros 10 días post-eclosión en *Solea senegalensis* (Ribeiro *et al.,* 1999), mientras se ha observado que un aumento en la actividad de la fosfatasa alcalina refleja el desarrollo de las membranas "brush border" de los enterocitos en el bacalao del Atlántico (*Gadus morhua*) (Wold *et al.,* 2007). Además, la adición PL en la dieta mejora el índice de maduración intestinal en esta especie, en base a la relación entre la fosfatasa alcalina del "brush border" y la aminopeptidasa citosólica leucina-alanina. La mejora de la maduración intestinal por PL de la dieta podría estar relacionada con una mayor disponibilidad intracelular de PL para la formación de membranas celulares y organelos, ya que los PL de la dieta promueven la re-acilación de los lípidos digeridos, aumentando así la disponibilidad intracelular de PL para la síntesis de lipoproteínas como ha sido observado en dorada (Caballero *et al.,* 2003; Liu *et al.,* 2002).

Es sabido que, generalmente los lípidos son bien digeridos por los peces (Olsen y Ringo, 1997). Normalmente en peces la digestibilidad de los lípidos de la dieta es alta, alrededor del 95%, sin embargo, la digestibilidad de los diferentes ácidos grasos varía de acuerdo al grado de insaturación y longitud de cadena. Así, saturados y monoinsaturados son menos digeribles que los ácidos grasos poliinsaturados (Lied y Lambertsen, 1982, Olsen y Ringo, 1997). Por lo tanto, se ha sugerido que la absorción de algunos ácidos grasos es más eficiente que la de otros (Olsen *et al.,* 1998).

Los lípidos de la dieta son hidrolizados en el lumen del tracto digestivo por enzimas lipolíticas que producen principalmente ácidos grasos libres, monoacilgliceroles y diacilgliceroles. Mientras que en los vertebrados superiores la hidrólisis de lípidos de la dieta es principalmente catalizada por una lipasa pancreática específica, que hidroliza los ácidos grasos sn-1 y sn-3 liberando 2-monoacilgliceroles, la principal enzima responsable de la digestión de lípidos neutros en peces es una lipasa no específica neutra dependiente de sales biliares ("bile salt activated lipase", BAL) (Iijima *et al.,* 1998; Izquierdo y Henderson, 1998), que hidroliza los enlaces éster de diferentes ácidos grasos que tienen una molécula de glicerol. A pesar de que esta

enzima se caracteriza por actuar en una amplia gama de sustratos, también parece tener una mayor especificidad por los ácidos grasos poliinsaturados (Lied y Lambrtsen, 1982; Iijima *et al.,* 1998, Izquierdo *et al.,* 2000.).

Han sido encontrados varios tipos de lipasas en el tracto digestivo de peces juveniles y adultos. Entre ellas, la BAL parece desempeñar un papel importante en la digestión de los lípidos neutros en la mayoría como en lubina, anchoa, trucha arco iris, besugo, rodaballo y dorada (Patton *et al.,* 1975; Ijima *et al.,* 1990; Iijima *et al.,* 1998; Izquierdo y Henderson, 1998; Caballero *et al.,* 2002). Esta enzima cataliza la hidrólisis no sólo de acilglicéridos, sino también la de otros lípidos de la dieta, incluyendo ésteres de colesterol y vitaminas. También hay pruebas de la presencia de una lipasa pancreática específica (MPL) en varias especies, que es activada en presencia de sales biliares y es específica para triacilgliceroles (Iijima *et al.,* 1998). Los lípidos que contienen ácidos grasos poliinsaturados de la serie n-3, esenciales para los peces marinos, son más resistentes a la hidrólisis de la MPL. Por el contrario, se ha demostrado que la BAL es específica para este tipo de ácidos grasos. Así, aunque la MPL hidroliza preferentemente los ésteres de 18:1 n-9 que los de 18:2 n-6 ó 18:3 n-3, la BAL muestra una preferencia por 20:5 n-3 y 20:4 n-6 más que por 18: 2 n-6 y 18:1 n-9 (Iijima *et al.,* 1998).

Los patrones de distribución de la actividad de la lipasa a lo largo del tracto digestivo de peces juveniles y adultos parecen diferir entre especies (Izquierdo y Henderson, 1998). Esta diferencia en la distribución de la actividad lipolítica en peces adultos casi desaparece en juveniles y larvas, lo que sugiere una menor diferenciación funcional en el tracto digestivo en las primeras etapas de desarrollo.

La digestión de los fosfolípidos de la dieta casi no ha sido estudiada en peces, pero se presume que los mecanismos en general son similares a los de los mamíferos, en los que los fosfolípidos son digeridos por la fosfolipasa intestinal A2, secretada por el páncreas al intestino, reconociendo específicamente el enlace fosfolipídico acilo sn-2 e hidrolizando el enlace resultante de la formación de 1-acil liso-fosfolípidos y ácidos grasos libres que son absorbidos por las células intestinales mucosas (Fig. 2) (Henderson y Tocher, 1987; Sargent *et al.,* 1989; Izquierdo y Henderson, 1998). Existen dos isoformas de la fosfolipasa A2 que han sido purificadas del hepatopáncreas de besugo (Ono y Iijima, 1998). La actividad de la fosfolipasa A2 ha sido detectada en larvas de peces marinos varios días después de la primera alimentación (Izquierdo *et al.*, 2000).

Ambas enzimas lipolíticas pancreáticas, lipasa y fosfolipasa A2, aumentan con el desarrollo en larvas de peces marinos (Izquierdo *et al.,* 2000) y son estimuladas por el aumento de sus respectivos sustratos: triglicéridos y fosfolípidos de la dieta. La actividad de la fosfolipasa A2 aumenta con el nivel de su correspondiente ARNm en lubina (Zambonino Infante y Cahu, 1999) lo que sugiere una regulación de la transcripción de esta enzima que se eleva cuando las dietas incorporan más de 4,5% de fosfolípidos.

9.1.4.2 Transporte y absorción de fosfolípidos

En peces la absorción de lípidos de la dieta es similar a la de los mamíferos (Sire *et al.,* 1981, Tocher *et al.,* 2008). Después de la hidrólisis en el lúmen, los lípidos de la dieta se incorporan en las células epiteliales intestinales por difusión, en forma de micelas de monoglicéridos y ácidos grasos libres (Izquierdo *et al.,* 2000; Tocher *et al.,* 2008). Los productos de la hidrólisis lipídica, ácidos grasos libres, monoacilgliceroles, diacilgliceroles y lisofosfolípidos se mezclan con las sales biliares para formar pequeñas micelas que son transportadas al interior de los enterocitos (Figura 9.2) y su contenido, que también incluye otros lípidos como vitaminas liposolubles y colesterol, atraviesa la membrana celular por difusión y se vierte en el interior del enterocito (Smith *et al.,* 1983). La reacilación se produce en el retículo endoplasmático y sus productos son liberados en la submucosa en forma de lipoproteínas de muy baja densidad (quilomicrones). La actividad de los enterocitos durante la absorción es el empaquetado de lípidos junto con proteínas para constituir moléculas estables y transportarlos a través de la sangre a los sitios de metabolismo y almacenamiento (Gurr y Harwood, 1991).

Como los ácidos grasos de cadena corta (menos de 12 átomos de carbono) son suficientemente solubles en el plasma, son liberados directamente desde el enterocito a la circulación portal y se metabolizan en el hígado (Green y Selivonchick, 1987). Los ácidos grasos de cadena larga y monoacilgliceroles absorbidos migran a las vesículas del retículo endoplasmático, donde son resintetizados a triglicéridos u otros lípidos por medio de un complejo multienzimático (Gurr y Harwood, 1991). Estos ácidos grasos difunden hacia el retículo endoplasmático asociados con proteínas de transporte FABP (proteína de transporte de ácidos grasos), que desempeñan un papel importante en el transporte intracelular de los ácidos grasos absorbidos. En los peces,

estas proteínas tienen una preferencia por los ácidos grasos insaturados de la misma forma que se produce en mamíferos (Gangl *et al.,* 1980). Sire y Vernier (1981) relatan que estas proteínas son más abundantes en peces alimentados con dieta altas en lípidos, lo que indirectamente puede afectar a la activación y reacilación de ácidos grasos.

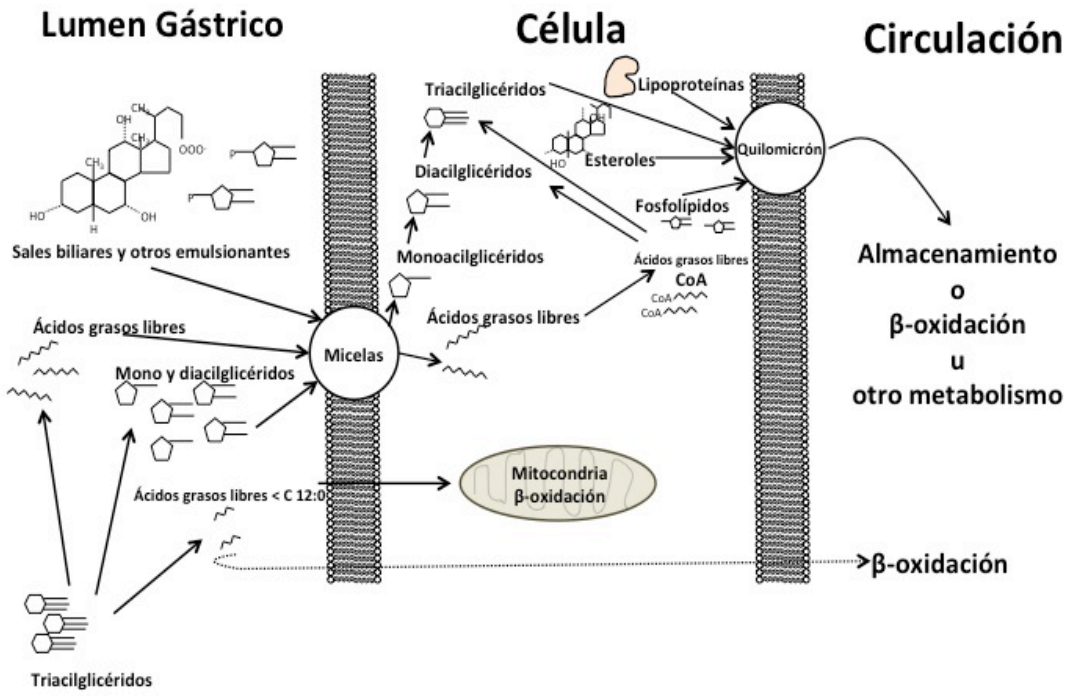

Figura 9.2 Absorción y metabolismo de triglicéridos (Adaptado de Smith *et al.,* 1983).

Las dos principales vías de reacilación de los ácidos grasos en los enterocitos son la vía del monoacilglicerol y la del glicerol-3-fosfato. Ambas rutas producen triglicéridos y fosfolípidos (Iijima *et al.*,1983; Caballero *et al.*, 2006a). La mayoría de las enzimas que participan en estas vías han sido identificadas y estudiadas en ratas, pero hay muy pocos estudios realizados en peces (Sire *et al.,* 1981; Iijima *et al.,* 1983; Caballero *et al.,* 2006a). Iijima *et al.* (1983) mostraron que el intestino de la carpa (*Cyprinus carpio*) es capaz de esterificar el ácido palmítico en triglicéridos y fosfolípidos. Por otro lado, Sire *et al.* (1981) observaron que en los enterocitos de trucha arco iris el ácido linoleico es esterificado con mayor rapidez a triacilgliceroles

que a ácido palmítico. Además, estos autores proponen que mientras la vía del monoacilglicerol es predominante en los mamíferos, debido a los productos de hidrólisis en el lumen son los 2-monoacilgliceroles, en peces la vía principal es la del glicerol-3-fosfato debido a que los principales productos de la hidrólisis son ácidos grasos libres y glicerol, reflejándose así que la síntesis de glicerofosfolípidos es más importante en peces que en mamíferos. Caballero *et al.* (2006) aislaron los microsomas de los enterocitos de dorada demostrando que la síntesis de fosfolípidos en esta especie se produce principalmente a través de la vía del glicerol-3-fosfato, cuya actividad es modulada a través de los lípidos de la dieta.

Hasta ahora no se han estudiado profundamente los mecanismos de absorción de los productos de la digestión de fosfolípidos en peces, pero se supone que serían similares a los de los mamíferos. Los productos de la hidrólisis, fosfolípidos 1-acil-lisosoma y los ácidos grasos libres, se asocian en micelas mixtas junto con todos los otros productos de la digestión de lípidos de las sales biliares, dichas miselas son absorvidas en la mucosa intestinal y penetran por difusión pasiva en los enterocitos. La concentración de lisofosfolípidos es muy baja en la mucosa intestinal, ya que la mayor parte de los 1-acil lisofosfolípidos son reesterificados con ácidos grasos libres activados en los microsomas antes del transporte desde los enterocitos al sistema circulatorio (Sargent *et al.,* 1989).

El transporte de lípidos y de otros componentes liposolubles desde el intestino a los tejidos es realizado principalmente a través de lipoproteínas (Babin y Vernier, 1989; Izquierdo *et al.,* 2001; Liu *et al.,* 2002; Caballero *et al.,* 2003, 2006b). Las lipoproteínas son partículas que difieren en su proporción de lípidos (triglicéridos, colesterol, ésteres de colesterol y fosfolípidos) y proteínas (Figura 9.3).

Figura 9.3 Representación esquemática de la estructura de una lipoproteína.

Las lipoproteínas se clasifican generalmente de acuerdo a su densidad en seis clases principales: los quilomicrones (CM), lipoproteínas de muy baja densidad (VLDL), de densidad intermedia (IDL), de baja densidad (LDL), de alta densidad (HDL) y de muy alta densidad (VHDL) (Liu *et al*., 2002; Caballero *et al*., 2003, 2006b). Estas lipoproteínas tienen apolipoproteínas como componentes estructurales y funcionales, que son cofactores enzimáticos y ligandos para los receptores de la superficie celular. En particular, la apoA1 es el principal componente proteico de las lipoproteínas de alta densidad; apoA4 se cree que actúa principalmente en la absorción intestinal de lípidos y la apoE es una proteína del plasma sanguíneo que media el transporte y la absorción de colesterol y lípidos por medio de su alta afinidad de interacción con diferentes receptores celulares, incluyendo un receptor de lipoproteínas de baja densidad (LDL) (Saito *et al*., 2004).

Las VLDL transportan principalmente lípidos y contienen preferentemente triacilglicerol endógeno, similar a los quilomicrones. Su principal lugar de síntesis es el hígado y su catabolismo es básicamente el mismo, en forma de quilomicrones, sobre los cuales actua una lipasa de lipoproteínas que hidroliza los triglicéridos (Figura 9.4). La interacción lipasa-VLDL es menos eficiente que la interacción entre lipasa- quilomicrones, por lo que la VLDL persiste más tiempo en la circulación (Sheridan, 1988). La HDL es la principal forma de almacenamiento de colesterol y

acepta el colesterol de los tejidos periféricos y los transporta al hígado para su degradación y reutilización.

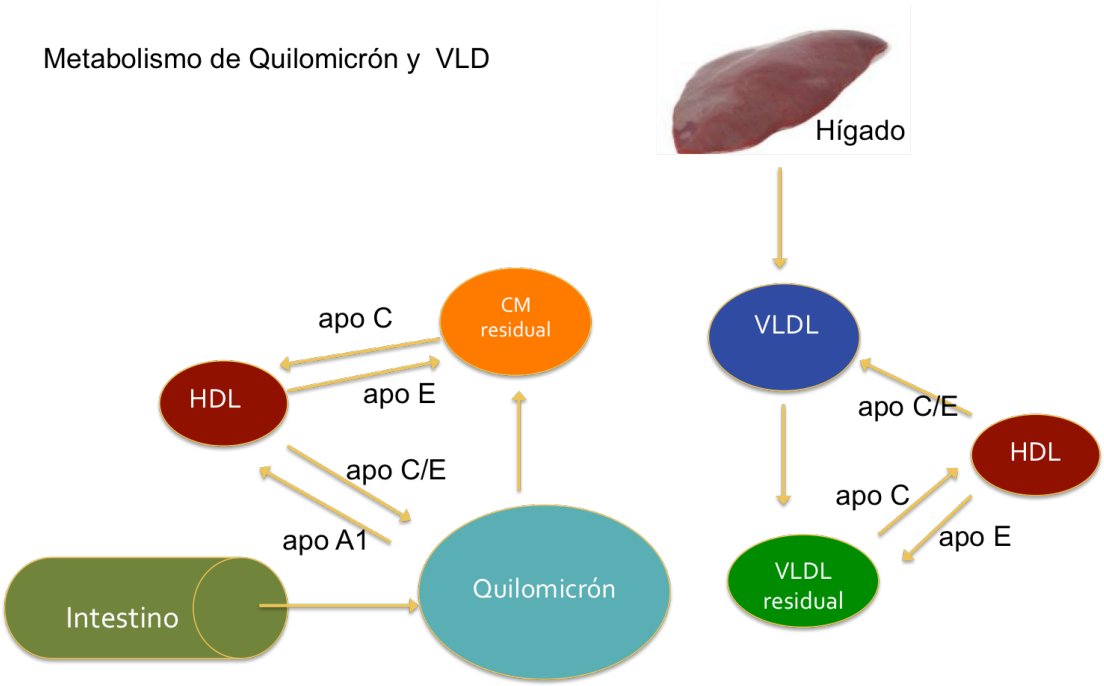

Figura 9.4 Representación esquemática de la estructura de una lipoproteína.

Hay pruebas que hacen suponer que el transporte de lípidos desde los enterocitos a los hepatocitos aumenta debido a los fosfolípidos suplementados en la dieta (Kanzawa *et al*., 1993). La alimentación de las larvas de dorada con microdietas sin suplementación de lecitina produce acumulación de vacuolas lipídicas en los enterocitos y esteatosis en el tejido hepático, indicativos de que la dieta induce problemas en el transporte de lípidos de la mucosa intestinal a la circulación corporal (Kanzawa *et al*., 1993). Ambos efectos se reducen mediante la adición de 2% de lecitina a la dieta, observándose el aumento de transporte de lípidos en el intestino y en el hígado (Kanzawa *et al*., 1993). Es sabido que niveles insuficientes de PL en la dieta (PL 23,7 g kg-1 peso seco, lípidos dietéticos 178,5 g kg-1 peso seco), aumentan la acumulación de gotas de lípidos en los enterocitos de larvas de peces marinos (Salhi *et al*., 1999; Morais *et al*., 2005), dependiendo del tipo y cantidad de PL en la dieta y su relación con el contenido de NL. En larvas de carpa la deficiencia de fosfolípidos se ha asociado con la acumulación de gotas de grasa en los enterocitos del intestino anterior, lo que sugiere que este tipo de grasa tiende a tener un papel específico en la síntesis de VLDL al igual que en los mamíferos (Fontagné, 1996).

Esta acumulación ha llevado a sugerir que las inclusiones de lípidos intestinales son una nueva forma de almacenamiento temporal de ácidos grasos re-esterificados cuando la tasa de absorción de lípidos es superior a la tasa de síntesis de lipoproteínas o incluso a una incapacidad para metabolizar los lípidos (Kjørsvik *et al*., 1991). El hígado es un órgano esencial que interviene en el metabolismo de las sustancias absorbidas por el intestino, involucrado también en el procesamiento de estas substancias a ser utilizadas por otros tejidos, y que es el lugar principal de regulación de lípidos, participando en la síntesis y degradación de ácidos grasos (Cross, 1993). El hígado es un órgano metabólicamente sensible que puede ser analizado para determinar los posibles efectos secundarios del metabolismo lipídico, debido a que en larvas de peces el almacenamiento de energía hepática responde sensible y rápidamente a los cambios nutricionales (Hoehne-Reitan y Kjørsvik, 2004).

9.1.5 Clases fosfolipídicas

Existen evidencias bioquímicas que sugieren que las vías de síntesis de los fosfolípidos son esencialmente las mismas que en mamíferos (Tocher, 1995), en los que los fosfolípidos pueden ser sintetizados *de novo* por dos mecanismos principales (Lykidis, 2007). Uno utiliza un difosfato de citidina (CDP) y un grupo de cabeza polar activado para su fijación al 1,2-diacilglicerol (DAG), y el otro utiliza CDP-DAG activado y un grupo de cabeza polar inactivada (Fig. 8) (Tocher *et al*., 2008). Ambos grupos proceden inicialmente de la esterificación del glicerol-3-fosfato con dos ácidos grasos activados (acil-CoA) para formar ácido fosfatídico (PA). La acción de la PA-fosfatasa en PA, resulta en la producción de DAG, el cual puede reaccionar entonces con CDP-colina o CDP-etanolamina para producir fosfatidilcolina (PC) y fosfatidiletanolamina (PE), respectivamente (Tocher *et al*., 2008). Alternativamente, el CTP reacciona con PA para formar CDP-DAG, que luego se puede combinar con serina e inositol para formar fosfatidilserina (PS) y fosfatidilinositol (PI), respectivamente (Figura 9.5).

De acuerdo a los pocos estudios en los que se han utilizado especies puras de fosfoglicéridos, el orden de importancia según su eficacia parece ser fosfatidilcolina, fosfatidilinositol, fosfatidiletanolamina y fosfatidilserina (Izquierdo y Koven, 2010).

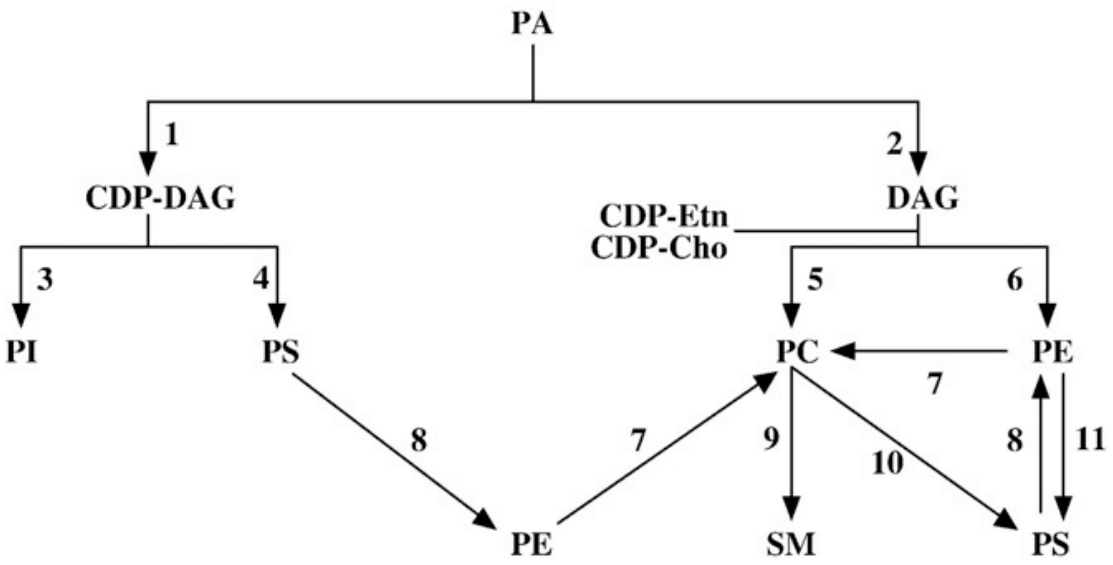

Figura 9.5 Vías de biosíntesis de las clases principales de fosfolípidos (Tocher et al., 2008). CDP-Cho, CDP-colina, CDP-DAG, CDP-diacilglicerol, CDP-Etn, CDP-etanolamina; DAG, diacilglicerol, PA, ácido fosfatídico: PC, fosfatidilcolina, PE, fosfatidiletanolamina, PI, fosfatidilinositol, PS, fosfatidilserina, SM , esfingomielina. Las enzimas son; 1, CDP-DAG sintasa; 2, PA-fosfatasa; 3, PI-sintasa; 4, PS-sintasa; 5, CDP-colina:DAG fosfotransferasa; 6, CDP-etanolamina:DAG fosfotransferasa; 7, PE metiltransferasa; 8, PS- descarboxilasa; 9, SM-sintasa; 10 y 11, PS-sintasa a través de intercambio de base.

Entre los diferentes fosfoglicéridos, PC parece ser particularmente importante, siendo el principal producto de la síntesis de PL en los enterocitos de peces (Caballero *et al*., 2006a) y que comprende hasta 95% de PL encontrado en VLDL (Lie *et al*., 1993). Además, PC induce la síntesis de la apolipoproteína B en mayor medida que otros fosfoglicéridos (Field *et al*., 1994; Campo y Mathur, 1995). El aumento de la síntesis de lipoproteínas puede ser el responsable de promover el crecimiento mediante el aumento de flujo de energía a partir de la mucosa intestinal a la sangre (Seiliez *et al*., 2006). De esta forma, se ha encontrado que PC promueve mejor el crecimiento que otras clases de PL en varias especies (Takeuchi *et al*., 1992; Kanazawa, 1993; Hadas, 1998; Geurden *et al*., 1997, 1998) y aumenta la actividad de alimentación (Koven *et al*., 1998).

El fosfatidilinositol (PI) tiene una rica diversidad de formas y funciones dentro de la célula, además de ser un componente estructural de biomembranas (Sargent *et al*., 1989). Actúa como un precursor de segundos mensajeros (inositol-3-fosfato, IP3) regulando la entrada de iones de calcio en la célula desde el retículo endoplasmático (Cahu *et al*., 2003a; Tocher *et al*., 2008), así como un ancla que se puede fijar sobre

una gran variedad de proteínas de la superficie celular. Por lo tanto, PI está involucrado en un sistema de señalización que controla los procesos biológicos en el desarrollo temprano de vertebrados (Berridge e Irvine, 1989).

9.1.6 Efecto de los fosfolípidos sobre el desarrollo esquelético

El sistema esquelético soporta la integridad estructural del cuerpo durante el desarrollo, proporciona sitios para inserción muscular y protege órganos vitales (Lall y McCrea-Lewis, 2007). Los peces teleósteos muestran un tejido esquelético descrito como un espectro continuo que va desde el tejido conectivo al cartílago y hueso (Hall y Witten, 2007). El esqueleto maduro es un órgano metabólicamente activo que sufre una remodelación continua por un proceso en que se sustituye el hueso viejo con nuevo (Figura 9.6). En ejemplares adultos sanos, la resorción y formación de hueso están en equilibrio y se mantiene un nivel constante de masa ósea (Kruger *et al*., 2010). Los osteoclastos responsables de la resorción ósea se originan a partir de células-madre hematopoyéticas conocidas como monocitos, mientras que los osteoblastos responsables de la formación de hueso se originan a partir de células estromales de la médula ósea (Kruger *et al*., 2010).

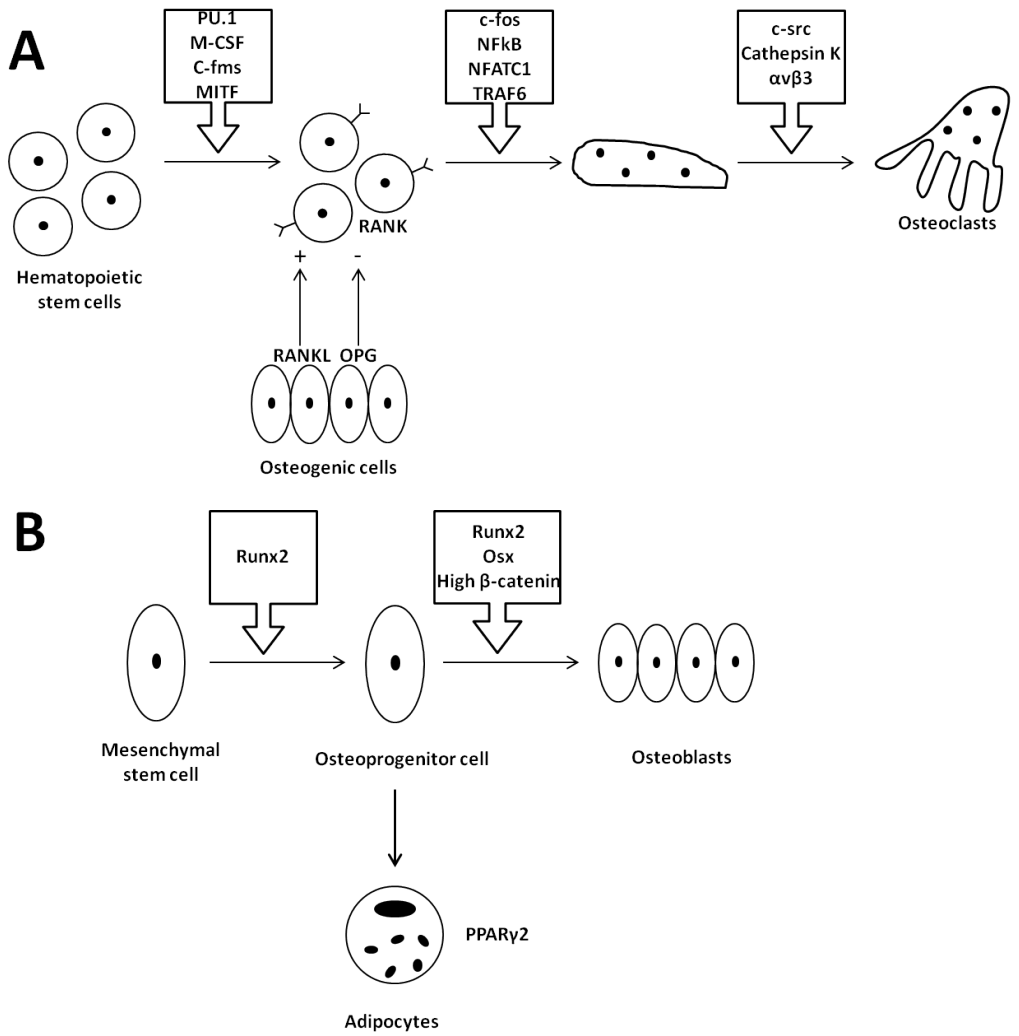

Figura 9.6 Diagrama simplificado de la diferenciación de precursores de osteoclastos (A) en los osteoclastos y los precursores de osteoblastos (B) en osteoblastos. PU.1, factor de transcripción PU.1; M-CSF, factor estimulante de colonias de macrófago, c-fms, genes c-fms; MITF, factor de transcripción asociado a microftalmia; c-fos, gen c-fos; NFαβ, factor nuclear αβ; NFATc1,factor nuclear de células T1 activadas; TRAF6, factor de necrosis tumoral asociado al receptor factor-6; c-src, gen del sarcoma celular-; α v ß 3, α v ß 3-integrina; RANKL, activador del receptor del factor nuclear de α v β; OPG , osteoprotegerina, OSX, osterix; PPARγ2, receptores activados por proliferador de peroxisoma, (Adaptado de Kruger *et al*., 2010).

El hueso es un tejido conectivo vascularizado especializado que consiste en células y una matriz extracelular mineralizada de colágeno que se mineraliza mediante la deposición de hidroxiapatita (Hall y Witten, 2007; Nordvick, 2007). Durante la primera alimentación varios nutrientes de la dieta desempeñan un papel central en el desarrollo óseo y la posterior aparición de malformaciones esqueléticas (Izquierdo *et al*., 2010; 2012). Varios estudios relacionados con el impacto de la primera alimentación en el desarrollo de peces mostraron que la incorporación en la dieta de

fosfolípidos totales reducen malformaciones, especialmente la torsión de la mandíbula y la escoliosis en larvas de ayu (*Plecoglossus altivelis*) (Kanazawa *et al*., 1981). Geurden *et al*., (1998) sugieren que la dieta suplemententada con PL, especialmente PI, reduce la deformidad esquelética en larvas de carpa. Estos hallazgos fueron reforzados por Cahu *et al*., (2003) quienes encontraron que la dieta PC/PI en la proporción de 2,18 impidió la aparición de deformidades durante el desarrollo larvario de lubina. Por otra parte, la osificación de la columna vertebral en larvas de bacalao, observada a los 21 días post-eclosión en los arcos neurales, se produjo significativamente más temprano en los grupos alimentados con dietas que contenían PL que en aquellos alimentados con NL, cuando se comparaba el tamaño y la edad de las larvas (Kjørsvik *et al*., 2009). Los PL tienen un papel como precursores de segundos mensajeros que regulan la entrada de calcio en las células y está implicado en un sistema de señalización que controla los procesos biológicos en el desarrollo temprano de los vertebrados (Berridge y Irvine, 1989). Sandel *et al*. (2010) encontraron una correlación muy alta entre la edad de desarrollo de dorada alimentada con PI y la expresión génica de osteocalcina, una proteína que se utiliza como un marcador para ver el crecimiento del hueso y cartílago y se ha correlacionado con la mineralización ósea (Simes *et al*., 2008). PI, al igual que el segundo mensajero inositol-3-fosfato (IP3), moviliza el calcio mediante la regulación de la entrada de este ión en la célula desde el retículo endoplásmico (Cahu *et al*., 2003; Tocher *et al*., 2008). Estos autores argumentaron que PI de la dieta puede aumentar la disponibilidad de calcio para la mineralización ósea, lo que estimula la producción de osteocalcina y el normal desarrollo del hueso. Concordando con esto, Nishimoto *et al*. (2003) trabajando con carpa encontraron que la unión de hidroxiapatita ósea de proteína Gla ósea (BGP) es mayor en la presencia de iones de calcio. Por otra parte, los cambios en PUFA en la dieta se reflejan en la composición de diversos tejidos, incluyendo células óseas, tales como los osteoblastos (Atkinson *et al*., 1997; Moyad, 2005). Se ha observado que varios de los LCPUFA afectan a las células óseas (osteoclastos y osteoblastos) por medio de diversas vías de señalización celular o factores de crecimiento, afectando la formación de hueso y la reabsorción y densidad ósea en animales y seres humanos (Salari *et al*., 2008). Cambiar el equilibrio de los PUFAs presentes en las membranas con ácidos grasos n-3 en lugar de ácidos grasos n-6 cambia la relación de n-6/n-3 en las membranas y afecta a la producción de varias citocinas, lo que podría afectar la resorción y formación ósea. Se ha demostrado que

LCPUFAs afectan a las proteínas celulares y al receptor activador del factor nuclear kβ (RANK), un receptor que se encuentra en el osteoclasto y que controla la osteoclastogénesis (Sun *et al*., 2003; Coetzee *et al*., 2007). Se han realizado un gran número de estudios que muestran que en animales en crecimiento los PUFAs afectan el contenido mineral, masa, espesor y área femoral de los huesos, y también, aparentemente, la formación y el crecimiento óseo. En ratas macho en crecimiento los PUFAs n-3 también aumentan la actividad de la fosfatasa alcalina (ALP), así como el factor de crecimiento insulínico I (IGF-1) y los niveles de proteínas transportadoras del factor de crecimiento insulínico (IGFBP) (Coetzee *et al*., 2007; Li *et al*., 1999).

Los factores de crecimiento insulínico (IGF), especialmente IGF-I, son importantes factores de crecimiento derivados de hueso y se cree que funcionan como factores de crecimiento sistémicos y locales del tejido óseo. Una vez secretados y depositados en la matriz ósea, IGFs son liberados durante la resorción ósea osteoclástica, actuando de forma autocrina o paracrina para estimular la formación de nuevas células y producción de la matriz ósea (Watkins & Seifert 2000). IGF-I actúa como un regulador de la función de las células óseas, ya que estimula la proliferación de pre-osteoblastos, aumentando así el número de células capaces de producir la matriz ósea. Además, IGF-I aumenta la expresión de colágeno y con esto la disminución de su degradación, causando un efecto anabólico en el tejido óseo (Schmid *et al*., 1992; Delany *et al*., 1994).

Los PUFAs de la dieta pueden aumentar o disminuir la producción de IGF-I en el hueso a través de su capacidad para modular las concentraciones locales de las prostaglandinas E2 (PGE2) que son metabolitos del ácido araquidónico (AA) ((Li *et al*., 1999; McCarthy *et al*., 1991; Delany *et al*., 1994). La PGE2 proviene del AA PUFA n-6 y es la principal prostaglandina en el hueso. Se ha demostrado que es un potente modulador de la remodelación ósea, afectando tanto a la resorción (Collins and Chambers, 1991) como a la formación ósea (Jee y Ma *et al*., 1997; McCarth y *et al*., 1991). PGE2 también promueve la expresión de diversas proteínas de unión de IGF (Schmid *et al*., 1992; McCarthy *et al*., 1994; DiBattista *et al*., 1996) sugiriendo que PGE2 podría mantener disponible IGF-1 para la estimulación de los osteoblastos en una fase posterior de la remodelación ósea (Schmid *et al*., 1992). McCarthy *et al*. (1991) sugirieron que la capacidad de PGE2 para mejorar la síntesis osteoblástica de IGF-I podría explicar su potencial anabólico, y, además, sugeriría un papel para la PGE2 en la remodelación ósea acoplada. Los efectos anabólicos de PGE2 pueden

ocurrir a través de la estimulación de la producción de IGF-I endógeno por los osteoblastos (Raisz *et al*., 1993) o por una mayor receptividad de células óseas a IGF-I (Hakeda *et al*., 1993)

En conclusión, enriqueciendo dietas con LCPUFA n-3 puede elevarse o conservarse la masa ósea a través del incremento del número de células madre mesenquimales (MSC), potenciando la expresión de factores de transcripción clave requeridos para la formación de hueso, tales como el factor α-1 de unión al núcleo (Cbfα1) y osterix (Watkins *et al*., 2003; Zhang *et al*., 2002) que aumentan la diferenciación de pre-osteoblastos en osteoblastos maduros. La expresión de ambos, Cbfα1 y osterix, se ve reforzada por IGF-1, el factor de crecimiento transformante β1 (TGF-β 1), la proteína morfogenética ósea (BMP) y por cantidades moderadas de PGE2 (Zhang *et al*., 2002), todos lo cual resulta de dietas ricas en LCPUFA n-3.

9.1.7 Estrés oxidativo: mecanismo de defensa antioxidante

Los productos de peroxidación de lípidos tales como hidroxiperóxidos, hidróxidos de ácidos grasos, aldehídos e hidrocarburos están implicados en varias condiciones patológicas que dañan las biomembranas celulares (Kanazawa, 1991, 1993) y las membranas subcelulares, tales como las de mitocondrias, causando varias patologías en peces (Kawatsu, 1969; Watanabe *et al*., 1970; Murai y Andrews, 1974; Sakai *et al*., 1989). Los peces tienen un sistema de defensa antioxidante endógeno (Filho *et al*., 1993) con una amplia gama de mecanismos antioxidantes para mantener un equilibrio oxidativo adecuado. Cuando este equilibrio se inclina a favor de los agentes oxidantes el estrés oxidativo produce efectos perjudiciales en moléculas que tienen gran importancia biológica. Las especies reactivas del oxígeno (ROS) son radicales libres y/o derivados de oxígeno producidos en todos los sistemas biológicos aeróbicos y en las funciones celulares normales (Mate´s, 2000). La desventaja biológica asociada con el estrés oxidativo puede ser considerada como la condición físico-química en la que hay un aumento en los niveles normales de especies oxidantes, por ejemplo, H_2O_2, HO^-, O^{-2}, $ONNO^-$, R^- y ROO^- (Ansaldo *et al*., 2000), lo que produce un aumento aleatorio de ROS, conduciendo a daños en diferentes componentes celulares. Si este proceso es continuo producirá enfermedades o incluso la muerte celular.

9.1.7.1 Defensas enzimaticas

El sistema de defensa antioxidante está en su mayoría formado por enzimas eliminadoras de radicales que desempeñan un importante papel fisiológico en la protección antioxidante (Blazer, 1982). Estos mecanismos de defensa antioxidante para desintoxicar especies reactivas de oxígeno se han desarrollado para contrarrestar los efectos potencialmente nocivos del oxígeno activado (Yu, 1994). Entre las enzimas antioxidantes se encuentran la superóxido dismutasa (SOD) que cataliza la dismutación de superóxido en oxígeno y peróxido de hidrógeno; la catalasa (CAT) que descompone el peróxido de hidrógeno a oxígeno molecular y agua; la glutatión peroxidasa (GPX) que desintoxica peróxido de hidrógeno en agua o peróxidos orgánicos en sus correspondientes alcoholes estables mediante la oxidación del glutatión reducido (GSH) en su forma oxidada (GSSG) y, por último, la glutatión reductasa (GR) que cataliza la reducción de GSSG en GSH (Halliwell y Gutteridge, 2007). La SOD desempeña un papel crucial ya que es la primera enzima que responde a los radicales de oxígeno, previniendo el inicio de la reacción en cadena de radicales que produce el anión superóxido (McCord y Fridovich, 1969; Winston y Di Giulio, 1991). Todas estas enzimas tan importantes en los mecanismos de defensa antioxidantes han sido detectadas en la mayoría de las especies de peces (Rudneva, 1997). Las actividades de las enzimas de defensa antioxidante mostraron variaciones en los diferentes órganos de peces (Wdzieczak *et al*., 1982; Lemaire *et al*., 1993), dependiendo de la conducta de alimentación (Radi y Markovics, 1988), período de alimentación (Mourente *et al*., 2002), condiciones ecológicas (Winston y Di Giulio, 1991) y el contenido antioxidante de la dieta. Todos estos mecanismos antioxidantes son responsables de mantener un adecuado "equilibrio oxidativo". Cuando este equilibrio se inclina a favor de los agentes oxidantes, debido a la excesiva generación de estos compuestos o por un debilitamiento de las defensas antioxidantes, o por ambos simultáneamente, surge el "estrés oxidativo" (Sies, 1985; Tocher *et al*., 2002).
Los antioxidantes pueden ser encontrados en el plasma celular, mitocondrias y membranas celulares (Figura 9.7) y pueden actuar en diferentes etapas del proceso de oxidación, siendo que algunos pueden tener más de un mecanismo de acción. Pueden ejercer su acción mediante diferentes mecanismos tales como la ruptura de la cadena de una secuencia inicial, eliminando oxígeno libre o disminuyendo las concentraciones locales de O2.

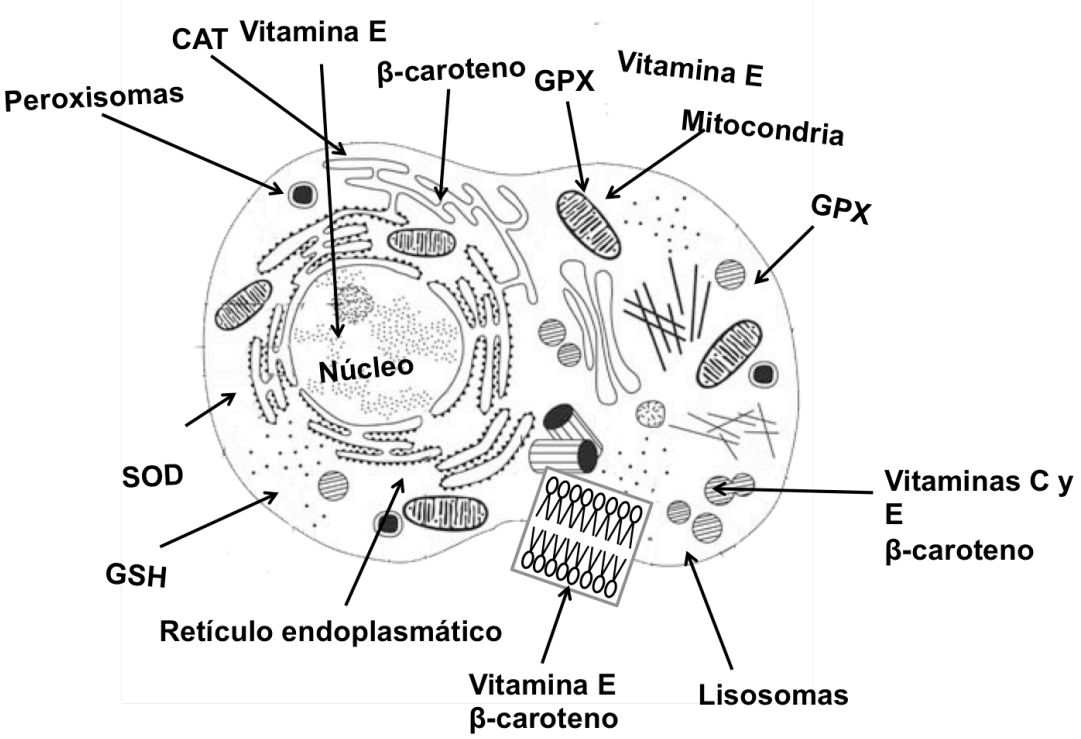

Figura 9.7 Mecanismos antioxidantes dentro de la célula. Adaptado de Machlin y Bendich (1987). Catalasa (CAT), Superóxido dismutasa (SOD), Glutatión peroxidasa, (GPX), Glutatión reducido (GSH).

9.1.7.2 Defensas no enzimaticas

Un antioxidante es una sustancia que cuando está presente en bajas concentraciones en comparación con la de un sustrato propenso a la oxidación, es capaz de interactuar con los radicales libres para acabar con la reacción (Halliwell y Gutteridge, 1990). Los niveles adecuados de compuestos antioxidantes pueden ser proporcionados por medio de suplementos nutricionales, incluyendo vitaminas E y C o minerales como el selenio, que establecen una segunda línea de defensa (Sen, 1995). Estos suplementos promoverían el máximo aprovechamiento de los nutrientes y evitarían problemas de oxidación de lípidos que podrían causar patologías (Sakai *et al*., 1989) como daño celular (Halliwell y Gutteridge, 1996), lesiones músculares (Betancor *et al*., 2011), crecimiento negativo, bajo consumo de alimento, pobre desarrollo y el aumento de la incidencia de deformidades (Lewis-McCr y Lall, 2007). En general, el daño oxidativo a moléculas de gran importancia biológica tales como lípidos, proteínas, carbohidratos y ADN causan alteraciones que pueden conducir a la muerte celular (Winston y Di Giulio, 1991; Halliwell y Gutteridge, 2007).

9.1.7.2.1 Vitamina E (α-tocoferol)

La vitamina E tiene una gran importancia como componente exógeno en la dieta de peces de acuicultura, actuando como una defensa antioxidante en la reducción de los productos de la peroxidación de lípidos y como eliminadora de radicales libres (Machlin, 1984, Betancor *et al*., 2011). Mourente *et al*. (2002) mostró una reducción de los productos de peroxidación en el hígado de doradas alimentadas con dietas que contenían aceite oxidado con un suplemento de vitamina E en comparación con los alimentados con dietas que carecían de esta vitamina.

La vitamina E es liposoluble y es absorbida en el tracto digestivo en asociación con moléculas de grasa. Putnam y Comben (1987) demostraron que la vitamina E es un componente estructural de las membranas celulares, donde actúa como un factor antioxidante de fosfolípidos por procesos antioxidantes de quiebre de cadena ("chain-breaking") (Sargent *et al*., 1997). Además, juega un papel en el mantenimiento de la calidad de carne en trucha arco iris (Frigg *et al*., 1990; Chaiyapechara *et al*., 2003; Yildiz, 2004), salmón del Atlántico (*Salmo salar*) (Hamre *et al*., 1998; Scaife *et al*., 2000), rodaballo (*Psetta maxima*) (Ruff *et al*., 2003; 2004) y lubina (Gatta *et al*., 2000; Pirini *et al*., 2000), mejorando la inmunidad al aumentar la resistencia de los glóbulos rojos a la hemólisis, y también la permeabilidad de los capilares y del músculo cardíaco (Halver, 2002). Además, se ha propuesto como el factor más importante en el mantenimiento de la estabilidad *post-mortem* de la membrana en filetes de pescado (Baker, 1997). Se ha estudiado el requerimiento diaria de vitamina E en diferentes especies de peces (NRC, 1993) y las recomendaciones de inclusión son de: 120 mg / kg de dieta (Hamre y Lie, 1995a) para el salmón del Atlántico; entre 30 y 50 mg / kg de dieta para bagre de canal (*Ictalurus punctatus*) (Murai y Andrews, 1974; Wilson *et al*., 1984); 200 a 300 mg / kg de dieta para la carpa común (*Cyprinus carpio*) (Watanabe *et al*., 1977); 1200 mg / kg para juveniles de dorada (Ortuño *et al*., 2000) y 99 mg / kg para alevines de mrigal (*Cirrhinus mrigala*) (Paul *et al*., 2004). En dorada incluir 250 mg α-tocopherol/kg de dieta es suficiente para satisfacer el requerimiento y obtener una reproducción exitosa (Fernández-Palacios *et al*., 1998).

Poco se sabe acerca de las necesidades exactas de de vitamina E en larvas de peces marinos, pero Atalah *et al*. (2008) utilizaron 1,5 y 3,0 g kg-1 de dieta seca de vitamina

E en larvas de dorada y lubina, y sugirieron que el nivel ideal era de 3 g kg-1 debido a que este alto nivel de vitamina redujo la mortalidad después del estrés. En el mismo estudio, el alto nivel de vitamina E alivió las lesiones musculares provocadas por el exceso de DHA en la dieta (Betancor *et al*., 2011). Los resultados de ambos estudios concuerdan con la opinión general de que la inclusión de vitamina E debe ser superior a 1 g kg-1 en dietas de larvas, mientras que el requisito dado por el NRC (1993) para los juveniles es de 50 mg kg-1. Sin embargo, cuando se utilizan las respuestas inmunológica y al estrés para medir las necesidades de esta vitamina, se encuentran frecuentemente cantidades mayores necesarias en la dieta de juveniles y adultos (Hamre, 2011).

El efecto del α-tocoferol en el desarrollo esquelético ha sido ampliamente estudiado en mamíferos terrestres, pero no en peces. La vitamina E es importante para un apropiado desarrollo del esqueleto (Xu *et al*., 1995; Jilka *et al*., 1996), ya que se asocia con la bicapa lipídica de las células óseas, permitiendo que sea la primera línea de defensa contra los radicales libres (Arjmandi *et al*., 2002). Son escasos los estudios sobre el efecto de ROS en el desarrollo óseo de peces. Lewis-McCrea y Lall (2007) encontraron un aumento en la incidencia de escoliosis en juveniles de fletán (*Hippoglossus hipoglossus*) alimentados con niveles crecientes de aceites oxidados. Esto puede ser el resultado del efecto de los productos de peroxidación lipídica, que estimulan la diferenciación osteoclástica e inhiben la actividad osteoblástica lo que puede causar la resorción ósea y conducir a la formación de malformaciones o anomalías óseas (Tintut *et al*., 2002; Parhami, 2003; Kruger *et al*., 2010). El mecanismo antioxidante del α-tocoferol intercepta el radical peroxilo (ROO-) más rápidamente que los PUFA, mediante la donación de su átomo de hidrógeno fenólico al radical, convirtiéndolo así en un producto hidroperóxido (Figura 9.8), rompiendo de esta manera con la cadena de reacciones implicadas en la autooxidación lipídica. El resultado de esta reacción es el radical tocoferoxilo que es suficientemente estable e incapaz de continuar la cadena de oxidación y que se retira del ciclo por reacción con otro radical peroxilo para formar productos inactivos no radicales.

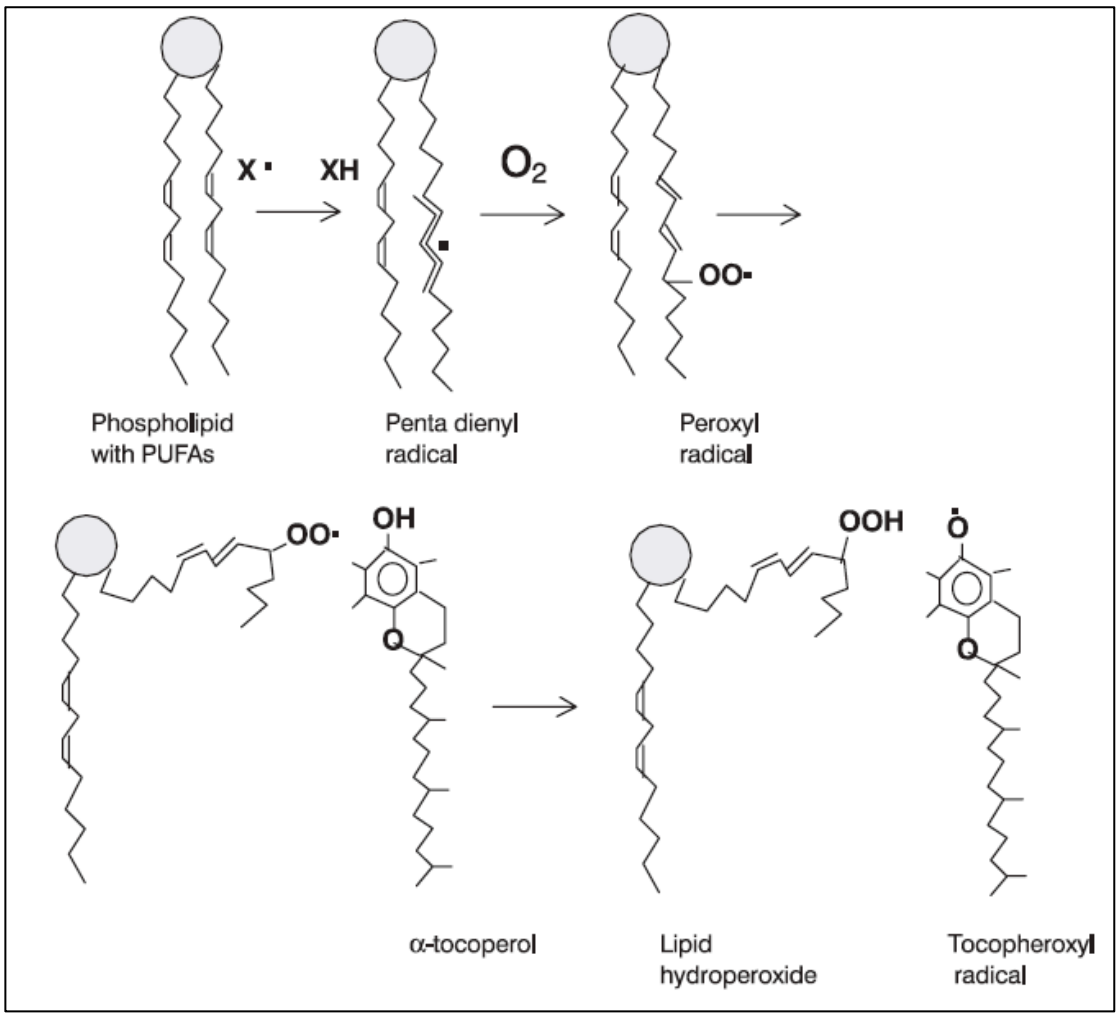

Figura 9.8 Mecanismo propuesto para la reacción del α-tocoferol con lípidos oxidantes. El grupo de radicales peroxilo formado durante la oxidación de lípidos es polar y flota a la superficie de la membrana donde puede reaccionar con el α -tocoferol, resultando en un hidroperóxido lipídico y el radical tocoferoxilo (Buettner, 1993).

9.1.7.2.2 Selenio

La investigación sobre los requerimientos de minerales en larvas de peces sólo se inició después de 2005 y el número de publicaciones es muy pequeño. El selenio (Se) es un oligoelemento y un micronutriente esencial para vertebrados (Johansson *et al*., 2005). En peces, el SE está relacionado también con hormonas de la glandula tiroides, la función de la insulina, el mantenimiento de la fertilidad así como con la regulación del crecimiento celular (Tabla 1,5; Lall, 2002; Kohlmeier, 2003). Una de las principales funciones de Se es ser un componente de las selenoproteínas GPX, isoenzimas que protegen los componentes lipídicos y membranas, a nivel celular y

subcelular, del daño oxidativo (Arteel y Sies, 2001). La deficiencia de Se puede conducir a un estrés oxidativo en los órganos (Gatlin *et al*., 1986; Bell *et al*., 1986, 1987), un crecimiento reducido (Wang y Lovell, 1997) y un aumento de la mortalidad (Gatlin *et al*., 1986; Bell *et al*., 1987) en varias especies de peces. Penglase *et al*. (2010) alimentaron larvas de bacalao incluyendo en su dieta seca rotíferos enriquecidos con hasta 3 mg de selenio kg^{-1}. Estos autores encontraron sólo efectos menores sobre el crecimiento y la supervivencia; en cambio, observaron que la expresión de genes y la actividad de la glutatión peroxidasa mejoraba, indicando un requerimiento de Se por encima del nivel usado como control.

La importancia del Se en el estrés oxidativo se debe al papel que juega en los sitios activos de las enzimas antioxidantes glutatión peroxidasa y glutatión peroxidasa fosfolípido hidroperóxido (Felton *et al*., 1996), que catalizan la reducción de hidroperóxidos y peróxidos de alcoholes menos reactivos y agua (Felton *et al*., 1996). Hay que tener en cuenta que el requerimiento de Se variará en peces de acuerdo con la forma de selenio ingerida y el contenido de PUFA y vitamina E de la dieta (Lall, 2002).

Como se ilustró anteriormente, las mediciones directas del requerimiento de vitaminas y minerales en larvas de peces están fragmentadas y son escasas, debido a que cada nutriente se mide sólo en unas pocas especies y no en todas. Son necesarios más estudios para determinar si el requerimiento de micronutrientes en larvas de peces es diferente al de juveniles y adultos.

9.2 Objetivos

Obtener alevines de buena calidad es esencial para el futuro desarrollo de la piscicultura marina. Su éxito y la consecuente obtención de una alta productividad se ve especialmente afectada por la calidad nutricional de las dietas utilizadas en los estadios larvarios iniciales (Kolkovski *et al.,* 2009; Izquierdo *et al.,* 2000). Uno de los principales objetivos en el área de la alimentación de larvas de peces es la formulación de una dieta eficaz y capaz de sustituir las presas vivas (Watanabe y Kiron, 1994). Los fosfolípidos de la dieta son componentes fundamentales de tales dietas de destete (Koven *et al.,* 1995; Izquierdo, 1996; Fontagné *et al.,* 2000; Izquierdo y Koven, 2010) debido a que tienen funciones esenciales en el desarrollo larvario (Izquierdo y Koven, 2010). Sin embargo, y a pesar de los muchos estudios que señalan la importancia de PL en la dieta, pocos de ellos han sido dirigidos a determinar específicamente sus requerimientos cuantitativos probando por lo menos cinco niveles de inclusión diferentes en la dieta. Adicionalmente, hay que considerar que el tipo de fosfolípido utilizado es un factor determinante de su valor nutritivo, pero pocos estudios han comparado diferentes fuentes y varios niveles de inclusión. A pesar de que el aumento de HUFA n-3 ricos en PL puede aumentar el riesgo oxidativo, estos aspectos no han sido todavía estudiados y no se encuentra información ni publicaciones que traten sobre la posible interacción de PL con nutrientes antioxidantes. Basados en todo lo anterior, el principal objetivo de esta tesis es definir los requerimientos de fosfolípidos de larvas de dorada y aportar conocimiento sobre los efectos de este nutriente en el crecimiento, supervivencia, resistencia al estrés, actividad enzimática digestiva, formación y mineralización del esqueleto así como del metabolismo oxidativo en esta fase de cultivo. Para lograr este objetivo principal se formularon los siguientes objetivos:

1. Determinar los niveles óptimos de fosfolípidos de krill (KPL) en la dieta de larvas de dorada (*Sparus aurata*) y su influencia en su desarrollo y en la actividad de sus enzimas digestivas.
2. Definir los niveles óptimos de lecitina de soja (SBL) en la dieta de larvas de dorada (*Sparus aurata*) y la influencia en su desarrollo y en la actividad de sus enzimas digestivas.
3. Comparar la efectividad de KPL vs lecitina SBL en microdietas usadas para el

destete temprano de larvas de dorada (*Sparus aurata*) en términos de rendimiento en el cultivo larvario, malformaciones esqueléticas, mineralización y desarrollo óseo, asi como en marcadores moleculares del estado oxidativo.

4. Evaluar el efecto combinado de niveles graduales de α-tocoferol con KPL o SBL en microdietas utilizadas para el destete temprano de larvas de dorada (*Sparus aurata*) en términos de rendimiento en el cultivo larvario, malformaciones esqueléticas, mineralización y desarrollo óseo y en marcadores moleculares del estado oxidativo.
5. Evaluar el efecto de niveles variables de Se, derivado de levaduras, en conjunto con KPL en microdietas usadas para el destete temprano de larvas de dorada (*Sparus aurata*) en términos de rendimiento en el cultivo larvario y en marcadores moleculares del estado oxidativo y el desarrollo esquelético.

9.3 Materiales y Métodos

9.3.1 Larvas

Se obtuvieron las larvas de dorada (*Sparus aurata*) de puestas naturales de reproductores existentes en las instalaciones del GIA (Grupo de Investigación en Acuicultura, Las Palmas de Gran Canaria, España) en donde se llevaron a cabo los experimentos presentados en esta Tesis. Las larvas fueron alimentadas previamente con rotíferos (*Brachionus plicatilis*) enriquecidos con Selco (DHA Protein Selco®, INVE, Dendermonde, Bélgica) hasta que llegaron a 16 días de edad. Al día 16 las larvas fueron distribuidas al azar en los tanques experimentales y fueron alimentadas con una de las dietas experimentales ensayadas por triplicado.

9.3.2 Cultivo en tanques experimentales

Los tanques experimentales son cilíndricos de fibra de vidrio y color gris de 200 L, fueron abastecidos por agua de mar filtrada (salinidad de alrededor de 37 ppm) a través de una malla de 50µm y entró del fondo del tanque a una tasa creciente de 0.4 - 1.0 L min^{-1} en un sistema abierto. El agua estaba continuamente aireada (125 ml min^{-1}), manteniendo niveles de oxígeno adecuados. La temperatura, el pH y el oxígeno se midieron diariamente. 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 mediante un sifón.

9.3.4 Dietas y alimentación

9.3.4.1 Rotíferos

Los rotíferos (*Brachionus plicatilis*) se cultivaron en tanques cilíndricos de 1700 L de capacidad. Estos tanques poseían un sistema central de aireación. El agua del cultivo se hizo 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 264 ind ml^{-1}. La alimentación de los rotíferos se basó en levadura de panificación (0,4 g 10^6 ind^{-1} d^{-1}), repartida en dos tomas diarias.

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 dos 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.

9.3.4.2 Microdietas

Para la realización de los experimentos de la Tesis se formularon dietas con distintos niveles y orígenes de PL, combinadas con vitamina E o Se.

9.3.4.2.1 Formulación de las microdietas

Las microdietas experimentales (250-500 µm) fueron suplementadas con aceite de krill (Qrill, high PL, Aker BioMarine, Fjordalléen, Noruega) y lecitina de soja (Acrofarma, Barcelona, España) como fuentes de PL, mientras que el α-tocoferol acetato (Sigma-Aldrich, Madrid, España) se usó como fuente de vitamina E. Además, se usó selenio orgánico extraído de levaduras (Sel-Plex, Alltech Inc, Lexington, KY, EEUU). Para igualar el contenido lipídico, se empleó un aceite que no es fuente de ácidos grasos esenciales, como es el ácido oléico (Merck, Darmstadt, Alemania). Como fuente de proteína, se empleó la harina de calamar, la cual fue desengrasada en algunas dietas para poder controlar el contenido de lípidos de las dietas.

9.3.4.2.2 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 250-500 µm). Las dietas

fueron analizadas para conocer su contenido proximal y de ácidos grasos, testando cada dieta en triplicado.

9.3.5 Alimentación

Las dietas se administraron cada 45 min desde las 8:00 hasta 19:00. Se alimentó a las larvas durante los dos primeros días de vida con rotíferos alimentados con levadura (a una densidad de 1 rotífero ml^{-1}), para así evitar cualquier aporte de LC-PUFA. Para garantizar la disponibilidad de alimento, la administración de las dietas experimentales fue al inicio 2.5 g y se fue incrementando 0.5-1 g cada semana.

9.3.6 Muestreos

9.3.6.1 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).

9.3.6.2 Composición Bioquímica

9.3.6.2.1 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.

9.3.6.2.2 Histología

En cada punto de muestreo se fijaron 30 larvas por tanque en formol tamponado al 10%.

9.3.6.2.3 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.

9.3.6.3 Test de actividad y supervivencia final

Antes del final de los experimentos se llevó a cabo un test de actividad, manteniendo 25 larvas en una red fuera del agua durante 1-1,5 min para después ponerlas de nuevo en el agua y observar la supervivencia a las 24 h, o poner 25 larvas en agua a 24ºC y observar la supervivencia a las 24 h. La supervivencia final se calculó tras contar todas las larvas remanentes en los tanques experimentales.

9.3.7 Análisis bioquímicos

9.3.7.1 Análisis proximales

9.3.7.1.1 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.

9.3.7.1.2 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).

9.3.7.1.3 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.

9.3.7.1.4 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.

9.3.7.2 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).

9.3.7.3 Clases lipídicas

La separación de clases lipídicas se realizó mediante el desarrollo doble de cromatografía de capa fina de alto rendimiento (HPTLC) usando el método descrito anteriormente (Tocher y Harvie 1988; Olsen y Henderson, 1989). Las clases lipídicas se visualizaron por carbonización a 160°C durante 15 min de placas de TLC después de la pulverización con 3% (w / v) de acetato cúprico acuoso con un 8% (v / v) de ácido fosfórico. Fueron cuantificadas por densitometría usando un escaner CAMAG-3 TLC (versión firmware 1,14 .16; CAMAG, Muttenz, Suiza) con el programa winCATS Planar Chromatography Manager. Las clases lipídicas fueron identificadas de manera individual al compararlas concomparación con los valores Rf de estándares analizados junto con las muestras en placas de HPTLC y desarrollada en las mismas condiciones. Los glicerofosfolípidos totales, incluyendo la lisofosfatidilcolina (LPC), la fosfatidilcolina (PC), la fosfatidiletanolamina (PE), el fosfatidilinositol (PI) y la fosfatidilserina (PS), se aislaron a partir de placas de HPTLC colectivamente y se sometieron a transesterificación catalizada por ácido de acuerdo con el método de Christie (1982). La extracción y purificación se realizaron siguiendo el método descrito por Tocher y Harvie (1988).

9.3.7.4 Determinación de actividades de enzimas digestivas

La determinación de las actividades enzimáticas se llevo a cabo en el Departamento de Biología Aplicada de la Universidad de Almería (Escuela Politécnica Superior de la Universidad de Almería, España). Las actividades de la fosfatasa alcalina, la tripsina y lipasa se expresan como unidades de fluorescencia relativa (RFU) y la de la

PLA2 se expresó como unidades (U); una uindad es capaz de hidrolizar 1.0 µmol of soybean L-α-phosphatidylcholine a L-α-lysophosphatidylcholine y ácido graso por minuto. Las larvas se homogeneizaron mediante un sonicador de ultrasonidos (Misonix Microson XL2007 homogeneizador ultrasónico) en hielo en 110µl de agua de alta purificación, se centrifugaron y el sobrenadante fue usado como la solución madre de la muestra.

La actividad de la fosfatasa alcalina se cuantificó por ensayo fluorimétrico utilizando un espectrofluorómetro (Thermolab Systems; Helsinki, Finlandia) a longitud de onda de excitación de 358 nm y longitud de onda de emisión de 455 nm. Después de la preparación de 140 µl de tampón de reacción pH 10,4 (100 mM de Glicina, 1 mM $MgCl_2$, 1 mM $ZnCl_2$), y 50 µl de solución de sustrato (200 mM de 6,8 difluoro-4 fosfato methyllumbelliferyl (DiFMUP) en DMSO), la reacción se inició mediante la adición de 10 µl de la solución madre de la muestra y las curvas cinéticas se registraron durante 20 min (Gee *et al.*, 1999).

La actividad de la tripsina se cuantificó mediante ensayo fluorométrico utilizando un espectrofluorimétro a longitud de onda de excitación de 380 nm y longitud de onda de emisión de 440 nm. Después de la preparación de 195 µl de tampón de reacción pH 8,0 (50 mM Tris-HCl, 10 mM $CaCl_2$), y 5 µl de solución de sustrato (20 µM de Boc-Gln-Ala-Arg-7 clorhidrato metilcumarina amido-4 en DMSO), la reacción se inició mediante la adición de 10 µl de solución madre de la muestra y las curvas cinéticas se registraron durante 5 min (Rotllant *et al.*, 2008).

La actividad de la lipasa neutra se cuantificó por ensayo fluorimétrico utilizando un espectrofluorómetro a longitud de onda de excitación de 355 nm y longitud de onda de emisión de 460 nm. En primer lugar se mezclaron 247,4 µl de tampón de reacción fosfato 0,1M pH 7,0 (19,5 ml de 0,2 M fosfato monobásico; NaH_2PO_4 1 H2O) y 30,5 ml de una solución de fosfato dibásico 0,2 M (NaH_2PO_4 7 H2O), completándose hasta los 100 ml de volumen mediante la adición 50 ml agua de alta purificación, a continuación se añadieron 2.6µl de solución de sustrato (40 mM de butirato 4Methylumbelliferol (MUB) en N, N dimetil formamida (DMSO)) y la reacción se inició tras la adición de 10 µl de la solución madre de la muestra. Las curvas cinéticas se registraron durante 5 min (Rotllant *et al*., 2008).

La actividad de la PLA2 se cuantificó por ensayo fluorimétrico utilizando un espectrofluorómetro a longitud de onda de excitación de 377 nm y longitud de onda de emisión de 450 nm. En primer lugar, se preparó un mix conteniendo 160 µl de

tampón de reacción pH 8 (50 mM Tris-HCl, 100 mM de NaCl, 2 mM de NaN3, 5µg/ml albúmina de suero bovino, y 10 µl 1-anilinonaphthalene-8-sulfonato de sodio), 20 µl de solución de sustrato (50 µl de 1 ,2-dimiristoil-sn-glicero-3-fosfocolina en solución 40 mM, metanol mezclado con 15 µl solución de ácido desoxicólico en 40 mM, metanol y rápidamente inyectado en 1 ml agua de alta purificación, se agita durante 1 min y se sónico durante 2 min) y 10 µl de solución 100 mM de $CaCl_2$, incubándose a 25 ° C durante 10 min. La reacción se inició mediante la adición de 20 µl de la solución madre de la muestra y las curvas cinéticas se registraron durante 40 min (Huang *et al.*, 2006).

9.3.7.5 Determinación de las sustancias reactivas al ácido tiobarbitúrico (TBARS)

La medida de TBARS se determinó por triplicado 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:

$$\text{nmol MDA/g tejido} = \frac{A}{0.156} \times \frac{50}{Pesomuestra}$$

9.3.7.6 Determinación de selenio

En la presente Tesis se determinó la concentración total de selenio tanto en larvas como en 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 un

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.

9.3.8 Estudios histológicos

9.3.8.1 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 a los 45 días post eclosión. Las larvas fueron fijadas en formol tamponado al 10% para examinar las deformidades esqueléticas y el grado de osificación. Fueron teñidas con rojo Alizarina para detectar el hueso según el protocolo de Vandewalle *et al*. (1998) (Tabla 9.2). Para evitar cualquier variabilidad técnica, las larvas de los distintos tratamientos se tiñeron a la vez.

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, las larvas fueron observados bajo la lupa (Leica DM2500, Nussloch, Alemania).

Tabla 9.2 Protocolo de tincíon según Vandewalle et al. (1998)

Paso	Duración	Importancia del paso	Soluciones
Azul alcián (8GX) en el protocolo dices que solo usaste rojo….	2 horas	Tinción de proteoglicanos (cartílago)	Para 100 mL: 80 mL alcohol 95% 20 mL ácido acético glacial 10 mg azul alcián
Etanol 95%	1 hora	Hidratación	Para 100 mL: 95 mL etanol absoluto 5 mL agua destilada
Etanol 95%	1 hora		
Etanol 95%	1 hora		
Etanol 75%	1 hora		Para 100 mL: 75 mL etanol absoluto 15 mL agua destilada

Etanol 40%	1 hora		Para 100 mL: 40 mL etanol absoluto 60 mL agua destilada
Etanol 15%	1 hora		For 100 mL: 15 mL etanol absoluto 75 mL agua destilada
Agua destilada	1 hora o toda la noche		
Solución de tripsina	1 hora	Digestión d tejido	90 mg de tripsina de pancreas porcino 70 mL agua destilada 30 mL de solución saturada de $Na_2B_4O_7$
Rojo alizarina	1 hora y 30 min	Tinción de calico (hueso)	1g L^{-1} rojo alizarina en una solución 0.5% de KOH
Glicerina:KOH 1:3	12 a 24 horas	Aclarado	Para 100 mL: 25 mL Glicerina 75 % KOH (0.5%)
Glicerina:KOH 1:1	12 a 24 horas	Aclarado	Para 100 mL: 50 mL Glicerina 50 mL KOH (0.5%)
Glicerina:KOH 3:1	12 a 24 horas	Aclarado	Para 100 mL: 75 mL Glicerina 25 mL KOH (0.5%)
Glicerina pura con algunos granos de timol		Almacenaje	

9.3.9 Biología molecular

Los análisis de biología molecular se llevaron a cabo en el IUSA (Instituto Universitario de Sanidad Animal de la Universidad de Las Palmas de Gran Canaria, España). El ARN total de las muestras de las larvas (peso promedio por muestra de 60 mg) fue extraído utilizando el RNeasy Mini Kit (Qiagen). Los tejidos se homogeneizaron usando el TissueLyzer-II (Qiagen, Hilden, Alemania) con el reactivo

de lisis QIAzol (Qiagen). Se centrifugaron las muestras con cloroformo para la separación de fases (12000g, 15 min, 4 ºC). La fase superior acuosa que contiene ARN se mezcló con 75% de etanol y se transfirió a una columna de RNeasy donde el ARN total quedó unido a una membrana y los contaminantes fueron arrastrados por RW1 y tampones de RPE (Qiagen). El ARN purificado se eluyó con 30 ml de agua libre de RNasa. La calidad y la cantidad de ARN se analizaron usando el espectrofotómetro NanoDrop 1000 (Thermo Scientific, Wilmington, DE, EE.UU.). La síntesis de cDNA se llevó a cabo usando el kit de síntesis de ADNc iScript (Bio-Rad) de acuerdo con las instrucciones del fabricante en un ciclador térmico iCycler (Bio-Rad, Hercules, CA, EE.UU.). 100 ng de ARN total se transcribieron inversamente a cDNA complementario. La eficiencia del cebador fue probada con diluciones seriadas de un conjunto de ADNc (1:1, 1:5, 1:10, 1:15, 1:20 y 1:25). El tamaño del producto de la amplificación obtenido tras la qPCR en tiempo real verificado por análisis de electroforesis utilizando PB322 cortado con HAEIII como un estándar. La PCR cuantitativa a tiempo real se realizó en un iQ5 Multicolor Real-Time PCR (Bio-Rad, Hercules, CA, EE.UU.), utilizando β-actina como gen de referencia (house-keeping gene) en un volumen final de 20 μl por pocillo de reacción. Cada muestra se analizó una vez por gen. Las condiciones de PCR fueron las siguientes: 95 º C por 3min y 30 seg seguido por 40 ciclos de 95 º C durante 15 segundos, 61 º C durante 30 segundos y 72 º C durante 30 segundos, 95 º C durante 1 minuto, y una etapa final de desnaturalización de 61 º C a 95 º C por 10 seg. Los datos obtenidos se normalizaron de acuerdo al método de Livak ($2^{-\Delta\Delta Ct}$) para determinar los niveles relativos de expresión de ARNm. Los cebadores específicos para dorada se diseñaron después de buscar wn la base de datos NCBI de nucleótidos y utilizando el software de análisis Oligo 7 Primer (Molecular Insights Biología, Cascade, CO, EE.UU.). La información detallada de secuencias de los cebadores y números de acceso se presenta en la Tabla 9.3.

Tabla 9.3 Genes estudiados en la presente tesis

Gene	Primer	N° de acceso
Catalasa (CAT)	Forward primer: 5´-ATGGTGTGGGACTTCTGGAG-3´ Reverse primer: 3´-AGTGGAACTTGCAGTAGAAAC-5´	FJ860003
Superoxido dismutasa (SOD)	Forward primer: 5´- AAGAATCATGGCGGTCCTACTGA-3´ Reverse primer: 3´- TGAGCATCTTGTCCGTGATGTCT -5´	AJ937872
Glutathion peroxidasa (GPX)	Forward primer: 5´- TCCATTCCCCAGCGATGATGCC-3´ Reverse primer: 3´- TCGCCATCAGGACCAACAAGGA-5´	DQ524992
Osteocalcina	Forward primer: 5´-AGCCCAAAGCACGTAAGCAAGCTA-3´ Reverse primer: 3´- TTTCATCACGCTACTCTACGGGTT-5´	AF048703
Osteopontina	Forward primer: 5´- AAGATGGCCTACGACATGACAGAC- 3´ Reverse primer: 3´- CCTGAAGAGCCTTGTACACCTGC-5´	AY651247
Osteonectina	Forward primer: 5´- AAAATGATCGAGCCCTGCATGGAC-3´ Reverse primer: 3´- TACAGAGTCACCAGGACGTT-5´	AY239014
[a]RUNX2	Forward primer: 5´- GCCTGTCGCCTTTAAGGTGGTTGC-3´ Reverse primer: 3´- TCGTCGTTGCCCGCCATAGCTG-5´	AJ619023
Alkalina phosphatasa	Forward primer: 5´- AGAACGCCCTGACGCTGCAA-3´ Reverse primer: 3´- TTCAGTATACGAGCAGCCGTCAC-5´	AY266359
Matrix Gla proteina	Forward primer: 5´- GTGCCCTCCTTCATTCCAC-3´ Reverse primer: 3´- TATGACCACGTTGGATGCCT-5´	AY065652
[b]BMP4	Forward primer: 5´- CCACCAGGGCAGACACGTCC-3´ Reverse primer: 3´- GCGTAGCTGCTCCCAGTCCTC-5´	FJ436409
β-actina	Forward primer: 5´- TCTGTCTGG ATC GGAGGCTC-3´ Reverse primer: 3´- AAGCATTTG CGGTGGACG -5´	X89920

a RUNX2 (Runt-related transcription factor 2).
b BMP4 (Bone morphogenetic proteina 4).

9.3.10 Análisis estadístico

Los análisis estadísticos realizados en la presente tesis se llevaron a cabo empleando en programa SPSS (SPSS for Windows 11.5; SPSS Inc., Chicago, IL, USA). 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.

9.4 Resúmenes de los experimentos

9.4.1 Capítulo 3:

Contenido óptimo de fosfolípidos de krill en microdietas de larvas de dorada (*Sparus aurata*)

Resumen

El objetivo del presente estudio fue determinar los niveles óptimos de fosfolípidos de krill (KPL) en dietas de larvas de dorada (*Sparus aurata*) y su influencia en el desarrollo larvario y en la actividad de algunas enzimas digestivas. Fueron utilizadas cinco microdietas formuladas con cinco niveles diferentes de KPL. La sustitución total de presas vivas con las microdietas experimentales produjo una supervivencia y tasas de crecimiento altas, en particular en aquellas larvas alimentadas con los niveles más altos de KPL. En el presente estudio el aumento de KPL de la dieta hasta el 12% de inclusión (10% total PL) mejoró significativamente la supervivencia y crecimiento larvario, sin embargo, un aumento adicional de PL no aumento ninguno de los dos. Adicionalmente, fue observado un aumento de la actividad de fosfatasa alcalina, tripsina y lipasa cuando la elevación de KPL fue hasta del 12%, denotando un mejor funcionamiento del sistema digestivo de las larvas. Además, hubo un efecto estimulador lineal del sustrato de KPL sobre la actividad de la fosfolipasa A2. Por último, el aumento de KPL en la dieta permitió una mejor asimilación de los HUFA n-3 especialmente deEPA, lo que se vió reflejado en el mayor contenido de estos ácidos grasos en los lípidos neutros y polares de las larvas. En resumen, los KPL resultaron ser una excelente fuente de lípidos para las larvas de dorada. Los niveles óptimos de inclusión de este ingrediente en microdietas al sustituir completamente las presas vivas en larvas fue del 12% de KPL.

9.4.2 Capítulo 4:

Contenido óptimo de lecitina de soja en microdietas de larvas de dorada (*Sparus aurata*)

Resumen

El objetivo del presente estudio fue determinar los niveles óptimos de inclusión de lecitina de soja (SBL) en dietas de larvas de dorada (*Sparus aurata*) y su influencia en el cultivo y en la actividad de algunas enzimas digestivas. Fueron utilizadas cinco microdietas formuladas con cinco niveles diferentes de SBL. La sustitución total de presas vivas con las microdietas experimentales en el día 16 post-eclosión produjo una supervivencia superior al 55%, en particular en aquellas larvas alimentadas con los niveles más altos de SBL. Además, un incremento superior al 80 g kg^{-1} de inclusión de SBT en la dieta mejoró significativamente el crecimiento, reflejándose en una longitud y peso corporal final mayores. Adicionalmente, fue observado un aumento de la actividad de fosfatasa alcalina cuando el nivel de inclusión de SBT se elevó a 80 g kg^{-1}, denotando un mayor desarrollo del sistema digestivo. También hubo un efecto estimulador de SBL sobre la actividad de la fosfolipasa A2. Por último, el aumento de SBL en la dieta permitió una mejor asimilación de los HUFA de la dieta, lo que se vió reflejado en el mayor contenido de este ácido graso en los lípidos neutros y polares de las larvas. En resumen, con un incremento superior al 80 g kg^{-1} de inclusión de SBT en la dieta fue observada una mejora significativa de la actividad enzimática, maduración de enterocitos, utilización y deposición de acidos grasos esenciales y del crecimiento, como consecuencia de una mejor digestión, absorción, transporte y deposición de los nutrientes de la dieta.

9.4.3 Capítulo 5:

Efecto de los fosfolípidos de krill vs lecitina de soja en microdietas de larvas de dorada (*Sparus aurata*) sobre marcadores moleculares del estrés oxidativo y el desarrollo óseo

Resumen

El objetivo del presente estudio fue comparar la eficacia de los fosfolípidos marinos (MPL) obtenidos a partir de krill y lecitina de soja (SBL) sobre el rendimiento y el desarrollo de larvas de dorada (*Sparus aurata*). Las larvas fueron alimentadas desde el día 16 al 44 post-eclosión (dph) utilizando cinco microdietas formuladas con tres niveles diferentes (5, 7 o 9%) de fosfolípidos (PL) obtenidos a partir de MPL o SBL. Las larvas alimentadas con MPL mostraron una supervivencia, resistencia al estrés y crecimiento superiores a las alimentadas con SBL, sin importar el nivel de PL de la dieta. En general, el aumento de MPL hasta 7% de PL totales en la dieta fue suficiente para mejorar el rendimiento en larvas de dorada, mientras que un nivel superior de inclusión de SBL (9% de PL) no fue capaz de proporcionar un éxito similar en el crecimiento o supervivencia. La inclusión de SBL aumentó notablemente el riesgo de peroxidación lo que se vió reflejado en los mayores TBARS en larvas, así como una mayor expresión de los genes CAT, GPx y SOD. Además, SBL tiende a producir larvas con un menor número de vértebras mineralizadas y una menor expresión de los genes OC, OP y BMP4. Finalmente, el aumento de la dieta de MPL o SBL llevó a una mejor asimilación de los ácidos grasos poliinsaturados en las larvas, HUFA n-3 (especialmente 20:5 n-3) o ácidos grasos n-6 (especialmente 18:2 n-6), respectivamente. En conclusión, MPL tuvo una eficacia superior en la promoción de la supervivencia, crecimiento y mineralización esquelética de larvas de dorada en comparación con SBL.

9.4.4 Capítulo 6:

Biomarcadores del desarrollo óseo y estrés oxidativo en larvas de dorada (*Sparus aurata*) alimentadas con microdietas con varios niveles de lípidos polares y α-tocoferol

Resumen

A pesar de que los fosfolípidos marinos son capaces de mejorar el rendimiento en el cultivo de larvas de peces marinos de mejor manera que la lecitina de soja, ambos tipos de fosfolípidos (PL) aumentan notablemente el riesgo oxidativo. La inclusión de un antioxidante liposoluble como la vitamina E α-tocoferol podría permitir un mejor control del estrés oxidativo. El objetivo del presente estudio fue determinar el efecto combinado de niveles variables de α-tocoferol con diferentes niveles y fuentes de fosfolípidos de krill (KPL) y lecitina de soja (SBL) sobre el crecimiento, supervivencia, resistencia al estrés, estado oxidativo, expresion de genes relacionados con el metabolismo óseo y composición bioquímica de larvas de dorada. Las larvas fueron completamente destetadas el día 16 post-eclosión y alimentadas durante 30 días utilizando siete microdietas con tres niveles diferentes de PL (0, 4 y 8%) y dos de α-tocoferol, 1.500 y 3.000 mg kg^{-1} de dieta. Las larvas de dorada alimentadas con dietas sin suplementación de PL mostraron la menor supervivencia, crecimiento y resistencia al estrés, mientras que el aumento en PL, especialmente KPL, promovió notablemente la supervivencia y crecimiento. Sin embargo, la alimentación con SBL incrementó notablemente los TBARS y la expresión del gen GPX, denotando el mayor riesgo de peroxidación en las larvas. Además, la inclusión de KPL mejoró la incorporación de HUFA n-3 y, en particular, de EPA en los tejidos de las larvas. Estos ácidos grasos están correlacionados positivamente con la expresión de los genes BMP-4, RUNX 2, ALP, OC y OP y en la mineralización del hueso en un determinado tamaño. El incremento de α-tocoferol tiende a mejorar el crecimiento en relación con los niveles de HUFA n-3 de la dieta, denotando la función protectora de esta vitamina frente a la oxidación. En efecto, el α-tocoferol de la dieta disminuyó el estrés oxidativo en las larvas, lo que se vió reflejado en la reducción de TBARS y la expresión génica de SOD y CAT, pero no de GPX. Por lo tanto, el aumento de α-tocoferol en la dieta previno eficazmente la formación de radicales libres a partir de

HUFA, en particular de EPA, pero no afectó a la incidencia de anomalías óseas o la expresión de genes relacionados con los procesos osteogénicos.

9.4.5 Capítulo 7:

Niveles de selenio en dietas de destete precoz de larvas de dorada

Resumen

La inclusión de factores antioxidantes complementarios, como el selenio (Se), podría contrarrestar el riesgo de oxidación en las dietas de destete precoz con un alto contenido de ácidos grasos poliinsaturados (PUFA). El objetivo del presente estudio fue investigar el efecto de niveles variables de Se derivado de levadura con krill PL (KPL), en el desarrollo esquelético, supervivencia, resistencia al estrés, estado oxidativo y composición bioquímica de larvas de dorada. Las larvas de dorada fueron completamente destetadas al día 16 post-eclosión y alimentadas durante 30 días utilizando cinco microdietas isoenergéticas e isoproteicas con diferentes niveles de Se: 2SE, 4SE, 6SE, 8SE y 12SE (1.73, 3.91, 6.41, 8.47, 11.65 mg SE kg-1 de peso seco de la microdieta, respectivamente).

El aumento de Se hasta un 11,65 mg kg-1 peso seco mejoró significativamente la supervivencia (54%), la resistencia al estrés de las larvas y no afectó su crecimiento. Las larvas alimentadas con dietas suplementadas con 12SE (11,65 mg kg-1) mostraron un aumento gradual de este mineral de acuerdo a los niveles dietéticos de Se, mostrando su progresiva absorción. El grado de oxidación de lípidos en las larvas fue observado a través del contenido del malondialdehído (MDA) y la expresión del gen AOE y fue significativamente menor en aquellas larvas alimentadas con las dietas 8SE y 12SE en comparación con las dietas 2SE y 4SE.

Además, fue observada una respuesta reactiva como consecuencia de la inclusión en Se a través del incremento de osteocalcina, osteonectina, osteopontina, fosfatasa alcalina y la expresión génica de la proteína de la matriz GLA en los tejidos de las larvas, sugiriendo un buen desarrollo esquelético. Estos resultados indican la alta eficiencia de Se como un factor antioxidante y la importancia de su inclusión en niveles adecuados (11,65 mg Se kg-1 peso seco de dieta) en dietas de destete precoz.

9.5 Conclusiones Generales

1. La deficiencia de PL reduce el rendimiento larvario, retarda el desarrollo intestinal y la mineralización ósea, inhibe la actividad de las enzimas digestivas y reduce la utilización de los lípidos de la dieta en larvas de dorada.

2. Los niveles óptimos de inclusión de PL de krill en microdietas que sustituyen completamente las presas vivas a partir del día 16 después de la eclosión es alrededor de 12% de PL de krill, proporcionando así un 10% del PL total.

3. Los niveles óptimos de inclusión de SBL en microdietas que sustituyen completamente las presas vivas a partir del día 16 después de la eclosión es alrededor de 8% de PL de krill, proporcionando así un 9% de PL total. Sin embargo, la inclusión de SBL aumentó notablemente el riesgo de peroxidación, indicado por la mayor TBARS en larvas, así como por una mayor expresión de los genes CAT, GPx y SOD.

4. Los PL de krill son una excelente fuente de lípidos para larvas de dorada, siendo marcadamente mejores que los de SBL.

5. Los requerimientos de PL de dorada en dietas que sustituyen completamente las presas vivas entre los días 16 y 45 después de la eclosión es de aproximadamente 9-10%. Estos valores parecen promover la digestión, transporte y deposición de los lípidos de la dieta y contribuyen a reducir las anomalías esqueléticas por la regulación de BMP-4, Runx2, ALP y las proteínas de la matriz extracelular del esqueleto, induciendo una mineralización temprana y la resistencia de los cuerpos vertebrales, reduciendo así anomalías como lordosis y cifosis.

6. El aumento de α-tocoferol en la dieta promueve efectos beneficiosos en el PL de la dieta, promoviendo el crecimiento de las larvas, mostrando su función como protector contra la oxidación y la reducción de TBARS de las larvas y

de la expresión de los genes SOD y CAT.

7. La inclusión de 11,65 mg de Se kg-1 de dieta en microdietas mejora la capacidad antioxidante de las larvas y la regulación de los genes implicados en el desarrollo del esqueleto. La mejora de la supervivencia y la resistencia al estrés debido al aumento de los niveles de Se orgánico en la dieta parece estar relacionado por una parte con la reducción de radicales libres tóxicos, como lo indican los bajos niveles de MDA y del contenido de AOE, y, por otro lado, con una mejor utilización de los lípidos de la dieta.

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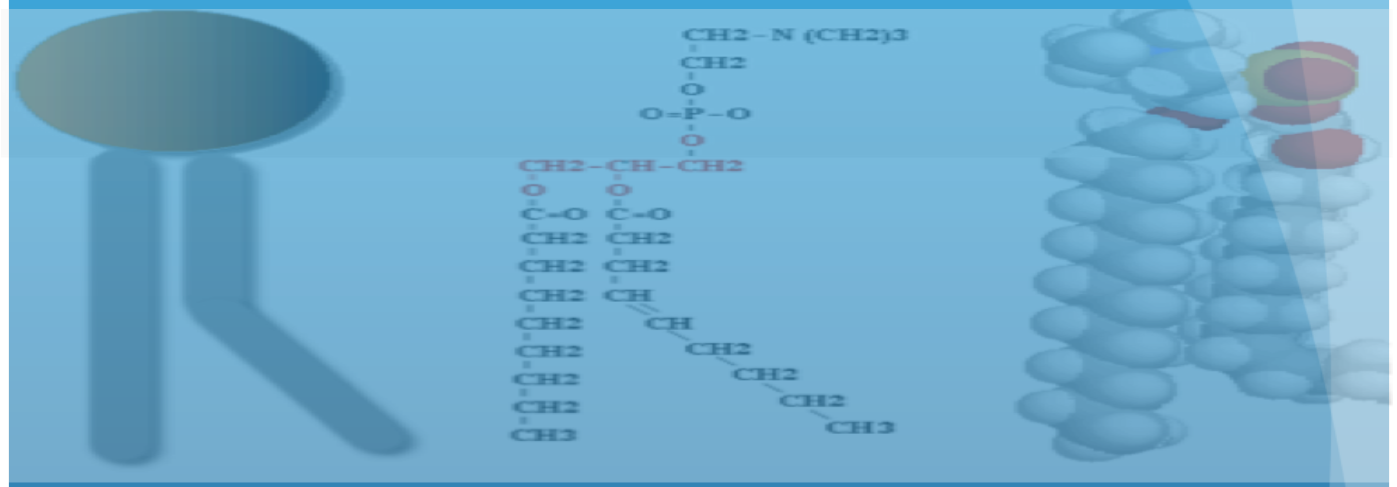

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