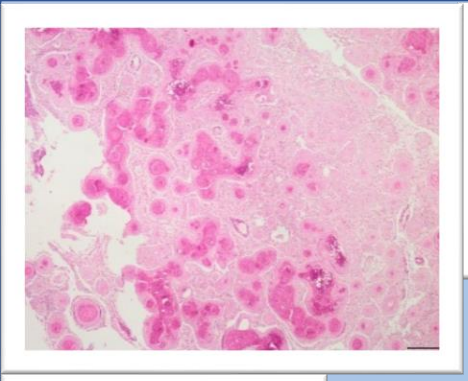
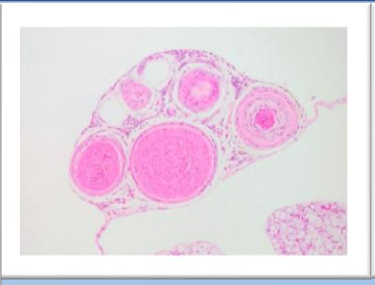
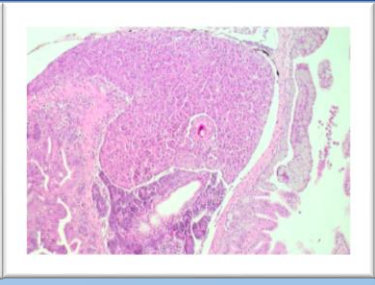
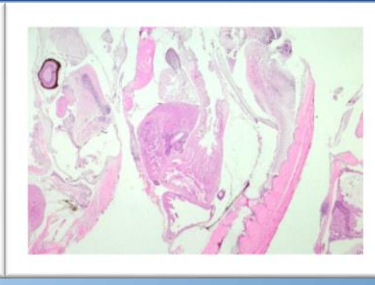
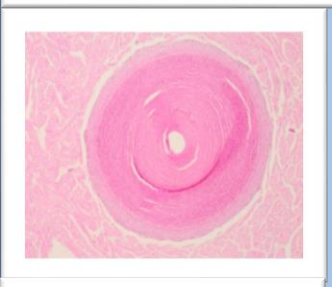
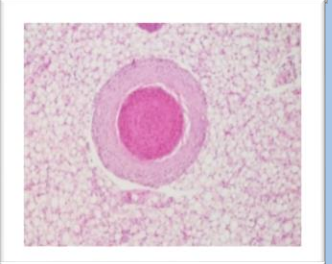
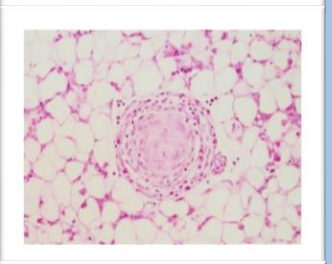
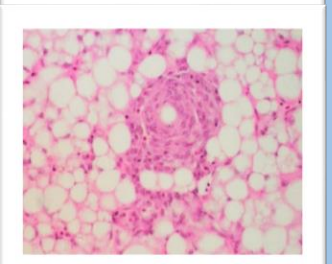
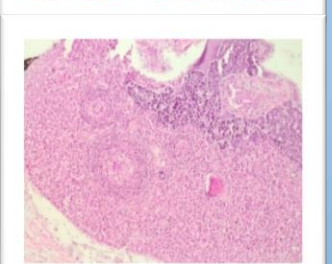
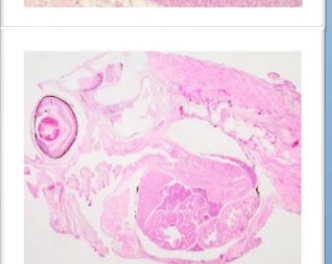
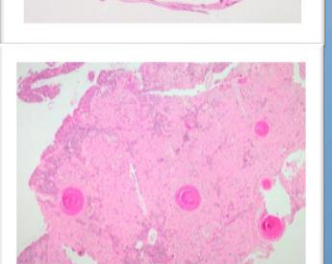

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Las Palmas de Gran Canaria, Spain
July 2019

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D. DANIEL MONTERO VÍTORES COORDINADOR DEL PROGRAMA DE DOCTORADO EN ACUICULTURA SOSTENIBLE Y ECOSISTEMAS MARINOS DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

INFORMA,

Que la Comisión Académica del Programa de Doctorado, en su sesión de fecha 15 de Julio de 2019 tomó el acuerdo de dar el consentimiento para su tramitación, con Mención Internacional de Doctorado a la tesis doctoral titulada **DETERMINING THE INCIDENCE OF GRANULOMATOSIS IN MEAGRE (*Argyrosomus regius*): MODULAR EFFECT OF THE NUTRITIONAL BALANCE OF MICRONUTRIENTS IN DIET. "ESTUDIOS DE DETERMINACIÓN DE INCIDENCIA DE GRANULOMATOSIS EN CORVINA (*Argyrosomus regius*): EFECTO MODULADOR DEL BALANCE NUTRICIONAL DE MICRONUTRIENTES EN DIETA**". presentada por el doctorando **D. MIGUEL ÁNGEL RUIZ GARCÍA** y dirigida por las Doctoras **CARMEN MARÍA HERNÁNDEZ CRUZ** y **MÓNICA BEATRIZ BETANCOR QUINTANA**

Y para que así conste, y a efectos de lo previsto en el Artº 11 del Reglamento de Estudios de Doctorado (BOULPGC 7/10/2016) de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria, a 16 de Julio de dos mil diecinueve.

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UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

ESCUELA DE DOCTORADO

Programa de doctorado ACUICULTURA SOSTENIBLE Y ECOSISTEMAS MARINOS

Título de la Tesis

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"ESTUDIOS DE DETERMINACIÓN DE INCIDENCIA DE GRANULOMATOSIS EN CORVINA (*Argyrosomus regius*): EFECTO MODULADOR DEL BALANCE NUTRICIONAL DE MICRONUTRIENTES EN DIETA".

Tesis Doctoral presentada por D. MIGUEL ÁNGEL RUIZ GARCÍA

Dirigida por el Dra. Dª. CARMEN MARÍA HERNÁNDEZ CRUZ

Codirigida por el Dra. Dª. MÓNICA BEATRIZ BETANCOR QUINTANA

El/la Director/a,	**El/la Codirector/a**	**El/la Doctorando/a,**
(firma)	**(firma)**	**(firma)**

Las Palmas de Gran Canaria, a 3 de Julio de 2019

A mis padres

Gracias por vuestro apoyo incondicional
en cada paso que he dado

List of contents

Abstract

The farming of traditional aquaculture species such as sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) has reached their maturity and has forced the search of new species which can cover other market niches. Meagre (*Argyrosomus regius*) is one of the fast-growing species proposed as a candidate for marine finfish diversification on commercial aquaculture in the Mediterranean and Eastern Atlantic coasts. Nevertheless, one of the most critical points in meagre production is the fish health, considering the systemic granulomatosis as the more frequent disease in this species. The objective of the present Thesis was to study the effect of antioxidant nutrients, such as vitamin E and C (Chapters 3, 5 and 6), different feeding sequences (Chapter 4), stocking densities (Chapter 6) and supplementation of manganese, selenium and zinc (Chapter 6), on the appearance of systemic granulomatosis in meagre larvae and juvenile.

In juvenile and adult meagre, the incidence of systemic granulomatosis can affect up to 100 % of the population, so, firstly, an experiment was carried out to elucidate the effect of the supplementation with vitamins E, C and K on the incidence of granulomas in juvenile meagre (Chapter 3). Juvenile meagre were fed six diets with different levels of vitamin E, C and K (150/20/0, 150/20/23, 300/70/0, 300/70/23, 450/230/0 and 450/230/23 mg kg^{-1}, respectively). In this study, a molecular approach was carried out, by studying the expression of several genes related to oxidative stress (*sod*, *cat* and *gpx*) and immune system *(cox-2* and *tnfα*). Supplementation with 450 mg kg^{-1} vitamin E and 230 mg kg^{-1} vitamin C strongly induced the expression of antioxidant enzymes and immune genes, in an attempt to reduced deleterious effect of reactive oxygen species. The incidence of granulomas in liver and heart was reduced when high levels of antioxidant vitamins E and C were added to the diet. An immunostaining for actin showed the presence of actin around some of the granulomas and the observation of irregular aggregated of cells around the blood vessels, what could suggest a possible origin of the granulomas in the blood vessels.

The results of this experiment showed that the incidence of granulomas in juvenile meagre is very high (86-100 %), and an adequate supplementation of vitamin E, C and K can retard and avoid the appearance of new granulomas. However, the granulomas already present cannot be eliminated, being necessary to act in earlier stages. For that reason, an additional study was carried out in meagre larvae (Chapter 4). Meagre larvae

were fed four different feeding regimes as follows: RS and RO (rotifer enriched with Easy DHA Selco or Ori-Green from 3-30 dph, respectively), AS and AO (rotifer enriched with Easy DHA Selco or Ori-Green from 3-21 dph and *Artemia* enriched with Easy DHA Selco or Ori-Green from 12-30 dph, respectively). It was observed that meagre larvae could be weaned from enriched rotifer to inert diet after 20 dph, but the lack of *Artemia* in the feeding sequence lead to poor survival and growth probably because of a high energy cost/benefit related to the small size of the prey, indicating that is necessary an intermediate feeding between rotifers and microdiet. Lipid peroxidation, as indicated by TBARS value, was higher in fish fed without *Artemia* in the diet. The results showed that granulomas first appeared in meagre larvae at 20 dph when fed rotifers only. Conversely, a reduced appearance of granulomas and lipid peroxidation occurs when *Artemi*a is included in the feeding sequence. In the light of these results, a new experiment was planned (Chapter 5), where meagre larvae were reared until 30 dph following the best feeding sequence obtained in the previous study and then, fed from 30 to 44 dph with five different diets with graded levels of vitamin C and E (40/100, 200/500, 400/1,000 and 800/500, respectively) and a fifth diet with 400/1,000 and substitution of fish oil by krill oil. Despite the fact that antioxidant vitamins did not affect growth performance, the appearance of granulomas was avoided when the microdiet was supplemented with 800 mg kg^{-1} of vitamin E and 2,000 mg kg^{-1} of vitamin C. This mitigation was simultaneous with the reduction of TBARS content in larvae, which were indeed highly correlated with the appearance of granulomas (R^2=0.892, y=0.0446x+0.0756). The results of this study showed that the occurrence of systemic granulomatosis seemed to be associated to the larvae peroxidation status, so that high dietary levels of vitamin E and C (800 and 2,000 mg kg^{-1}, respectively), reduced lipid peroxidation and completely prevented the appearance of granulomas in meagre larvae at 44 dph. At this point in the Thesis, it was observed that the systemic granulomatosis can first appear in very early stages of life (20 dph), but with the adequate feeding sequence and vitamins supplementation in the microdiet it can be avoided, almost until 44 dph.

In the last study it the effect of different levels of vitamin C (100, 600, 1,200 and 3,200 mg kg^{-1}), minerals Se, Zn and Mn (1.5 mg kg^{-1}, 200 mg kg^{-1} and 40 mg kg^{-1}, respectively) and two different densities (3.20 and 6.20 kg m^{-3}) on the appearance of systemic granulomatosis in juvenile meagre was evaluated (Chapter 6). Supplementation of vitamin C and E and minerals Mn, Zn, and Se did not affect meagre growth in terms

of final weight, length, FCR and SGR, with growth parameters only affected by the stock density, being lower in fish reared at a high density (6.20 kg m^{-3}). High supplementation of vitamin C (1,200-3,200 mg kg^{-1}) significantly reduced the percentage of granulomas in any tissue, but only when juvenile meagre were reared at 3.20 kg m^{-3}). The high stock density can disturb the balance between the production of ROS and its removal, and this imbalance can in turn lead to a status of oxidative stress which may cause different diseases and lesions. In the present study, the levels of TBARS were affected by the stock density, being higher in fish farmed at high density. Therefore, the present results suggest that the high stock density is a stressful factor that can lead to oxidative stress, lipid peroxidation and promote the appearance of granulomas. The present results show that high levels of vitamin C (1,200-3,200 mg kg^{-1} C) and low stock density (3.20 kg m^{-3}) favour the growth of juvenile meagre, reducing the lipid peroxidation indicators and decreasing the incidence of granulomas, which confirms that this pathology is mostly triggered by the deficiency of antioxidant nutrients, particularly vitamin C. These studies have shown that the granulomas can begin to appear in meagre larvae at 20 dph, but they could be avoided with the correct feeding sequence and supplementation of vitamins, almost until 44 dph. The incidence of granulomatosis in juvenile meagre is high and the supplementation of vitamin E and C in the diet can retard and avoid the appearance of new granulomas but the granulomas already present cannot be eliminated, being necessary to act in earlier stages. High stock density is a stressful factor that can reduce growth, increase the appearance of systemic granulomatosis and oxidative stress. The results show that the combination of high dietary content of antioxidant vitamins E and C can have an influence on the incidence of the granulomatosis in meagre, which suggests that this pathology could be mediated by nutritional factors.

List of abbreviations

ANOVA	Analysis of variance
APROMAR	Asociación Empresarial de Productores de Cultivos Marinos
ARA	Arachidonic acid (20:4n-6)
ASA	Ascorbic acid
ATP	Adenosine triphosphate
BACT	β-actin
BHIB	Brain Heart Infusion broth
BHT	Butylated hydroxytoluene
BP	Base pairs
CAT	Catalase
cDNA	Complementary DNA
COX-2	Cyclooxygenase 2
CT	Cycle threshold
DEPC	Diethyl pyrocarbonate
DHA	Docosahexaenoic acid (22:6n-3)
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DPA	Docosapentaenoic acid (22:5n-3)
Dph	Days post hatching
DW	Dry weight
EF1a	Elongation factor 1α
EPA	Eicosapentaenoic acid (20:5n-3)
FAMES	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FCR	Food conversion
FeCAT	Monofunctional catalase
FFBE	Formalin fixed paraffin-embedded tissues
FIED	Flame ionization detector
FI	Feed intake
GC-MS	Gas chromatography–mass spectrometer
GnRH	Gonadotropin Releasing Hormone

GOI	Gene of interest
GPX	Glutathione peroxidase
GR	Glutathione redactase
GSH	Reduced glutathione
GSSG	Glutathione disulfide
GULO	L-Gulono-g-lactone oxidase
H&E	Haematoxylin and eosin
HK	Housekeeping
HPLC	High performance chromatography
HRP	Horseradish peroxidase
HSI	Hepatosomatic index
H_2O_2	Hydrogen peroxide
ICCM	Instituto Canario de Ciencias Marinas
IFAPA	Instituto de Investigación y Formación Agraria y Pesquera
IMIDA	Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario
IRTA	Instituto de Investigación y Tecnología Agroalimentarias
K	Condition factor
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low-density lipoprotein
LIMIA	Laboratorio de Investigaciones Marinas y Acuicultura
MDA	Malondialdehyde
Mn	Manganese
MnCAT	Manganese catalase
MnSOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
n-3	Omega-3
NADH	Nicotinamide adenine dinucleotide
NRC	National Research Council
NTC	Non template control
OH	Hydroxyl radical
O^-_2	Superoxide anion
O_2	Oxygen
PCR	Polymerase chain reaction

PIn	Peroxidation index
PLANCOR	Plan Nacional de la cría de la corvina
PUFA	Polyunsaturated fatty acid
RNA	Messenger ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Quantitative time real time PCR
SD	Standard deviation
Se	Selenium
SGR	Specific growth rate
SOD	Superoxide dismutase
TBARS	Thiobarbituric reactive substances
TBE	Tris-borate-EDTA
TL	Total length
Tm	Melting temperature
TNFα	Tumour necrosis factor alpha
TUB	Tubulin
ULPGC	Universidad de Las Palmas de Gran Canaria
UPV	Universidad Politécnica de Valencia
UV	Ultraviolet
VKD	Vitamin K-dependent proteins
VSI	Viscerosomatic index
ZN	Ziehl-Neelsen
Zn	Zinc
α-TOH	α-tocopherol

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Glossary index of common and scientific fish names (Source: Fishbase)

Common name	Nombre común	Scientific name
Amur sturgeon	Esturión	*Acipenser schrenckii*
Asian sea bass	Lubina de Asia	*Lates calcarifer*
Asian stinging catfish	Pez gato de aguijón	*Heteropneustes fossilis*
Atlantic cod	Bacalao	*Gadus morhua L.*
Atlantic halibut	Halibut	*Hippoglossus hippoglossus*
Atlantic salmon	Salmón Atlántico	*Salmo salar*
Blue tilapia	Tilapia azul	*Oreochromis aureus*
Brown shrimp	Quisquilla	*Crangon crangon*
Channel catfish	Pez gato	*Ictalurus punctatus*
Common carp	Carpa común	*Cyprinus carpio*
Common dentex	Dentón Común	*Dentex dentex*
Dover sole	Lenguado	*Solea solea*
European sea bass	Lubina	*Dicentrarchus labrax*
Gibel carp	Pez rojo	*Carassius auratus gibelio*
Gilthead sea bream	Dorada	*Sparus aurata*
Golden shiners	Carpita dorada	*Notemigonus crysoleucas*
Greater amberjack	Pez limón	*Seriola dumerili*
Hurta	Hurta	*Pagrus auriga*
Hybrid tilapia	Tilapia híbrida	*O. niloticus* × *O. aureus*
Hybrid striped bass	Lobina de mar	*Morone chrysops x M. saxatilis*
Indian major carp	Rohu	*Labeo rohita*
Major carp	Mrigal	*Cirrhina mrigala*
Meagre	Corvina	*Argyrosomus regius*
Mummichog	Fundulo	*Fundulus heteroclitus*
Neon tetra	Tetra neon	*Hyphessobrycon innesi*
Nile tilapia	Tilapia del Nilo	*Oreochromis niloticus*
Pufferfish	Pez globo	*Takifugu obscurus*
Rainbow trout	Trucha arcoiris	*Oncorhynchus mykiss*
Red drum	Corvina roja	*Sciaenops ocellatus*

Red sea bream	Pargo	*Pagrus major*
Red porgy	Bocinegro	*Pagrus pagrus*
Senegalese sole	Lenguado Senegalés	*Solea senegalensis*
Striped trumpeter	Trompeta rayada	*Latris lineata*
Turbot	Rodaballo	*Scophtalmus maximus*
Walleye	Lucioperca Americana	*Sander vitreus*
Wuchang bream	Carpa de Wuchang	*Megalobrama amblycephala*
Yellow croaker	Verrugato de Manchuria	*Larimichthys polyactis*
Yellow perch	Perca amarilla	*Perca flavescens*

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Chapter 1

General introduction

1.1. Global aquaculture production

Aquaculture comprises a set of activities focussed on breeding and farming of aquatic species (fish, molluscs, crustaceans and plants), covering its biological cycle. It has the objective of concentrating species to feed them, protecting them from predators, controlling diseases, facilitating genetic selection and, therefore, to improve the production. Aquaculture plays a crucial role to erradicate hunger and malnutrition, providing foods rich in protein, essential fatty acids, vitamins and minerals to a broad sector of the population. Indeed, one of the world's greatest challenges to be faced is how to feed more than 9 billion people by 2050 in a context of climate change, economic and financial uncertainty, and growing competition for natural resources. Fisheries captures have not increased significantly since the early 1990´s (90-95 million tons). One of the main reasons for this is the overexploitation of traditional fishing areas and the limitations imposed by the changes in the policy and agreements on international fishing areas, what in turn caused a chronic restriction in marine products supply. Aquaculture has been responsible for the impressive growth in the supply of fish for human consumption from only 7 % in 1974, to 26 % in 1994, 39 % in 2004 and 46 % in 2016 (FAO, 2018). In 2016 the global aquaculture production was higher (110.2 million tons) than the extractive fisheries (92 million ton), with a value of more than 216 million euros (Figure 1.1). This exponential increase in production has been accomplished thanks to the research and innovation initiatives towards optimizing the aquaculture efficiency and productivity, both in small and large-scale systems. These investigations should improve the knowledge about the maintenance of the good health of the farmed animals, the optimization of the feeds and their raw materials, improvements in the management of the aquaculture facilities, as well as for domestication of new species.

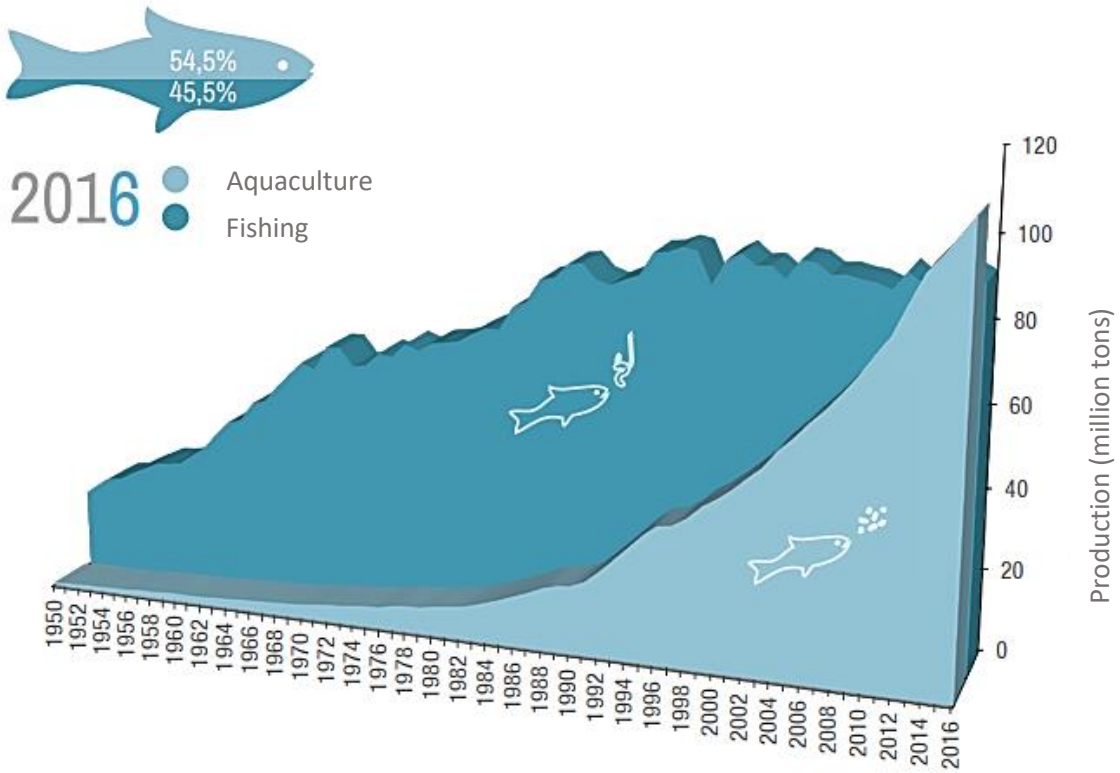

Figure 1.1. Evolution of aquaculture production and global Fishing from 1950 to 2016 (FAO, 2018).

Regarding global aquaculture production, China remains the undisputed leader with 63,721,768 million tons of production in 2016, 4.5 % higher than in 2015, followed by Indonesia (16,616,002 million tons), India (5,703,002 million tons) and Viet Nam (3,634,531million tons). The total aquaculture production in the European Union in 2016 was 1.3 millions of tons, being Spain the main producer with 283,831 tons (22 %), followed by the United Kingdom (194,492 tons, 15 %) and France (166,640 tons, 12.9 %) (APROMAR, 2018). The Spanish aquaculture industry has been mainly focused on gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). However, the production of these species has saturated the market, what has led to a decrease in the price of the final product. The aquaculture sector of sea bream and sea bass has reached its maturity and has forced production regions to look for new alternative species which will cover other market niches.

Meagre (*Argyrosomus regius*) is a species with great potential for the diversification of the Mediterranean aquaculture production. The Mediterranean production of meagre in 2017 was 7,934 tons, being a 17.2 % higher than in 2016. The

main producers in the European Union are Turkey (3,500 tons), Spain (2,298 tons) and Greece (2,200 tons) (APROMAR, 2018). Among the different species produced in Spain, meagre can be found in the eighth place (2,298 tons), being the most produced mussels (*Mytilus sp.*; 215,855 tons), sea bass (23,445 tons) and sea bream (13,740 tons) (Figure 1.2).

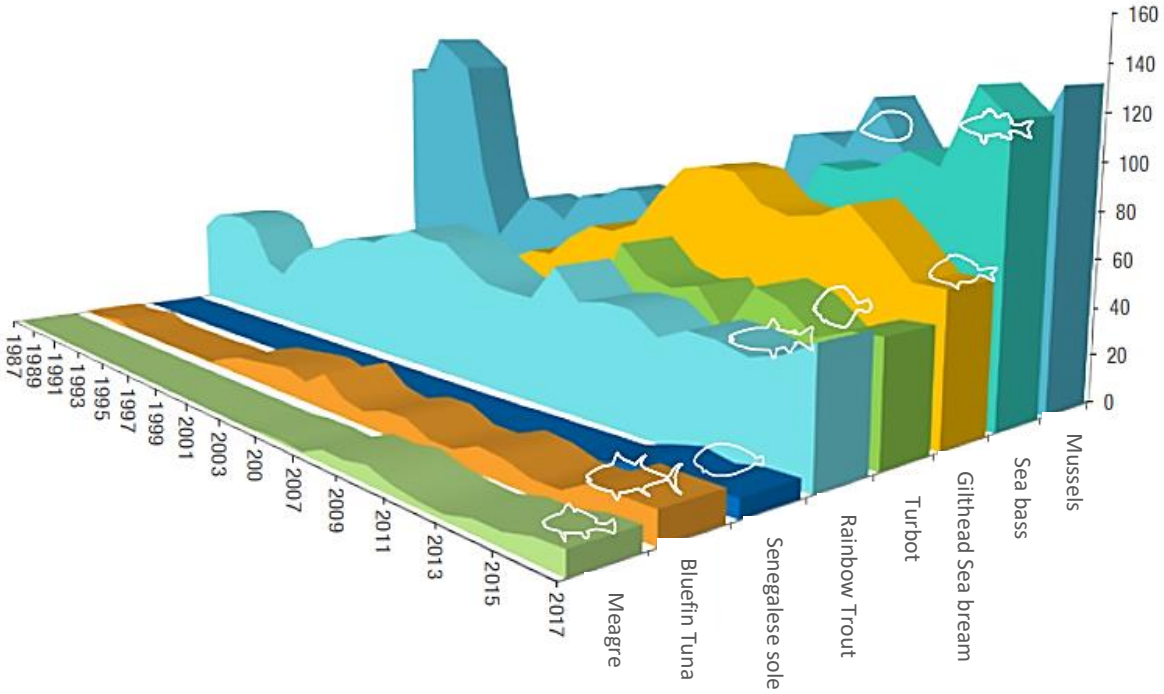

Figure 1.2. Main species farmed in Spain from 1960 to 2017 (APROMAR, 2018).

Meagre is a highly-prized species in some European regions, where it has been traditionally consumed. However, given its reduced fishing volumes and the recent start of its production in aquaculture, it is still poorly known in most markets. Nevertheless, meagre has a great potential for aquaculture diversification due to its high flesh quality and good flavour (Poli et al., 2003), rapid growth and good feed conversion rates (0.9 to 1.2) (Jiménez et al., 2005; Duncan et al., 2013) as well as good growth at a wide range of salinities (5-45 g L^{-1}) (Márquez, 2010). It also provides low-fat flesh even under intensive farming conditions (Piccolo et al., 2008) and has a great capacity to adapt to captivity (El-Shebly et al., 2007).

1.2. Meagre general description

The meagre, *Argyrosomus regius*, is a Teleost species belonging to the family Sciaenidae within the Order Percomorphi (Perciformes), suborder Percoidei (Figure 1.3). This family includes about 70 genera and 270 species distributed in temperate and tropical regions around the world (Nelson, 1994). Meagre is found in the Mediterranean, the Black Sea and along the Atlantic coasts of Europe and the west coast of Africa (Chao, 1986; Haffray et al., 2012).

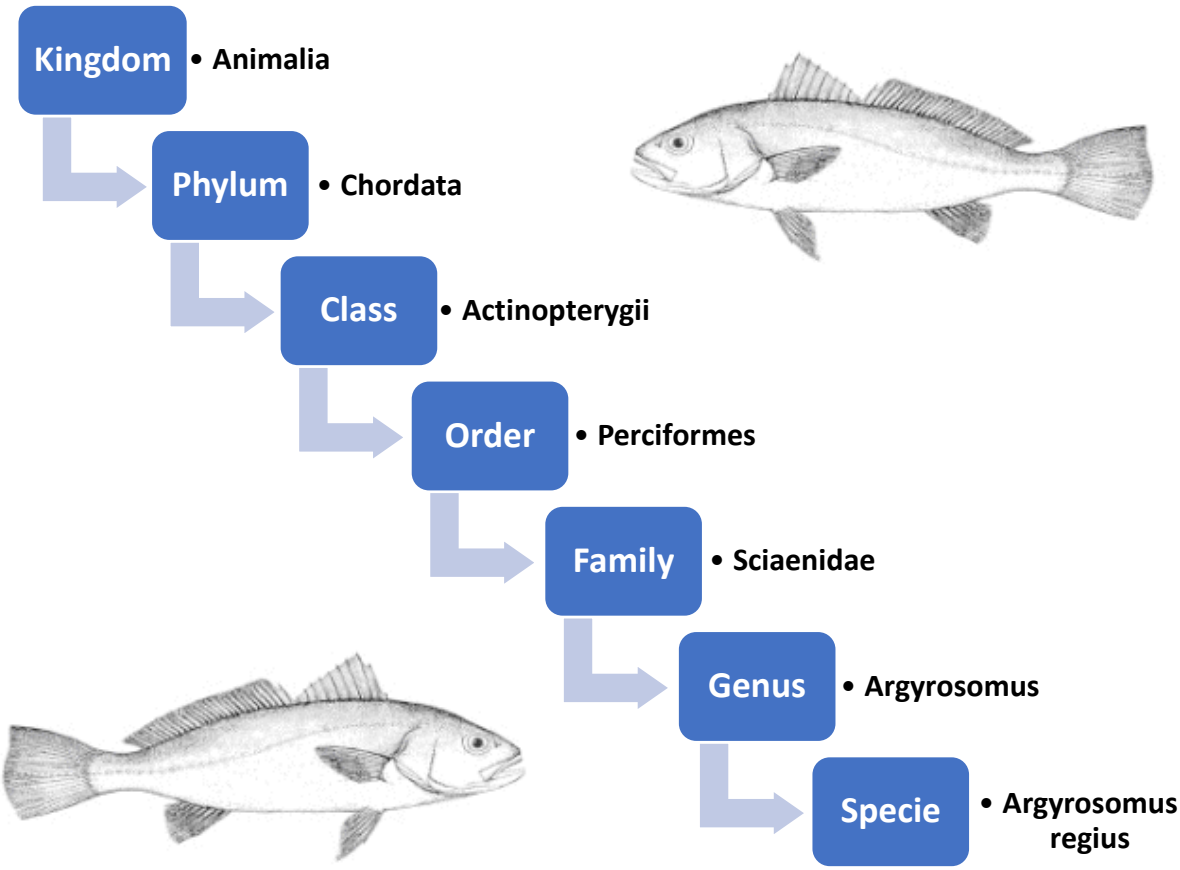

Figure 1.3. *Argyrosomus regius* classification. Data obtained from Fishbase.

Meagre has an elongated and slightly compressed body of grey-silver colour, with a relatively large head and wide but slightly oblique terminal mouth (Cárdenas, 2010; Figure 1.4), similar to sea bass in appearance but higher in size. The cavity of the mouth is golden and has small teeth arranged in several rows. This species is corpulent and very agile (Piccolo et al., 2008, Poli et al., 2001). It has large ctenoides scales throughout the body, except in some parts of the head where they are small and cycloid. The lateral line

is quite visible, due to its intense brightness. The dorsal fin is divided into two parts by a small slit, the front part has 9-10 hard radii and the back 1 hard and 26 soft radii. According to FAO (2005) the meagre can reach a size of 2 meters and weigh up to 50 kg, although the largest ever captured individual was 2.3 meters long and 103 kg in weight (Quero and Vayne, 1987). This species has the biological characteristics required for commercial aquaculture (Jiménez et al., 2005; Piccolo et al., 2008).

Figure 1.4. External morphology of meagre (*Argyrosomus regius*) (Asso, 1801).

1.3. Habitat and distribution

Meagre is distributed from the eastern Atlantic (from the English Channel to Senegal, including the Canary Islands) to the Mediterranean basin, the Black Sea and the Azov Sea (Chao, 1986, Haffray et al., 2012) (Figure 1.5). They are usually found in coastal waters, but can reach depths of up to 200 m. They inhabit rocky bottoms and fields of *Posidonia*. It is a eurythermal and euryhaline species (they resist sudden changes in temperature from 2 to 38 °C and salinity from 5 to 39 g L^{-1}), which allows them to enter the estuaries of rivers, where the offspring remain up to its juvenile state (Catalán, et al., 2006). Once they become juveniles, meagre migrate to deeper waters until they reach sexual maturity (Morales-Nin et al., 2012).

Figure 1.5. Meagre global distribution. Source Fishbase.

1.4. Reproduction

Meagre is an iteroparous species (presents several reproductive cycles during its life), gonochoric (separated sexes), with seasonal reproduction and multiple spawning with an asynchronous development of the oocytes (Grau et al., 2009). This species reaches sexual maturity between 3 and 6 years of age, with a size of about 61 cm long for males and 70-110 cm for females (González-Quirós et al., 2011). The sex ratio varies with the size of the fish, with a tendency for a greater number of females when the range of sizes is large.

From March to August, adults enter the estuaries to reproduce (González-Quirós et al., 2011). During the spawning period, meagre emit two different types of sounds: regular long grunts, which are the most common calls, and short grunts. It is suggested that long grunts serve for the formation of spawning aggregations, while short grunts serve to announce the beginning of courtship (Lagardere and Mariani, 2006). At the end of the summer the juveniles migrate to coastal waters between 20 and 40 m deep, to spend the winter until May, when they return to the estuaries, where they feed (Catalán et al., 2006).

The trophic and reproductive migrations of this species are determined by the temperature of the water. The temperature at which the spawning period begins is 16-17

°C (Quero, 1985). The females have a fertility potential in nature of between 2.1 and 31.1 million oocytes per year. This fertility potential depends exponentially on the size of the females (Gil et al., 2013). Reproduction in captivity remains as a bottle neck, because only few females mature spawn spontaneously, being necessary the use of injections with gonadotrophin hormone (GnRH) to ensure the spawning (Mylonas et al. 2013a).

1.5. Feeding

Meagre is a carnivorous species that feeds mainly on polychaetes, crustaceans, echinoderms and molluscs, as well as other smaller fish species such as clupeids ormugilids (Jiménez et al., 2005). Meagre larvae need temperatures higher than 20-21 ºC in order to start feeding. The juveniles (1 year old) feed on small demersal fish and crustaceans, mainly *Mysidacea* and brown shrimp (*Crangon crangon*) (Cabral and Ohmert 2001). When they reach a length of 30-40 cm, they begin to feed on largest preys such as pelagic fish, decapods and cephalopods (Monfort, 2010).

Nutritional requirements for meagre are still not well known. There is not available commercial feeds for this species, being diets formulated for sea bream and sea bass often used (Duncan et al., 2013; Parisi et al., 2014). Meagre needs a protein content in the extruded diets of approximately 45-50 %, similarly to sea bream or sea bass, but the lipid requirements seem to be lower, approximately 17 % (Chatzifotis et al., 2010, 2012). Normally, meagre is fed at a feeding rate of 1-1.5 % of their biomass, 2-3 times per day (FAO, 2005; Bajandas et al., 2009).

1.6. Meagre culture challenges

In recent years, meagre and other species such as greater amberjack (*Seriola dumerili*) or Atlantic halibut (*Hippoglossus hippoglossus*) have been identified as new candidate species for the diversification of the Mediterranean aquaculture. These species were selected based on their biological and economical potential. Nevertheless, several bottlenecks have been identified in the hatchery, on growing and reproduction of this species.

1.6.1. Hatchery

Initially, the hatchery protocols for meagre were adapted from other fish species. Nevertheless, nowadays there are specific protocols for this species, finding survival rates between 15-40 % at 30 days post hatching (dph) and 15 % at 60 dph (PLANACOR 2009). The best observed survival in meagre larvae has been obtained by Roo et al. (2010) with survival rates of 36-53 % at 30 dph using a feeding sequence consisting of phytoplankton (*Nannochloropsis* sp.) and enriched rotifers (*Brachionus* sp.) between days 2-15, instar I (*Artemia* sp.) between days 12-15, enriched instar II (*Artemia* sp.) between days 14-30 and microdiet from 20 to 30 dph (Figure 1.6). In addition, it was observed that the stocking density also affected growth and survival, obtaining the best results at a low stock density (50 larvae L^{-1}).

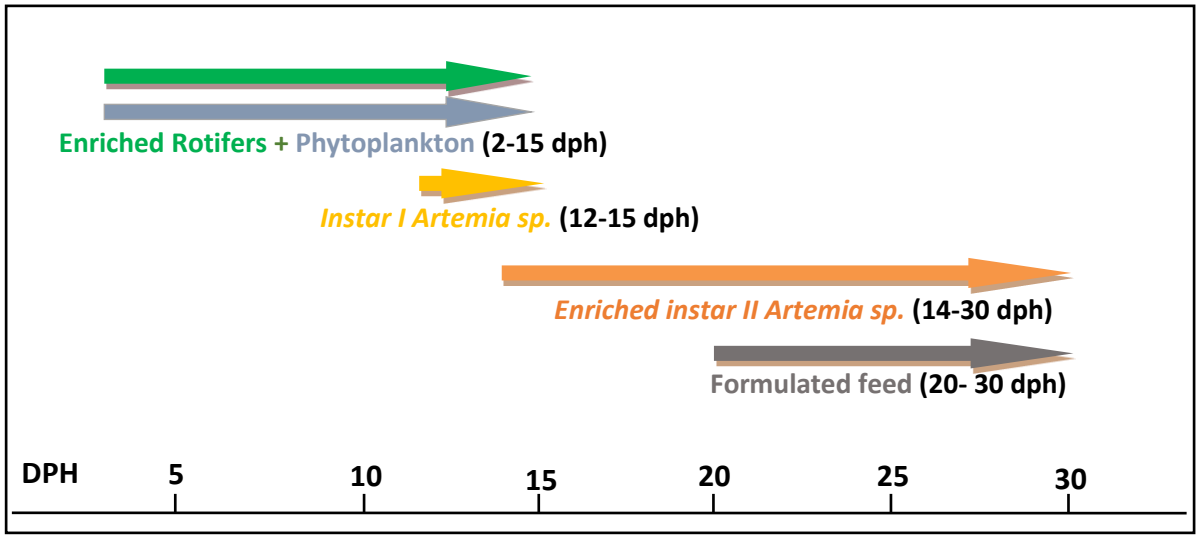

Figure 1.6. Meagre larvae feeding sequence from 2 to 30 days post hatching (dph). Adapted from Roo et al. (2010).

One of the more important causes of mortality at the initial life stages of meagre is the cannibalism, increasing drastically after 21 dph with the inclusion of artificial feed. Cannibalism has already been described for other species (Paller and Lewis 1987; Folkvord, 1991), observing two types of cannibalism (Hecht and Appelbaum, 1988; Van Damme et al., 1989; Baras, 1999; Hatziathanasiou et al., 2002). Firstly, due to the difference in size, the largest larvae attack the smallest ones biting their tail, producing

damage in the fin and finally dying, and secondly, the dominant larvae will ingest the dead larvae starting from the head.

The culture of meagre larvae is typically performed in tanks of 250 and 1,000 L volume. At a temperature of 22-24 °C, the exogenous feeding is expected to start at 3 dph (Gamsliz and Neke, 2008; Jiménez et al. 2007). Variable photoperiod is preferred during the hatchery phase as the use of long photoperiods, despite of leading to greater growth, can also cause high stress and an increase in the cannibalistic behaviour (Valles et al., 2009). A specific growth rate (SGR) of 19 % per day has been previously described in meagre larvae (Rodríguez-Rúa et al., 2007), growing to twice the size of *Pagrus auriga* larvae in the same time period (Lozano et al., 2004). It must be noted that *Pagrus auriga* has a higher growth than gilthead sea bream and red porgy (*Pagrus pagrus*).

1.6.2. On-growing

Nowadays, there is not any specific commercial diet for meagre larvae or on-growing juveniles. Moreover, most of the nutritional requirements for this species are not known, what restricts the fast-growing potential of this species. Indeed, the use of non-specific diets for meagre is related with poor growth and economic losses.

The minimum protein requirements for meagre is around 50 % (Martínez-Llorens et al., 2011, Chatzifotis et al., 2012) being higher than those for other sciaenids (McGoogan and Gatlin, 1999; Lee et al., 2002, Turano et al., 2002, Pirozzi et al., 2010), while the lipid requirement is lower than that of other carnivorous species (approximately 17 %). The increase of fat content from 17 % to 21 % resulted in higher fat accumulation in the whole body and muscle and a poor growth performance (Chatzifotis et al., 2010, Martínez-Llorens et al., 2011). Limited information is available about the requirements of vitamins in meagre. El Kertaoui et al. (2017) observed that high dietary levels of α–tocopherol (α-TOH) and ascorbic acid (ASA; 1,500 and 1,800 mg kg^{-1}, respectively) improved growth and protection against oxidative stress in meagre larvae, suggesting that the optimal requirements of these vitamins could be higher. The deficiency of these vitamins and micronutrients can lead to metabolic disorders and diseases through their negative influence on the fish physiology (Percival, 1995; Watanabe et al., 1997; Lin and Shiau, 2005). Moreover, the omega 3 (n-3) long chain polyunsaturated fatty acid (LC-

PUFA) requirements in meagre have been estimated to be, at least, 2 % of dry matter of the diet (Carvalho et al., 2019). In meagre juveniles it has been suggested that a supplementation with 451 mg kg^{-1} of dietary α-TOH is necessary in order to provide good growth performance and improve fish quality and storage stability (Rodriguez-Lozano et al., 2017). Nevertheless, a deficiency or excess may deteriorate fish health.

The pre-growing and on-growing of meagre can be carried out in circular or rectangular tanks or in marine cages. Temperature seems to be a more important factor in growth, than stocking density.The optimum feeding rate for larvae of this species is between 2. and 5 % of their biomass per day, with higher feeding rates leading to poor food conversion ratio (FCR) (Bajandas et al., 2009). On the other hand, with a feed rate of 0.5 %, fish barely grew, what in turn indicated that this value is close to the maintenance rate (Velazco et al., 2009). In the on-growing phase, the optimal feeding rate is over 1 % (Rodríguez-Rua et al., 2009). In order to reduce production costs and optimize the feed supplied during the on-growing phase, García-Mesa et al. (2009) conducted an experiment on weekly feeding frequencies, subjecting meagre of about 170 g of initial weight, to periods without feeding of 0, 1 or 3 days per week. The best results of growth and body composition were obtained by feeding 6 days a week and leaving 1 fasting day. As previously indicated, meagre is an euryhaline species and it has been observed that at low salinities (12-13 g L^{-1}), close to the isosmotic equilibrium, better SGR and FCR are obtained (Tinoco 2009a, b, c).

1.6.3. Reproduction

Meagre has reproductive dysfunction in captivity, being necessary the use of gonadotrophin hormones to ensure the spawning. From 2005 to 2008 the National Plan for the Breeding of the "Corvina" (PLANACOR) of the JACUMAR project was developed, carried out by six I+D centres (IFAPA, LIMIA, ICCM, IRTA, IMIDA and UPV), whose principal objective was the experimental production of meagre larvae. Within this project, the first spawnings were obtained in 2006 (Grau et al., 2007, Duncan et al., 2007a, b). Fecundities of 498,141 eggs kg^{-1} of female, 59 million fertilized eggs, 36 million larvae and survivals ranging from 10 to 63% after 30 dph were obtained, inducing spawning by GnRH (Cárdenas, 2007).

Based on these results, different studies have been carried out in order to develop protocols for the effective and efficient hormonal spawning induction and to enhance the fecundity and larval survival, resulting in a higher fecundity and quality of the eggs (Fernández-Palacios et al., 2009a; Mylonas et al., 2011; Duncan et al., 2012). Some other studies have been performed within the DIVERSIFY project from 2013 to 2018, with the objective to develop *in vitro* fertilization protocols and to study the genetic variability of commercial broodstock, as well as the genetic basis of growth in meagre (Mylonas et al., 2013b, 2016, 2017). Mylonas et al. (2013a) observed that a hormonal spawning induction with GnRHa (gonadotropin-releasing hormone agonist), resulted in a larval survival of 73 % of the total number of eggs spawned, demonstrating that it is an effective and efficient method, leading to a high fecundity and quality of the eggs.

1.6.4. Health on meagre culture

Health is one of the most important aspects in fish farming, and this is not different in the intensive culture of meagre. Infectious diseases caused by trematodes (*Calceostoma* spp. and *Sciaenocotyle* spp.) (Duncan et al., 2008; Ternengo et al., 2010), nematodes (*Philometra* sp.) (Moravec et al., 2007), dinoflagellate (*Amyloodinium ocellatum*) (Soares et al., 2012), monogenean (*Sciaenocotyle pancerii*, *Microcotyle pancerii*, *Benedenia sciaenae* and *Diplectanum sciaenae*) (Toksen et al., 2007; Merella et al., 2009; Quilichini et al., 2009; Ternengo et al., 2010; Andree et al., 2015) and bacteria (*Vibrio tapetis*, *Photobacterium damselae* subsp. *piscicida*, *Nocardia* spp.) (Bottari et al., 2009; Labella et al., 2010; Cárdenas, 2011; Elkesh et al., 2013) have all been described in meagre. Furthermore, most farmed populations are known to be affected by systemic granulomatosis, which is the pathology with the largest impact on meagre culture (Ghittino et al., 2004). Indeed, systemic granulomatosis has a chronic course, causing low mortality but with a high prevalence and intensity, which makes this disease one of the causes that impede the expansion of production. Although systemic granulomatosis is a disease of unknown etiology, two possible causes are thought to be related to the appearance of the disease: nutrients deficiency/imbalances or infectious agents.

On the one hand, systemic granulomatosis was first described in gilthead sea bream (Paperna et al., 1980) and later in turbot (*Scophthalmus maximus*) (Coustans et al., 1990). The cause of this disease has been associated with a nutritional imbalance of

vitamins (ASA and B-complex vitamins) (Ghittino et al., 2004). Systemic granulomatosis is characterized by the presence of multiple granulomas in internal organs, which progressively produces a necrotic centre surrounded by a layer of epithelial cells and macrophages (Figure 1.7). This disease mostly affects the kidney and liver, where macroscopic nodules of varying diameter are usually observed. In later stages, these nodules can also appear in other tissues such as spleen, heart, skin and eyes what can lead to exophthalmia and cataracts (Ghittino et al., 2004).

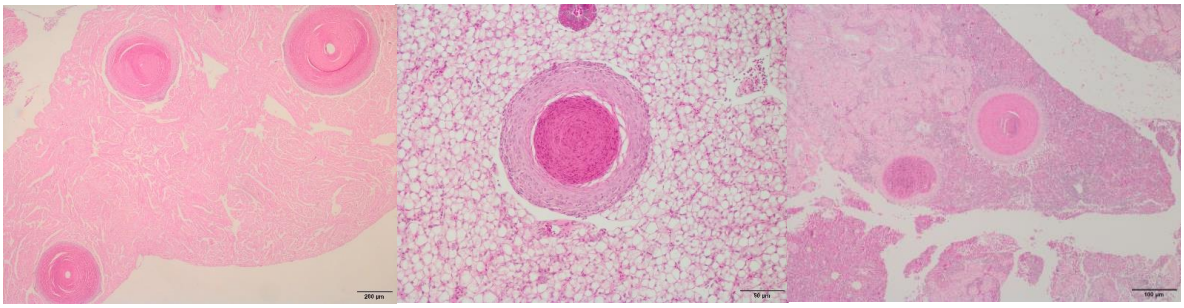

Figure 1.7. Developed granulomas in meagre A) heart, B) liver and C) kidney.

On the other hand, granulomas can be also produced by pathogens such as *Mycobacterium* spp. and *Nocardia* spp. (Bowser, 2009; Labrie et al., 2008). Mycobacteriosis is a chronic systemic granulomatous disease that affects different species of marine fish (Decostere et al., 2004). In fish it is mainly caused by three pathogenic species: *Mycobacterium marinum*, *Mycobacterium fortuitum* and *Mycobacterium chelonae* (Frerichs, 1993). These are immobile, Gram-positive, aerobic and acid-resistant bacteria (Kato-Meda et al., 2011). External clinical signs are nonspecific and include loss of scales and skin ulceration, pigmentary changes, abnormal behavior, emaciation and ascites (Gauthier and Rhodes, 2009). Internal signs of infection include thickening of the spleen, kidney and liver, and the appearance of white-grey granulomas in internal organs (Frerichs, 1993). Visceral granulomas are usually composed of a thick capsule of epithelial cells that surround necrotic centers, where a large number of acid-fast bacilli are concentrated (Toranzo et al., 2005). These bacteria are able to prevent degradation by phagosomes by producing superoxide dismutase (SOD) that removes oxygen radicals. In addition, they can survive inside of macrophages (Kato-Meda et al., 2010).

Nocardiosis is a disease of marine and fresh water fish caused by actinomycetes of the genus Nocardia (Woo and Bruno, 2011). The first case of nocardiosis reported in fish was caused by *Nocardia asteroides* in neon tetra (*Hyphessobrycon innesi*) (Valdez and Conroy, 1963). In 2013, Elkesh et al. described for first time the presence of Nocardia in a Mediterranean population of farmed meagre in Crete (Greece). The infection by nocardia can become misinterpreted and confused with mycobacteriosis, since both produce similar clinical signs and general pathology. Nocardiosis is a disease caused by Gram-positive, aerobic and filamentous bacteria (Brown-Elliott et al., 2006). Currently there are only three pathogens, belonging to the genus Nocardia, which can cause disease in fish: *Nocardia asteroides*, *Nocardia seriolae* and *Nocardia salmonicida* (Elkesh et al., 2013). It is a systemic disease that produces lesions on the skin and internal organs, forming the typical structure of granulomas (Wang et al., 2005).

Given that several infectious agents can course with granulomatosis, it is necessary to carry out a series of tests to discard a posible infectious origin, such as microbiological (Rhodes et al., 2004; Pourahmad et al., 2009), histological, immunohistochemical (Chinabut, 1999; Noga, 2000) and molecular tests (Puttinaowarat et al., 2002; Roth et al., 2003) for *Mycobacterium* sp. or in the case of *Nocardia* sp. microbiological (Chen et al., 2000; Elkesh et al., 2013), histological, immunohistochemical (Labrie et al., 2008) and molecular tests would also be necessary (Kono et al., 2001; Itano et al., 2006).

1.6.5. Elucidate the origin of the systemic granulomatosis in meagre

Limited information is avaliable about the possible origin of the granulomas, nevertheless the inability to determine an infectious origin and the relation observed between the appearance of the systemic granulomatosis and the feeding sequence in meagre larvae reinforce the hypothesis of a nutritional origin of the disease (Cotou et al., 2016). Visceral granulomatosis has been observed in turbot fed diets deficient in ASA (Messager et al., 1986). Also in turbot, Coustans et al. (1990) confirmed the role of a deficiency of ASA in the appearance of visceral granulomatosis and showed that the pathological condition was exacerbated by B - complex hypovitaminosis. In sea bream, the deficiency of ASA has also been responsible of the appearance of granulomatosis in kidney (Alexis et al., 1997). ASA, is also involved in α-TOH metabolism, reducing α-

tocopheroxyl radicals and regenerating them back to α-TOH (Niki et al., 1985). Consequently, the optimum dietary α-TOH levels are influenced by the levels of ASA (Hamre et al., 1997; Chen et al., 2004; Atalah et al., 2010). The imbalance between prooxidants and antioxidants can lead to a status of oxidative stress (Rando, 2002), and lead to different diseases (Cowey et al., 1984; Lall and Lewis-McCrea, 2007; Betancor et al., 2012) such as the systemic granulomatosis.

1.6.5.1. Oxidative stress

In this sense, oxidative stress is caused by the presence of reactive oxygen species (ROS), which are continuously produced as products of various metabolic pathways in all organisms. Aerobic multicellular organisms require oxygen (O_2) which is susceptible to radical formation due to its electronic structure. At low concentrations, ROS may be beneficial or even indispensable in processes such as defence against micro-organisms, contributing to phagocytic bactericidal activity. However, when an imbalance between ROS generation and ROS removal occurs, a state of oxidative stress arises (Nita, et al., 2016). This status may lead to the oxidation of various cellular constituents like lipids, proteins or DNA, causing alterations that produce a range of cellular damages which can ultimately lead to cell death (Halliwell and Gutteridge, 1995). Fish and, specially, fish larvae, are highly at risk of suffering peroxidative attack to their membranes, as they contain great quantities of LC-PUFA, highly susceptible to oxidation. Aerobic organisms have integrated antioxidant systems, which include enzymatic and non-enzymatic antioxidants that are usually effective in blocking harmful effects of ROS.

ROS are highly reactive compound derived from oxygen. The three major ROS that are of physiological significance are superoxide anion ($O^{-}_{2}.$), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2). Oxygen is the terminal electron acceptor in mitochondrial respiration, being ultimately reduced to water during the oxidative phosphorilation, the major source of Adenosine triphosphate (ATP) in aerobes. Oxygen can be converted to other reactive ROS forms either by energy transfer or by electron transfer reaction, resulting in the formation of singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radical (Klotz, 2002). Superoxide anions are generated by the addition of one electron to the molecular oxygen (Michelson et al., 1977; Miller et al., 1990). Other enzymes that can produce superoxide include xanthine oxidase (Kuppusamy and Zwejer, 1989),

lipoxygenase and cyclooxygenase (Kontos et al., 1985; Mcintyre et al., 1999). Both O^-_2 and H_2O_2 are moderately reactive, whereas hydroxyl radical is the most reactive and dangerous free radical (Bedwell et al., 1989), that can cause significant biological damage reaction with molecules, including DNA, proteins, lipids and carbohydrates and can cause more damage to the cells than any other ROS can do (Halliwell, 1987). Hydroxyl radical can be generated in the mitochondria by the Fenton reaction, in which H_2O_2 reacts with metal ions (Fe^{+2} or Cu), often bound in complex with different proteins molecules (Fenton 1894). ROS are constantly produced in different cells organelles, such as mitochondria (Zorov et al., 2014), endoplasmic reticulum (Gross et al., 2006), peroxisomes (Río and López-Huertas, 2016), cytosol (Kukreja et al., 1986), plasma membrane (Crane and Low, 2008) and extracellular space (McNally, 2003). One of the major sources of molecular oxygen is the electron transport chain, that occurs in the mitochondria and endoplasmic reticulum (Salvador et al., 2001; Turrens, 2003; Andreyev et al., 2005; Balaban et al., 2005).

ROS action can be inhibited or delayed by the effect of the antioxidants, which avoid the initiation or propagation of oxidizing chain reactions (Figure 1.8) (Pokorny and Korczak, 2001). Antioxidants can react with a single free radical and neutralize it by donating one of their own electrons, ending the carbon-stealing reaction (Halliwell and Gutteridge, 1990). To prevent cell and tissue damage, there are a variety of antioxidant components that can act against free radical to neutralize them (Yu, 1994), being classified into two categories according to their origin: endogenous and exogenous (Jacob, 1995). The endogenous or enzymatic antioxidants mainly observed in fish are superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR) and catalase (CAT) (Rudneva, 1997). The exogenous or non-enzymatic antioxidants cannot be synthesized in the body and need to be supplied through dietary intake. Among them are ASA, α-TOH and carotenoids (McCall and Frei, 1999).

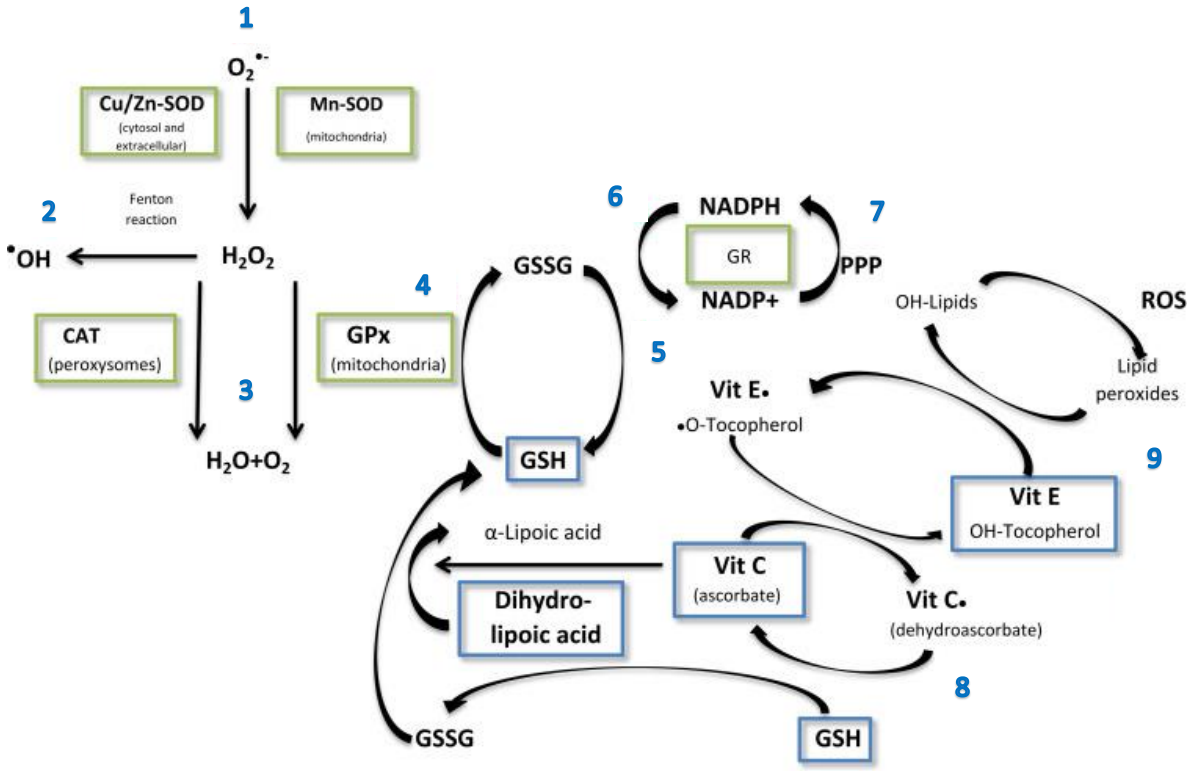

Figure 1.8. Antioxidant protection mechanism (Adapted from Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013). 1) Superoxide radical (O^{-}_2) is catalysed by superoxide dismutase, producing the removal of O^{-}_2 by dismutating it into O_2 and hydrogen peroxide (H_2O_2). 2) Hydroxyl radicals could be generated in mitochondria by the Fenton reaction, in which H_2O_2 reacts with metal ions (Fe^{+2} or Cu), often bound in complex with different protein molecules. 3) Catalase (CAT) participates in the dismutation reaction of hydrogen peroxide to oxygen and water. Glutathione peroxidase (GPX) metabolizes H_2O_2 to oxygen and water. 4) Reduced glutathione reacts with free radicals of proteins or other macromolecules, restoring them to the reduced form. 5) Hydrogen peroxide is subsequently metabolized to oxygen and water by the selenium-containing enzyme GPX, which uses glutathione (GSH) as a cofactor in the reaction. 6) Glutathione reductase (GR) can regenerate glutathione from the oxidized glutathione using NADPH as a reducing agent. 7) NADPH is generated in the first oxidative phase of the pentose phosphate pathway, in which two molecules of NADP+ are reduced to NADPH. 8) Vitamin C has a role scavenging ROS by oxidating itself. Ascorbic radical and dehydroascorbic radical are the oxidized products of vitamin C and are regenerated by glutathione, NADH or NADPH. Vitamin C can also reduce the oxidized forms of glutathione and vitamin E. 9) Vitamin E interacts and scavenges the lipid hydroperoxides. Vitamin C and E participate in the regeneration of glutathione by interaction with α-lipoic acid.

1.6.5.2. Enzymatic antioxidants

SOD, GPX and CAT are antioxidant enzymes that prevent the cascade of oxidant reactions, intercepting and inactivating the reactive intermediates of oxygen. Superoxide dismutase catalyzes the removal of O^{-}_{2} by dismutating it into O_2 and H_2O_2 (Figure 1.9). This removes the possibility of $OH^{\bullet}$ formation by the Haber-Weiss reaction. SOD can be found in living organisms in three isoforms, based on the metal ion it binds to: Mn-SOD (in mitochondria), Fe-SOD (in chloroplasts) and Cu/Zn-SOD (in cytosol, peroxisomes and chloroplasts) (Mittler, 2002). These three isoenzymes differ in their sensitivity to H_2O_2 and potassium cyanide (Bannister et al., 1987). SOD has been found to be up-regulated by abiotic stress conditions (Boguszewska et al., 2010).

Figure 1.9. O^{-}_{2} removal by superoxide dismutase (SOD) into O_2 and H_2O_2.

Catalase is a tetrameric heme-containing enzyme and can be classified into three types of enzymes: monofunctional catalases (FeCAT), biofunctional catalases (Catalases-peroxidases) and manganese catalases (MnCAT) (Loewen, 1997). The CAT structure consists of looped polypeptide chains arranged in form of dimers or tetramers with a core bearing an iron or a manganese ion (Vainshtein et al., 1981). Catalase participates in the dismutation reaction of hydrogen peroxide (a harmful compound for the living cells) to oxygen and water (Figure 1.10). This enzyme is present in the peroxisomes, which are the hotspot of H_2O_2 production due to β-oxidation of fatty acids, photorespiration, purine catabolism and oxidative stress, although it has been also found in cytosol, chloroplast and mitochondria (Mhamdi et al., 2010).

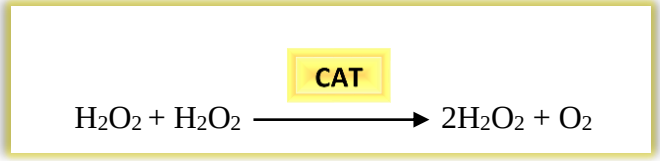

Figure 1.10. Dismutation reaction of H_2O_2 to O_2 and H_2O_2 by catalase (CAT).

Glutathione peroxidases are a family of tetrameric enzymes that contain the unique amino acid selenocysteine within the active sites and use low-molecular-weight thiols, such as glutathione, to metabolize H_2O_2 to oxygen and water and reduce lipid peroxides to their corresponding alcohols (Figure 1.11) (Flohé, 1988). Eight GPX have been described: GPX1 (cytosolic) is ubiquitous and reduces H_2O_2 and fatty acid peroxides (Arthur, 2000). GPX2 (gastrointestinal) is localized in gastrointestinal epithelial cells where it reduces dietary peroxides (Chu et al., 1993). GPX3 (plasma) resides in the extracellular compartment. GPX 4 (phospholipid hydroperoxidase) reduces esterified lipids and can use several different low-molecular-weight thiols as reducing equivalents. GPX1-4 and GPX6 are selenoproteins with selenocysteine in their catalytic center, whereas GPX5, GPX7 and GPX8 contain cysteine instead of selenocysteine in the active center (Matoušková et al., 2018). Finally, glutathione reductase (GR) can regenerate glutathione from the oxidized glutathione using NADPH as a reducing agent (Marcel, 2013).

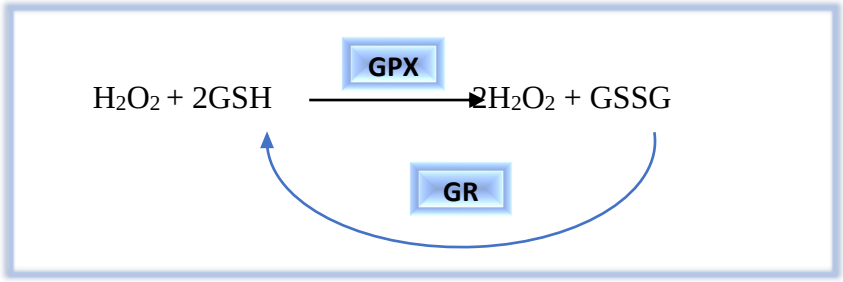

Figure 1.11. Reduction of H_2O_2 by glutathione peroxidase (GPX). Glutathione reductase (GR) can regenerate glutathione from the oxidized glutathione (Glutathione disulphide; GSSG) using NADPH as a reducing agent.

The antioxidant status in fish can accurately reflect the activity of SOD, GPX and CAT (Cheng et al., 2017). In this sense, SOD, GPX and CAT activity has been used as a biomarker of the effect of stressful factors in fish. In this sense, *Heteropneustes fossilis* exposed to fluoride-induced toxicity showed significantly increased levels of lipid peroxidation as well as enhanced CAT, SOD and GPX activity in the tissues (Yadav et al., 2015). Similarly, catfish (*Ictalurus punctatus*) exposed to liquid crystal doses displayed significantly increased activities of CAT, SOD and GPX (An et al., 2008). It

has also been described, that the activity of these antioxidant enzymes can be affected by the dietary contents of ASA and α-TOH (Monget et al., 1996; Kiron et al., 2004; Mahmoud et al., 2016). On the other hand, the enzymatic activity of CAT, GPX and SOD rises, whereas, the level of low molecular weight antioxidants falls along with larval development in larvae of several fish species, (Rudneva, 1999; Zhang et al., 2009). For instance, both the activity of GPX and CAT increased in unfed *Dentex dentex* larvae at 9 dph along with decreased contents in α-TOH (Mourente et al., 1999).

1.6.5.3. Non-enzymatic antioxidant

1.6.5.3.1. Ascorbic acid

Ascorbic acid (ASA, vitamin C) is a white, odourless, crystalline compound and water-soluble ketolactone with two ionizable hydroxyl groups. ASA is a neutrally charged molecule which can be protonated and become ascorbate. Ascorbic acid can lose the hydrogen ions attached to one of its two ionizable groups depending on the pH of the medium, generating ascorbate monoanion or dianion (Tolbert et al., 1975; Markarian and Sargsyan, 2011; Du et al., 2012). The oxidation of ascorbic acid depends on the pH and can be accelerated by catalytic metals (Buettner and Jurkiewicz, 1996). ASA oxidation is quite low at pH 7.0 (Buettner, 1988), but the increase of pH rapidly increases the autoxidation rate and vitamin activity is lost. L-Gulono-g-lactone oxidase (GULO) is a critical enzyme present in most mammalian species that catalyses the last step in ASA biosynthesis (Gabbay, et al., 2010). Teleost fish cannot synthesize ascorbate due to the lack of GULO (Ching et al., 2015) and this vitamin must therefore be provided by the diet. However, ASA is quite labile and vulnerable to degradation in fish diets and can be destroyed by multiples factors such as contact with oxygen, chlorate water, heat or light. After pelleting and storage, it is estimated that approximately 25-50 % of ASA activity is lost (Harper, 1988; Gadient and Fenster, 1994), being necessary to add bigger amounts to ensure that the requirements of the fish are covered.

ASA is a powerful antioxidant and scavenger of free radicals in biological systems (Rose and Bode, 1993; Duarte and Lunec, 2005). Ascorbate can donate an electron to potentially damaging oxidizing radicals, such as hydroxyl radical, peroxyl radical or thiol radical (Buettner, 1993; Padayatty et al., 2003; Mahfouz and Kummerow, 2004). The

product of the one-electron donation of ascorbate is dehydroascorbate and the ascorbate radical, which is relatively unreactive, less than the α-tocopherol radical or the glutathione radical. Ascorbate can be recovered by NADH and NADPH-dependent reductases (Linster and Schaftingen, 2007). ASA also acts as a defence against membrane oxidation (Retsky et al., 1999), and has been shown to protect against low-density lipoprotein (LDL) oxidation induced by macrophages, endothelial cells and copper ions (Lynch et al., 1994; Niki and Noguchi, 1997). The antioxidant effect of ASA has been observed is several fish species, such as in Wuchang bream juvenile (*Megalobrama amblycephala*) (Wan et al., 2014; Liu et al., 2016b), juvenile Pufferfish (*Takifugu obscurus*) (Cheng et al., 2017), *Oreochromis niloticus* (El-Sayed et al., 2016), red sea bream (*Pagrus major*) (Mahmoud et al., 2016) and sea bass (Betancor et al., 2012), in which the supplementation of ASA in the diet enhanced the activity and/or expression of SOD, GPX and CAT.

The requirement of ASA given by the National Research Council (NRC, 2011) for juvenile fish is 30 mg kg^{-1}. However, when stress and immune responses are used to measure requirements of this vitamin, higher estimations of dietary vitamin are most often found, also in juvenile and adult fish (Hamre, 2011). The requirements of this vitamin appear to be much higher during the hatchery phase than post-larval phase, probably due to the higher metabolism rate (Matusiewicz et al., 1994). The requirements of ASA in Asian sea bass (*Lates calcarifer*) has been stablished in 30 mg kg^{-1} to improve survival and growth (Phromkunthong et al., 1997). For larvae of other teleost species such as turbot and sea bass lower levels have been estimated for normal growth and post-larval survival (20 mg kg^{-1}; Merchie et al., 1997). In common carp (*Cyprinus carpio*) a higher dose (45 mg kg^{-1}) is necessary for maximum growth (Guillou-Coustans et al., 1998), being all these levels in agreement with the NRC recommendations. Deficient dietary levels of ASA can cause a reduction in growth as well as alteration of the immune response, increasing the appearance of malformations or incidence of parasitosis (Martins, 1995; Martins, 1998; Chagas, 2006; Zhou et al., 2012). Chen et al., (2003) found that the minimum dietary ASA level of 19.5 mg kg^{-1} was necessary to prevent deformities in golden shiners (*Notemigonus crysoleucas*), whereas 40.3 mg kg^{-1} diet was necessary to maximize survival rate. According to these studies, low dietary levels of ASA are necessary to ensure normal growth. Nevertheless, it has been observed that the supplementation can have other beneficial effects. For instance, it has been observed that the increase from 20 up to 2,500 mg kg^{-1} of ASA in the diet improved stress resistance in

sea bass and turbot (Merchie et al.,1997). Similar results were obtained by Kolkovski et al. (2001) in walleye (*Sander vitreus*), where the dietary increase of ASA from 300 to 1,500 mg kg^{-1} improved stress resistance. In yellow croaker (*Larimichthys polyactis*) and Indian major carp (*Labeo rohita*) a positive effect of ASA on immune response and disease resistance has also been described (Ai et al., 2006; Nayak et al., 2007).

On the other hand, ASA is a cofactor in the hydroxylation of proline and lysine, which catalyze the conversion of procollagen to mature collagen, contributing to bone and skin formation and therefore to growth (Barnes and Kodiced, 1972; Lim and Lovell, 1978; Padh, 1991; Nusgen et al., 2001). Indeed, the deficiency of ASA produces a decrease in alkaline phosphatase, producing a reduction in osteoblastic activity and resulting in a poor bone calcification (Tietz et al., 1983; Johnston et al., 1994) and has been associated with skeletal deformities, such as kyphosis, scoliosis and lordosis (Lim and Lovell, 1978).

ASA is also involved in the tyrosine metabolism. Tixerant et al. (1984) were the first to link the so called "Granulomatous Syndrome" observed on farmed turbots to a disorder in tyrosine metabolism. ASA is involved in the second pathway of tyrosine catabolism, via two mixed-function oxidases. The first is 4-hydroxyphenylpyruvate dioxygenase, which catalyzes the oxidation and decarboxylation of 4-hydroxylphenylpyruvic, an intermediate of tyrosine degradation (Messager, 1986). The second enzyme ascorbate-depending, homogentisate 1,2-dioxygenase, catalyzes the next step in the tyrosine degradation (Combs, 2012). According to the lysosomial theory of Goldsmith (1978), the ASA deficiency can cause hypertyrosinaemia and deposits of tissual tyrosine, these tyrosine crystals are then able to induce local inflammatory granulomatous response. Later on, Baudin-Laurencin et al. (1989) and Coustans et al. (1990) confirmed that a deficiency in ASA could be the cause of hypertyrosinaemia and of tissue tyrosine deposits in turbot. Granulomas have also been observed in the liver, kidney, heart, spleen and other tissues in sea bream, together with inflammatory response and necrotic lesions when diets deficient in ASA were offered to the fish (Paperna et al. 1980).

ASA is also involved in α-TOH metabolism, reducing α-tocopheroxyl radicals and regenerating them to α-TOH (Niki et al., 1985). During the α-TOH regeneration by

ascorbate, semidehydroascorbyl radical is generated, which can then be reduced to ascorbate and dehydroascorbarte to semidehydroascorbate reductase. Dehydroascorbate can be reduced to ascorbate by using thiols such as glutathione or dihydrolipoate, which are reduced by NADPH (Hacişevki, 2009) (Figure 1.12). Indeed, the liver concentrations of α-TOH increased along with the dietary ASA levels in yellow perch (*Perca flavescens*) and channel catfish (Lee and Dabrowski, 2003; Yildirim-Aksoy et al., 2008). Betancor et al. (2012) also observed that the increase in ASA from 1,800 to 3,600 mg kg^{-1} in European sea bass larvae increased tissue contents of α-TOH, denoting its sparing effect over dietary α-TOH. Consequently, the optimum dietary α-TOH levels are influenced by the levels of ASA (Hamre et al., 1997; Chen et al., 2004; Atalah et al., 2010).

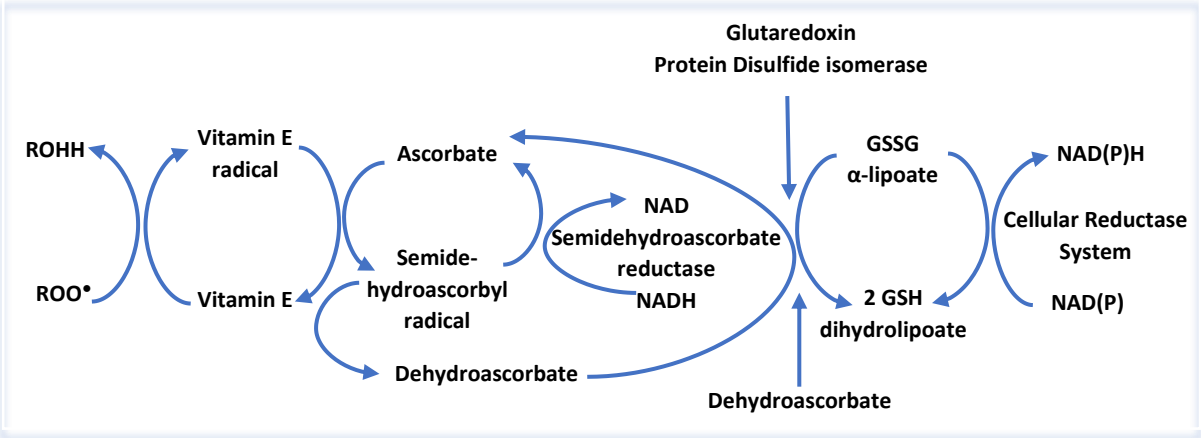

Figure 1.12. Antioxidant network showing the interaction between vitamin E and C. Adapted from Packer et al., 1979.

1.6.5.3.2. Vitamin E

Vitamin E (α-tocopherol; α-TOH) is a collective term given to a group of lipid soluble compounds (tocopherols and tocotrienols) that protect polyunsaturated lipids against oxidation. α-TOH is composed by eight lipid soluble compounds, four tocopherols (*α*-Tocopherol, *β*-Tocopherol, *γ*-Tocopherol, *δ*-Tocopherol) and four tocotrienols (*ζ*2-Tocopherol, *η*-Tocopherol, *ε*-Tocopherol, *ζ*1-Tocopherol) (NRC, 1993; Ahsan et al., 2014). In aquaculture α-TOH is usually supplied in the diet as α- tocopherol acetate, due to its higher stability and oxidation resistance (Hamre and Lie., 1995; Peng et al., 2008).

During the metabolism of α-TOH, the non-α-TOH forms are metabolized in preference to α-TOH (Swanson et al., 1999; Sontag and Parker, 2002). The α-TOH forms are oxidized by cytochrome P450, followed by ß-oxidation and finally excreted in urine (Brigelius-Flohé and Traber, 1999) or bile (Kiyose et al., 2001). In animals, the activity of α-TOH is much higher than other α-TOH homologues (Hamre, 2011). The preference of the different tocopherol forms is due to hepatic discrimination favouring the metabolism of these forms of α-TOH (Traber and Atkinson, 2007). α-tocopherol is the major membrane-bound lipid-soluble antioxidant (Machlin and Bendich, 1987). α-TOH has a methylated 6-chromanol nucleus with a free hydroxyl group (-OH), which can trap unpaired electrons (Nakamura and Omaye, 2009). α-TOH and PUFA compete for donating a hydrogen atom to the lipid peroxyl radical, thereby breaking the chain of reactions involved in lipid peroxidation (Hamre, 2011) (Figure 1.13). α -TOH can trap much faster the lipid peroxyl radical protecting a large amount of fatty acids.

α-TOH is a structural component of cell membranes where it has an important role as an antioxidant, controlling peroxidation of unsaturated fatty acids and protecting membranes from oxidative damage (Putnam and Comben, 1987; Tocher et al., 2002; Huang et al., 2003; Wilhelm-Filho, 2007; Gao et al., 2012). In fish, α-TOH has been found to improve growth, immune response anddisease resistance, in addition to protecting biological membranes and lipoproteins (Hamre et al., 1997; Hamre et al., 1998; Betancor et al., 2011; Li et al., 2013). In this sense, the supplementation from 0 to 100 mg kg^{-1} of α-TOH significantly improved growth after 8 weeks of feeding in red sea bream (Gao et al., 2012). Lipid peroxidation protection has been observed by Stéphan et al. (1995) where the supplementation with 320 mg kg^{-1} of α-TOH in turbot reduced thiobarbituric acid reactive substances (TBARS) values in diets containing high levels of DHA.

The requirements of α-TOH have been studied in some fish species, such as Atlantic salmon (*Salmo salar* ;60 mg kg^{-1}; Hamre and Lie, 1995), channel catfish (50 mg kg^{-1}; Wilson et al., 1984), rainbow trout (*Oncorhynchus mykiss* ;100 mg kg^{-1}; Watanabe et al., 1981), common carp (100 mg kg^{-1}; Watanabe et al., 1970) or red drum (*Sciaenops ocellatus*; 31 mg kg^{-1}; Peng and Gatlin, 2009). The optimum requirement for α-TOH to obtain normal growth, improve carcass composition and reduce erythrocyte fragility in *Labeo rohita* was 131.91 mg kg^{-1} (Sau et al., 2004). In *Pagrus major* a requirement of

200 mg kg^{-1} of α-TOH has been stablished to reduce lipid peroxidation and improve health condition (Linn et al., 2014).

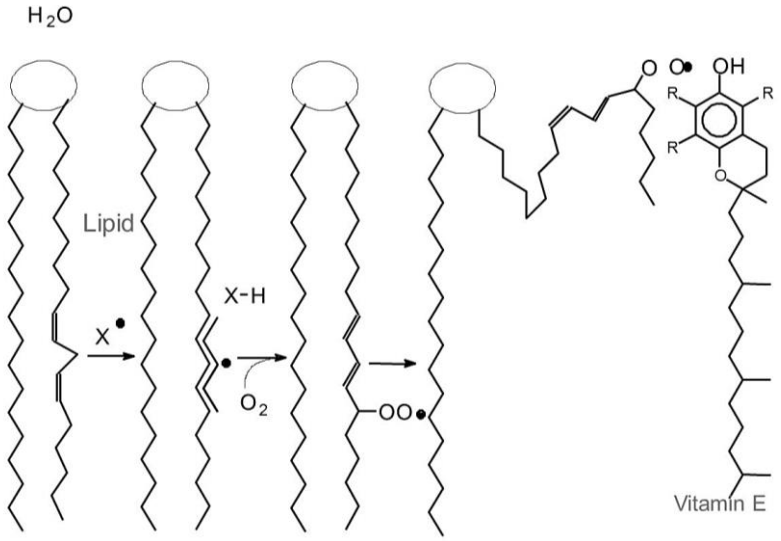

Figure 1.13. Mechanism of reaction of α-tocopherol with oxidizing lipids. The peroxidation process begins with the formation of pentadienyl radical, which react very fast with O_2, forming a peroxyl radical. The peroxyl radical is polar and floats to the surface of the membrane, where it can react with α-tocopherol, rendering a lipid hydroperoxide and the tocopheroxyl radical (Buettner, 1993).

The antioxidant effect of α-TOH has been observed in several fish species, such as sea bass, where the supplementation in microdiet with 3,600 mg kg^{-1} of α-TOH was shown to be successful in compensating the adverse effects of lipid oxidation (Betancor et al., 2012), similarly to what was previously observed in turbot (Tocher et al., 2002). The supplementation of α-TOH improves fillet quality in rainbow trout, protecting flesh from oxidation and improving shelf life (Chaiyapechara et al., 2003; Yildiz, 2004). A positive correlation has been observed between the levels of α-TOH in the diet and the activity of some antioxidant enzymes (SOD and GPX) (Monget et al., 1996; Kiron et al., 2004). The supplementation of 1,200 mg kg^{-1} of α-TOH enhanced serum haemolytic activity and the phagocytosis of head kidney leucocytes in gilthead sea bream (Ortuño et al., 2000).

Additionally, α-TOH is involved in numerous biological functions, and it has been observed that the deficiency of this vitamin leads to reduced growth (Lin and Shiau, 2005;

Peng and Gatlin, 2009), increased haematocrit and haemoglobin levels, increased lipid peroxidation (Kocabas and Gatlin, 1999; Bai and Lee, 1998; Abdel-Hameid et al., 2012), increased the appearance of muscular dystrophy (Betancor et al., 2012) and TBARS content (Lin and Shiau, 2005; Wang et al., 2016). As previously stated, the dietary α-TOH requirements depend on the interactions with other nutrients such as ASA (Hamre et al. 1997; Lee and Dabrowski 2003; Lee et al., 2004; Yildirim-Aksoy et al., 2008). ASA promotes a sparing effect on α-TOH (Tappel, 1972; Shiau and Hsu, 2002), and oxidized α-TOH could be regenerated to its reduced form by ascorbate. This sparing action has been suggested in some fish species having an influence in growth, tissue composition or immune response (Ortuño et al., 2001; Mourente et al., 2007; Yildirim-Aksoy et al., 2008; Hamre, 2011; Betancor et al., 2012).

1.7. Other nutrients

The exogenous or non-enzymatic antioxidants do not only protect different components of the cell from damage, but also play a vital role in fish growth, control of peroxidation of unsaturated fatty acids, immune response and malformations (Putnam and Comben, 1987; Kumari and Sahoo, 2005; Zhou et al., 2012). Nevertheless, there are other nutrients that play key biological roles in fish growth and health, such as fatty acids, vitamin K and minerals Manganese, Zinc and Selenium.

1.7.1. Fatty acids

Eicosapentaenoic acid (20:5n-3; EPA), docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; ARA) are considered essential fatty acids in marine fish due to the limited capacity to synthesize them from its precursors, specifically to the limited activity of Δ6- and Δ5-desaturase and elongase enzymes (Tocher, 2010). These essential fatty acids are involved in the maintenance of structural and functional integrity of cell membranes (Izquierdo and Koven, 2011), normal growth (Rodríguez et al., 1994; Salhi et al., 1997) or immune system (Izquierdo, 1996). Marine fish larvae have high requirements for LC-PUFA, specially EPA and DHA. Given that these fatty acids are highly unsaturated and thus at risk of suffering oxidation, fish larvae are more susceptible to suffering peroxidative attack than adults (Hamre et al., 2010).

Farmed fish have traditionally been fed a diet consisting of fishmeal and fish oil, rich in n-3 LC-PUFA. Krill oil is other marine source, which has a uniquely high content of phospholipid-bound n-3 LC-PUFA. Phospholipid composition is different in both oils, in fish oil fatty acids are mainly stored as triglycerides, whereas in krill 30–65 % of the fatty acids are incorporated into phospholipids (Tou et al., 2007). The main phospholipid in krill oil is phosphatidylcholine (Winther et al., 2011), which is involved on how lipids, including cholesterol, are handled by the body and also, can influencefish growth (Halver, 2002). It is thought that the bioavailability of n-3 LC-PUFA in krill oil is higher and is involved in the regulation of more metabolic pathways than the triacylglycerol-bound EPA and DHA found in fish oils (Ulven and Holven, 2015).

Therefore, adequate levels of EPA, DHA and ARA must be included in marine fish diets. A deficiency in these fatty acids leads to poor growth and increases mortality (Izquierdo et al., 1989; Salhi et al., 1994; Glencross, 2009), the appearance of several alterations such as erosion of the caudal fin, myocarditis and shock syndrome (Castell et al., 1972), immune-deficiency and cortisol levels (Izquierdo, 1996). Recently, the requirements of these fatty acid in meagre fingerlings has been studied, being required around 2.1% n-3 LC-PUFA in diets containing 16.5% lipids, 0.9 EPA/DHA and 0.4% ARA of total fatty acids contents for normal growth (Carvalho et al., 2018).

1.7.2. Vitamin K

It was not until 1974 that the biochemical role of vitamin K was revealed as a cofactor for the so-called carboxylation reaction, in which a specific glutamyl residue is converted to γ-carboxylglutamyl residues (Nelsestuen et al., 1974; Stenflo et al., 1974; Wu et al., 1991). Three forms of vitamin K are known, phylloquinone (vitamin K1), the menaquinones (vitamin K2) and menadione (vitamin K3) (Figure 1.14). The phylloquinone is synthesized by plants and algae and can be found in foods such as soy and olive oil (Bolton-Smith, 2000). The main source of phylloquinone in the wild for fish comes from diatoms or green algae, being metabolized in the liver (Udagawa et al., 1993). Menaquinones can be synthesized by bacteria (Collins and Jones, 1981; Fernandez, 1987). Menadione is a chemically synthesized vitamin K used as a compound in animal feeds, with menadione bisulphite being the main form of vitamin K supplementation in feed for aquaculture fish.

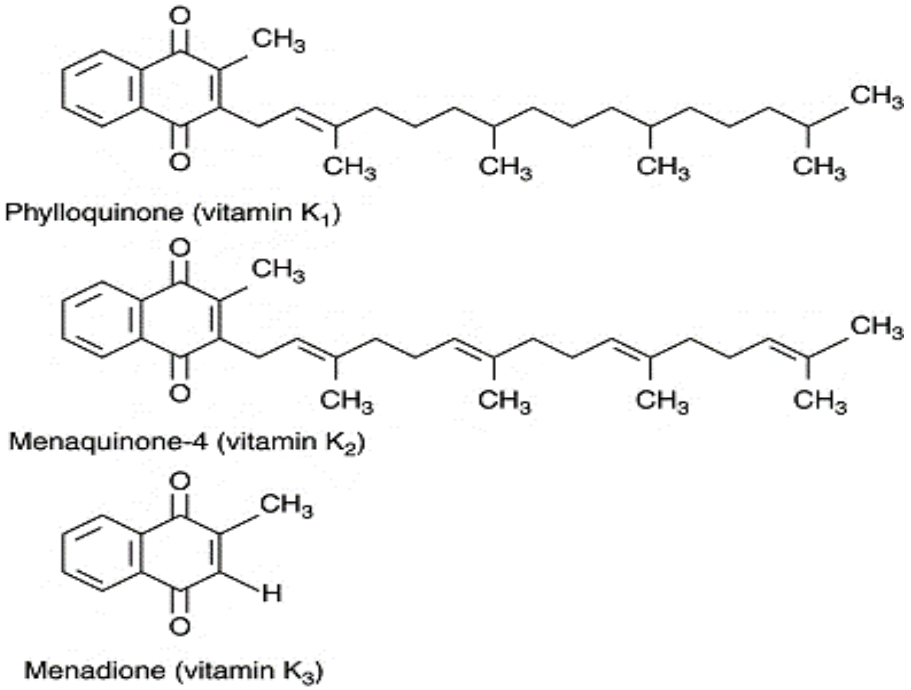

Figure 1.14. Chemical structures of vitamin K1 (phylloquinone), vitamin K2 (menaquinones) and vitamin K3 (menadione) (Chhabra, 2012).

Vitamin K is a lipid soluble vitamin involved in blood coagulation (Phillips et al., 1963; Olson, 1999; Sheehan et al., 2001), immune response (Choi et al., 2016) and bone formation (Udagawa, 2006; Roy and Lall, 2007; Udagawa, 2008). Vitamin K is involved in the posttranslational activation and modification of the vitamin K-dependent proteins (VKD) (Boskey et al., 1998; Lee et al., 2007). VKD has a diverse range of function and is implicated in a broad of biological functions (Berkner, 2008). Deficiency of vitamin K has been related with vertebral deformities in mummichog larvae (*Fundulus heteroclitus*), causing the formation of a thinner and weaker bone, which leads to bone abnormalities (Udagawa, 2004). Other symptoms associated with vitamin K deficiency in fish are the increase of blood coagulation time, haemorrhages, loss of fin tissue, reduction of growth, deformities and increase in mortality (Taveekijakarn et al., 1996; Udagawa and Hirose, 1998; Udagawa, 2004; Lall and Lewis-McCrea, 2007). Recently, a correlation between vitamin K and the occurrence of systemic granulomatosis has been observed in meagre larvae, in which larvae fed diets without supplementation of vitamin K displayed a higher incidence of hepatic granulomas (Vidal et al., 2016). However, limited information is

available on the vitamin K requirements in fish (Lall, 2005), the requirement for most of the fish species not being yet stablished (NRC, 2011).

1.7.3. Selenium

Selenium (Se) is an essential micronutrient (Johansson et al., 2005), necessary for normal growth and development. It is involved in the metabolism and detoxification of oxygen (Nève, 1991), giving place to the vital selenoenzymes. The most commonly used source of Se to supplement diets is sodium selenite, an inorganic form. The concentrations for selenium deficiency and toxicity are very close to each other (Kobayashi, et al., 2002), because it can be rapidly accumulated and reach toxic levels (Lemly, 2002). Indeed, excessive dietary selenium contents (20 mg kg^{-1}) resulted in a lower growth, feed efficiency ratio and survival in hybrid striped bass (*Morone chrysops* x *M. saxatilis*), all attributed to the toxicity of this micronutrient (Jaramillo et al., 2009).

Se can also be incorporated as selenocysteine at the active site of a wide range of proteins, being an efficient biological catalyst (Arthur et al., 1997). Se is fundamental for the function of several Se - containing proteins (selenoproteins) (Hatfield, 2002; Papp et al., 2007). The number of selenoproteins varies among the species (Lobanov et al., 2009), being teleost the animals with the highest number of identified selenoprotiens, up to 41 (Mariotti et al., 2012). One of the major functions of Se is as a component of glutathione peroxidase, which reduce hydroperoxides at the expense of reduced glutathione (Arteel and Sies, 2001), protecting lipid components and membranes from oxidative damage by the reduction of pro-oxidants. The supplementation of Se (3.98 mg Se-yeast kg^{-1}) improved growth performance, antioxidant balance and innate immune status in juvenile meagre (Mansour et al., 2017). Selenium is also involved in the regulation of cell growth, thyroid hormone and insulin function (Lall, 2002; Kohlmeier, 2003) and is an exogenous antioxidant involved in the prevention of oxidative stress (Felton et al., 1996; Biller-Takahashi et al., 2015; Silva-Brito et al., 2016).

Deficiency of Se has been associated to elevated lipid peroxidation, leading to alterations in the integrity of cell membranes what in turn can influence several cell functions (Stadtman, 1990). The deficiency in Se has also been related with oxidative stress in different organs (Gatlin et al., 1986; Bell et al., 1985, 1986). Sea bass supplemented with organic Se showed enhanced cell antioxidant capacity, increased

protection against muscular lesions and reduced TBARS values (Betancor et al., 2012). The requirement of Se may be affected by several factors/nutrients. In this sense, it has been observed that α-TOH and selenium have synergistic effects on antioxidant physiology (Wise et al., 1993).

1.7.4. Zinc

Zinc (Zn) is a dietary essential trace element involved in numerous metabolic processes such as catalysis and is a structural component and regulatory ion (King and Cousins, 2014). Zn does not participate in redox reactions (Williams, 1987), being a stable ion in a biological medium and an ideal cofactor in reactions that require a redox-stable ion (Butler, 1998), such as proteolysis and the hydration of carbon dioxide. More than 300 metalloenzymes that require Zn as a catalyst have been described. Zn also regulates genes through the metal-response element binding transcription factor-1 (MTF-1) and controls numerous cell-signalling pathways (Liuzzi and Cousins, 2004; Andreini, et al., 2006; Lukacik et al., 2008; Sousa, et al., 2009).

Zn is an essential nutrient for normal growth and skeletal development (Watanabe et al., 1997; Yamaguchi, 1998; Ovesen et al., 2001; Yamaguchi and Fukagawa, 2005) and is involved in several cellular functions, immune response and defence against ROS (Bray and Bettger, 1990; Powell, 2000). Zn has antioxidant properties and protects tissue from oxidative damage (Ho and Ames, 2002) and is necessary for reproduction and metabolic processes (Eisler, 1993). This essential nutrient has been demonstrated to have a stimulatory effect on mineralization and bone formation, activating the aminoacyl-tRNA synthetase in osteoblastic cells, and it stimulates cellular protein synthesis (Yamaguchi, 1998).

The Zn present in the water is not absorbed in enough amounts to meet the requirements of fish, being the more important source of this mineral the dietary supplementation (NRC, 1993). The requirement of Zn in the diet depends on the fish species, age, growing stage and reproductive cycle (Carpene et al., 2003). In Atlantic salmon larvae the supplementation with 57-97 mg kg^{-1} of Zn improved growth performance (Maage, 1991). A supplementation of 40 mg kg^{-1} of Zn is necessary for normal growth of rainbow trout (Satoh et al., 1987a). Zn has an important role enhancing

the antioxidant status and decreasing lipid peroxidation (Anderson et al., 2001). The supplementation of Zn in turbot significantly increased SOD activity (Ma et al., 2014), probably due to the fact that Zn is a component of Cu-Zn-SOD (Shiau and Jiang, 2006).

On the other hand, dietary deficiency of Zn can reduce growth (Ogino and Yang 1978; Do Carmo e Sa et al. 2005; Ma et al., 2014), retard ectopic bone formation (Calhoun et al., 1975), as well as reduce the digestibility of proteins and carbohydrates (Do Carmo e Sa et al., 2005). Zn deficiency in rainbow trout has been observed to produce dwarfism, cataract, erosion of fins and skin and reduction of wound healing (Satoh et al., 1983; Watanabe et al., 1997). Indeed, Zn is an essential component of enzymes involved in wound-healing process, such as collagen synthesis and cell division (Tenaud et al., 1999, 2000).

1.7.5. Manganese

Manganese (Mn) can be observed in a wide variety of oxidation states (Fraústo and Williams, 2001), is thermodynamically stable due to its electronic configuration (Irving and Williams, 1948) and can be a powerful oxidizing agent (Dean, 1985). Mn is an essential trace metal that is found in all tissues and is necessary for normal growth, reproduction and prevention of skeletal deformities in fish (Lall, 1989).

Dietary Mn is important, even in small quantities, as it is involved in many cellular processes including lipid, protein and carbohydrate metabolism and can act as a cofactor or activator for many enzymatic systems, such as Mn superoxide dismutase (Mn-SOD) (De Rosa et al., 1980; Andreini et al., 2008). Mn is an integral part of certain metalloenzymes (Watanabe et al., 1997; Lall, 2002). The supplementation of dietary Mn can improve growth, feed utilization and protection against oxidative stress (Wang and Zhao, 1994; Maage et al., 2000; Zhang et al., 2016). There is also some evidence that Mn is involved in immunological functions (Hurley and Keen, 1987; Cossarini-Dunier et al., 1988).

The requirements for Mn have been studied in many fish species, such as in channel fish, (2.4 mg kg^{-1}; Gatlin and Wilson, 1984), Atlantic salmon (7.5-10.5 mg kg^{-1}; Maage et al., 2000) or in gibel carp (*Carassius auratus gibelio*) (13.7 mg kg^{-1}; Pan et al., 2008), ranging all of them from 2.4 to 24.93 mg kg^{-1} (Ogino and Yang, 1980; Tan et al.,

2012; Liu et al., 2013; Ma et al., 2014; Zhang et al., 2016). The deficiency of Mn in fish diets can decrease survival, delay growth, produce skeletal deformities, cataracts and reduce the activity of Mn-SOD (Satoh et al., 1989; Lall, 2002; NRC, 2011). Mn deficiency has been described to produce dwarfism linked to disturbances in bone formation and cataracts in rainbow trout and carp (Satoh et al., 1987b, 1991). Indeed, a reduction of Mn content in bone has been linked to an insufficient dietary supply of Mn (Satoh et al., 1983).

Objectives

Meagre has been proposed as a good candidate to the aquaculture diversification due to its good biological characteristics. However, the intensive culture of meagre is affected by systemic granulomatosis. Eventually, the disease has been associated with infectious agents in farmed meagre. Nonetheless, it has been hypothesized that granulomas can also be caused by a nutritional imbalance, given that in the vast majority of cases in which the disease was observed, no infectious agent has been detected. Therefore, it seems that by using balanced feeds, the systemic granulomatosis could be tackled in farmed meagre. However, little is known about the nutritional requirements in this species. As meagre is a fast-growing species, it is expected to have higher dietary requirements than other farmed teleost. For that reason, the objectives of the present PhD dissertation were:

1.- To determine which dietary nutrients modulate the appearance of systemic granulomatosis in meagre. The role of several nutrients in the appearance of the disease were evaluated.

1.1- *Antioxidant vitamins E (α-TOH) and C (ASA)*: α-TOH and ASA are powerful antioxidants able to neutralize reactive oxygen species. The deficiency of these vitamins can lead to several alterations, including the appearance of granulomas. The addition of different levels of α-TOH and ASA and its antioxidant effect was tested both on larvae and on-growing meagre. The incidence and severity of the disease, the modulation of the vitamins on gene expression as well as fish performance was evaluated.

1.2- *Other nutrients*: In order to reach this objective diets containing graded levels of vitamin K, and minerals Se, Zn and Mn were fed to on-growing meagre to determine effects on appearance of systemic granulomatosis.

2.- To determine the first appearance of systemic granulomatosis in meagre larvae. When granulomas first appear in meagre larvae has not been previously studied. By determining its first appearance it will be possible to modulate the incidence of the disease

in later life stages. This has been approached through:

2.1- *Feeding protocol*: In order to prevent the appearance of granulomas it is necessary to establish an adequate feeding protocol to avoid the appearance of the disease. To reach this objective, the effect of the lack of *Artemia* in the feeding sequence was evaluated, by feeding larvae directly from rotifer to inert diets.

2.2- *Dietary nutrients*: The effect that different commercial enrichment media, with different vitamin profiles, have on the appearance of systemic granulomatosis was studied.

3.- To evaluate the role of daily farming conditions on the appearance of systemic granulomatosis on meagre. Farmed meagre are exposed to daily stressors that might potentially lead to the appearance of granulomas. In the present dissertation, the effect that density has on the disease in on-growing meagre was evaluated.

Chapter 2

Material and methods

2.1. Experimental animals and conditions

2.1.1. Fish

Meagre eggs were obtained from induced spawning from broodstock from the ECOAQUA facilities at University of Las Palmas de Gran Canaria (ULPGC; Telde, Canary Islands, Spain) where the experiments were carried out. In the larval trials, eggs were volumetrically counted and set randomly into experimental tanks (Chapter 4) or were counted and seeded into 2,000 L tanks and reared until 30 dph and then transferred to the experimental tanks (Chapter 5).

For on-growing experiments, larvae where reared in 2,000 L tanks and then transferred to 10,000 L tanks, until they reach 15.7 g (Chapter 6) or 79.3 g (Chapter 3), when they were randomly transferred to the experimental tanks. Prior to the start of the experiment, fish were fed with a commercial diet (Skretting, Burgos, Spain) for 14 days to acclimatize to the experimental conditions.

2.1.2. Experimental tanks

For larval trials, grey colour cylindrical fibreglass tanks of 170 L were used. All tanks were equipped with continuous aeration and supplied with filtered UV-sterilized seawater, previously stored at a 500 L tank for degasification, at an increasing rate from 5 % h^{-1} to a 100 % h^{-1}, to guarantee good water quality during the trial. Water entered the tanks from the bottom and exited at the surface. Oxygen (4.5-6.5 g L^{-1}), salinity (34 g L^{-1}) and temperature (24.6 to 25.3 ºC) was daily measured by using an Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, USA). Photoperiod was kept at 12 h light: 12 h dark by fluorescent lights. Tanks were manually cleaned daily by a siphon system.

For on-growing trials, meagre juveniles were transferred randomly to fibre glass tanks of 500 L with 50 fish per tank at an initial stocking density of 7.9 kg m^{-3} (Chapter 3) or with 100 fish per tank (3.20 kg m^{-3}) and 175 fish per tank (6.20 kg m^{-3}), depending on the experimental diet (Chapter 6). All tanks were covered with a net to prevent escapes. The temperature and dissolved oxygen concentration were measured twice a week with values ranging from 17.6 to 21.6 °C and 5.8 to 6.6 mg L^{-1}, respectively, by using an Oxy Guard-handy beta instrument (Zeigler Bros, Gardners, USA). Tanks were weekly cleaned manually by a siphon system.

2.2. Diets and feeding

2.2.1. Larvae trials

2.2.1.1. Rotifers

Rotifers were cultured in 1,700 L troncononical-tanks at a density of 400 rotifers mL^{-1}. Water of the culture was composed by 80 % sea water and 20 % freshwater. Tanks were constantly aerated with a porous stone and oxygen automatically measured at real time. Rotifers were fed twice a day with 0.6 g fresh yeast extract for each 10^6 rotifers. Each 3-4 days rotifers were filtered to clean the tank and resuspended in clean water at an optimum density. Prior to being supplied to meagre larvae, rotifers were enriched. The tanks used for the enrichment were well aerated (5 mg L^{-1} O_2) with filtered and UV-treated seawater (37 g L^{-1}). Rotifers were enriched with either Ori-Green (Skretting, Burgos, Spain) (0.15 - 0.25 g $million^{-1}$ rotifers) for 2 h before being fed to the meagre larvae or with Easy DHA Selco (INVE, Dendermonde, Belgium) (0.6 g L^{-1}) for 24 h. The enrichment time was that indicated by the different manufacturers. Larvae from all the dietary treatments (Chapter 4 and 5) were fed twice daily (9:00 and 14:00) from 3 to 21 or 30 dph, depending on the dietary treatment, with enriched rotifers. Before each feeding, rotifers were counted and added to maintained at a density of 10 ind L^{-1} in the experimental tanks. In addition, 1 h before feeding with rotifers, phytoplankton (*Nannochloropsis gaditana*) was supplied in each tank at a density of 300,000 cell m^{-3}.

2.2.1.2. *Artemia*

Artemia cysts (SEP-Art; INVE, Salt Lake City, Utah) were hatched in conical 50 L tanks at 27 °C with continuous aeration and 30 g L^{-1} salinity at a stocking density of 2 g *Artemia* non-decapsulated cysts L^{-1}. *Artemia* cyst required 18-24 h for decapsulation. These *Artemia* cysts are coated with a ferro-magnetic material, so that nauplii and cysts can be separated from non-burst cysts and debris by means of a magnetic separator. The separator attracts the magnetic empty cysts shells and unhatched cysts but not the *Artemia* nauplii. Then, nauplii were rinsed with sea water and transferred to a culture tank at 24 °C, where *Artemia* was enriched with either Ori-Green (Skretting, Burgos, Spain) (0.8 g million^{-1} *Artemia*, RAO dietary treatment, Chapter 4) for 12 h or with Easy DHA Selco (INVE, Dendermonde, Belgium) (0.6 g million-^{1} *Artemia*, RAS dietary treatment, Chapter 4) for 24 h before being fed to the larvae. Larvae were fed *A*rtemia twice daily (11:00 and 15:00) from 12 to 30 dph (Figure 2.1).

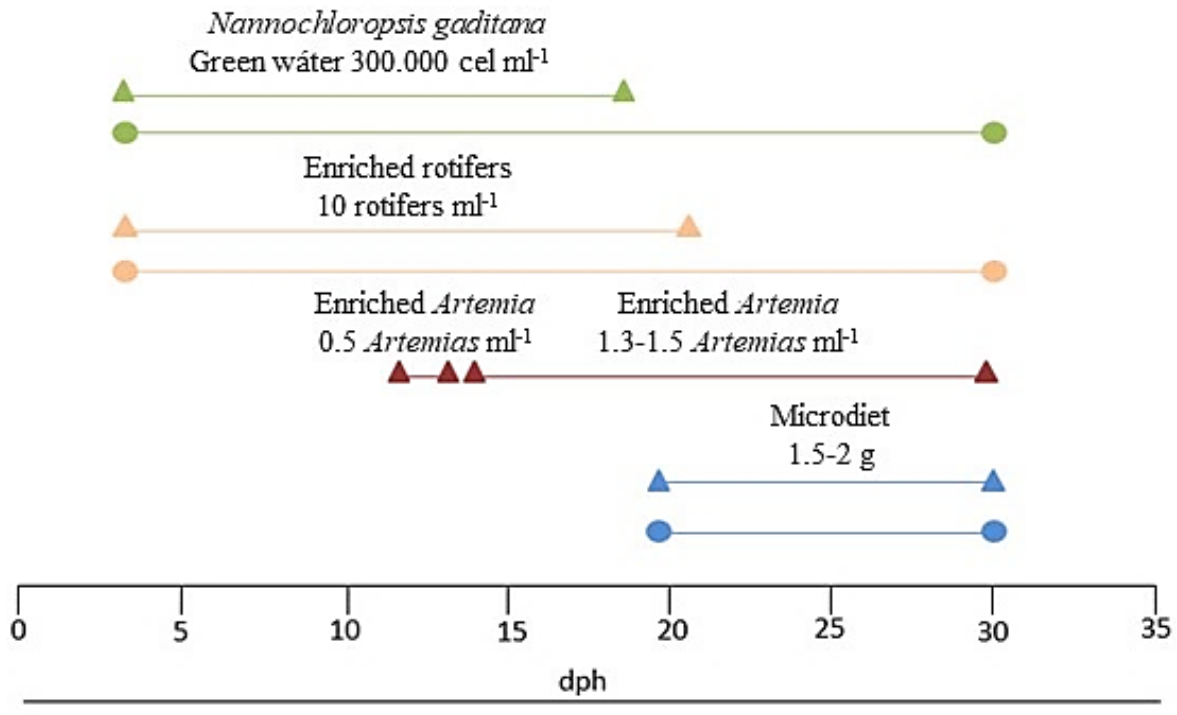

Figure 2.1. Meagre (*Argyrosomus regius*) larvae feed sequence from 3 dph to 30 dph.▲ Diets RAO and RAS. ● Diets RAO and RAS.

2.2.1.3. Microdiet

Isonitrogenous and isolipidic experimental microdiets (pellet size <250 and 250-500) were formulated and prepared at the ULPGC facilities (Chapter 5). Additionally, a commercial inert microdiet (Gemma Micro 150 and 300 µm; Skretting, France) was also used (Chapter 4). In the experimental diets, krill meal was the source of protein. The desired lipid content was completed with fish oil and only for one diet with krill oil. The krill meal was defatted (three consecutive times with a chloroform: krill meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiets. α-TOH in α-tocopheryl acetate form was obtained from Sigma-Aldrich (Madrid, Spain). ROVIMIX Stay-C-35 (ascorbyl monophosphate; Roche, Paris, France) was used as the ASA source. Soybean lecithin (Acrofarma, Barcelona, Spain) was used as a source of phospholipids, which contains around 50 % of polar lipids. The hydrosoluble and liposoluble vitamins mixture and mineral mixture was prepared as described by Teshima et al. (1982) and the attractants mixture was like that described by Kanazawa et al. (1989) (Table 2.1). Ingredients, proximal composition and fatty acid content of the experimental diets are shown in Tables 5.1 and 5.2 (Chapter 5).

Table 2.1. Composition of vitamins, minerals and attractants mix used to prepared experimental microdiets.

Vitamins	mg/100g DW
Hydrosoluble	
Cyanocobalamin (Vit. B_{12})	0.03
Astaxanthin	5
Folic Acid (Vit. B_9)	5.44
Pyridoxine-HCl (Vit. B_6)	17.28
Thiamine-HCl (Vit. B_1)	21.77
Riboflavin (Vit. B_2)	72.53
Calcium Pantothenate (Vit. B_5)	101.59
p-aminobenzoic acid (Vit. B_4)	145
Nicotinic acid (Vit. B_3)	290.16
myo-Inositol (Vit. B_8)	1450.9
Ascorbic polyphosphate	180
Choline chloride (Vit. B_5)	2965.8
Liposoluble	
Retinol acetate (Vit. A)	0.18
Ergocalciferol (Vit. D)	3.65
Menadione (Vit. K)	17.28
α-Tocopherol acetate (Vit. E)	40
Minerals	
NaCl	215.13
$MgSO_4.7H_2O$	677.54
$NaH_2PO_4.H_2O$	381.45
$K_2HPO_4.H_2O$	759.0
$Ca(H_2PO_4).2H_2O$	671.61
$FeC_6H_5O_7$	146.88
$C_3H_5O_3.1/2Ca$	1617.21
$Al_2(SO_4)3.6H_2O$	0.69
$ZnSO_4.7H_2O$	5
$CuSO_4.5H_2O$	1.25
$MnSO_4.H_2O$	3.00
KI	0.74
$CoSO_4.7H_2O$	10.71

Attractants	mg/100g DW
Inosine-5-monophosphate	500
Betaine	660
L-Serine	170
L-Tyrosine	170
L-Phenylalanine	250
DL-Alanine	500
L-Sodium aspartate	330
L-Valine	250
Glycine	170

2.2.1.4. Microdiets preparation

The microdiet was prepared according to the protocol established by Liu et al. (2002). Firstly, the fish or krill meal was mixed with water-soluble ingredients (attractants, minerals and water-soluble vitamins). Oil and fat-soluble vitamins were mixed and blended with the dry mixture. Finally, gelatine dissolved in warm water was added to the mix. The paste was pelleted and dried at 38 °C for 24 h. The final pellets were ground and sieved in two different particle sizes (<250 µm and 250-500 µm). Diets were kept at 5 °C during the feeding period in order to avoid deterioration.

2.2.2. On-growing trials

2.2.2.1. Experimental diets

Two different trials with meagre juveniles were carried out in the present Thesis. In a first trial (Chapter 3) six isolipidic (16 % lipid) and isoproteic (50 % protein) fish meal and fish oil based feeds were prepared by Skretting (Stavanger, Norway) by adding different levels of α-TOH (0, 300 and 700 mg kg^{-1}), ASA (0, 100 and 600 mg kg^{-1}) and vitamin K (0 and 35 mg kg^{-1}). For the second on-growing experiment other five isolipidic (16.7 % lipid) and isoproteic (49.6 % protein) fish meal and fish oil based feeds were prepared by Skretting (Stavanger, Norway) adding different levels of ASA (100, 600, 1,200 and 3,200 mg kg^{-1}), Mn (0 and 40 mg kg^{-1}), Zn (0 and 200 mg kg^{-1}) and Se (0 and 1.5 mg kg^{-1}) (Chapter 6). Ingredients, proximal composition and fatty acid content of the experimental diets are shown in Tables 3.1, 6.1 and Supplementary Table 6.1 (Chapters 3 and 6).

2.2.3. Feeding

Larvae from all the dietary treatments were co-fed with *Artemia*, rotifer or both and inert microdiets from 20 to 30 dph (Chapters 4 and 5). From 30 to 44 dph meagre larvae were fed with microdiet only every 45 min from 8:00 to 20:00 every day with 15 % biomass day^{-1} (Chapter 5).

The juvenile meagre were fed "*ad libitum*" 3 times per day (8:00, 11:30, 15:00), 6 days per week during 15 (Chapter 3) or 13 weeks (Chapter 6) with the different experimental diets. All the uneaten feed was daily collected from each tank and dried in order to accurately calculate the daily feed intake.

2.3. Sample collection

2.3.1. Growth performance

Before each sampling meagre larvae were unfed for 12 h. Larvae growth was evaluated at the beginning, intermediate and final point at each experiment. Larvae were sacrificed using clove oil and measured for total length (TL), using a profile projector (Mitutoyo PJ- 3000A, Kanagawa, Japan), as well as dry weight. For the determination of dry weight, the whole larvae were placed in an oven at 100 °C for 24 h and weighed. A second measurement was performed after placing back the larvae in the oven for an additional hour at 100 °C. At the beginning and at the end of the experimental trials all the alive larvae were counted to calculate final survival.

At on-growing experiments, juvenile meagre were sampled at initial, intermediate and final point. Meagre were unfed for 24 h before sampling, anesthetized with clove oil and weight and total length measured. Liver, viscera and eviscerated fish were weighed to determine productivity parameters. The data were analysed according to the following equations: Survival (%) = 100*(final number fish – initial number fish)/ initial number fish; Growth (%) = ((final mean weight – initial mean weight)/initial mean weight)*100; Weight gain = (final mean weight- initial mean weight); SGR (specific growth rate) = 100 x (ln final mean weight – ln initial mean weight)/ number of days; FI = feed intake (g)/fish per day; FCR (feed conversion ratio) = feed intake (g)/ weight gain (g); K (condition factor (%)) = 100*(fish weight/ (fish length)3); HSI (hepatosomatic index (%)) = 100*(liver weight / fish weight); VSI (viscerosomatic index (%)) = 100* (fish weight - eviscerated fish weight)/fish weight.

2.3.2. Histopathology

In each sampling point, whole meagre larvae (n = 150; Chapter 4 and 5) or sections of liver, heart, kidney, spleen and muscle (n = 21, Chapter 3 or n = 30, Chapter 6) of juvenile meagre were collected and fixed in 4 % buffered formalin for histological analysis. Formalin fixed tissues (liver and kidney) and formalin fixed paraffin-embedded tissues (FFBE) (liver, heart, spleen, kidney) were collected and sent to the University of Stirling for Polymerase chain reaction (PCR) identification of Nocardia.

2.3.3. Biochemical analysis

Feed samples were hold at -20 °C for proximal composition, fatty acids and vitamins content analysis at the beginning of the experimental trial. Live prey (enriched rotifers and *Artemia*) were collected during 4 different days, pooled and stored at -80 °C until they were analysed. At the beginning and at the end of each experiment samples of whole meagre larvae (12 h unfed, n = 50) were collected, washed in distilled water and quickly frozen (-80 °C) until further analysis performed. In the on-growing meagre trials, samples of liver, kidney, heart and spleen (24 h unfed, n = 15 per treatment) were collected, washed with distilled water and held at -80 °C until they were analysed.

2.3.4. Gene expression

At the end of the on-growing experiment (Chapter 3 and 6) kidney, liver and heart of juvenile meagre were aseptically collected from four fish per tank (n = 12 per treatment), washed with DEPC (Diethyl pyrocarbonate) water, stabilized with RNAlater (Sigma-Aldrich, Madrid, Spain) for 24 h at 4 ºC, and then stored at -80 ºC until RNA extraction.

2.4. Histopathology

Collected samples were fixed in 4 % buffered formalin until further analysis. Samples were dehydrated in a series of different concentrations of ethanol (70-96 º), then in xylene and finally embedded in a paraffin block with a Histokinette 2000 tissue processor (Leica, Nussloch, Germany). The samples were cut at 4 μm on a microtome (Leica, RM2135, Leica Instruments, Nussloch, Germany), fixed to the microscope slide, heated to eliminate the remaining paraffine and finally stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco (Fite et al., 1947) or Gram stain (Gregersen, 1978). All the tissues were analysed for the presence of macroscopic granulomas. The severity of the granulomas was individually scored in each organ depending on the number of granulomas observed during the microscopy evaluation. The average severity was classified in liver, kidney and heart according to the criteria shown in Table 2.2.

Table 2.2. Severity score of granulomas in liver, kidney and heart.

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

2.4.1. Haematoxylin and eosin (H&E)

Haematoxylin and Eosin stain is a popular staining method in histology, whereas nuclei are stain blue and collagen, muscle, erythrocytes and cytoplasm are a red, pink or orange colour. The staining protocol used for all samples was:

- Xylol-2'→ Xylol-2'→ OH 100-2'→ OH 100-2'→ OH 70-2'→ Distilled water-2'→Distilled water-2' → Distilled water-2'→ Haematoxylin-13'→ Distilled

water-20-30''→ Chlorhydric alcohol - shaking 5´´→ Distilled water-2'→ Ammonia water-shaking 10´´ (until the samples were slightly blue)→ distilled water 5'→OH 96-1'→ Eosin-6'→ OH 96-2'→ OH 96-2'→ OH 100-2'→ OH 100-2'→ Xylol-2'→ Xylol-2'→ Xylol-2'→ Cover the slides with a glass and DPX

2.4.2. Gram stain

Gram staining is a common technique that differentiates two groups of bacteria based on their different cell wall constituents, discerning between Gram-positive and Gram-negative groups. The protocol used for Gram staining was:

- Xylol-2'→ Xylol-2'→ OH 100-2'→ OH 100-2'→ OH 70-2'→ Distilled water-2'→Distilled water-2' → Crystal violet-1`→ Distilled water-2'→ Lugol-1´→ Distilled water-2'→ OH-acetone (70 % alcohol 96 and 30 % acetone)-15''→ Distilled water-2'→ Safranin-1´→ Distilled water-2'→ Let dry and cover the slides with a glass and DPX

2.4.3. Ziehl-Neelsen (ZN) and Fite-Faraco staining

The Ziehl-Neelsen method is used to stain acid-fast bacilli such as *Mycobacterium* spp. and *Nocardia* sp. The walls of these bacteria species contain a waxy substance composed of mycolic acids (ß-hydroxy carboxylic acids). These bacteria cannot be stained with Gram stain, but they are with carbol fuchsin combined with phenol. The Ziehl-Neelsen protocol used in the present Thesis was:

- Basic fuchsin – 30`room temperature → Distilled water – 2´→ Acid OH – 5`→ Distilled water – 5`→ Light Green – 1` → Distilled water 2` → OH 96-2'→ OH 96-2'→ OH 100-2'→ OH 100-2'→ Xylol-2'→ Xylol-2'→ Xylol-2'→ Cover the slides with a glass and DPX

The Fite-Faraco staining is a more sensitive method than Ziehl-Neelsen and is also used to stain acid fast bacilli. Fite-Faraco method is a modified stain from Ziehl-Neelsen, differing only in the use of 10 % sulphuric acid instead of acid OH (100 ml of 70 % alcohol and 1 ml of chlorydric acid). The staining protocol used was the following:

- Basic fuchsin – 30`room temperature → Distilled water – 2´→ 10 % Sulphuric acid – 2`→ Distilled water – 5`→ Light Green – 1` → Distilled water 2` → OH 96-2'→ OH 96-2'→ OH 100-2'→ OH 100-2'→ Xylol-2'→ Xylol-2'→ Xylol-2'→ Cover the slides with a glass and DPX

2.4.4. Actin immunohistochemical staining

Additionally, an immunohistochemistry staining was performed using monoclonal anti-actin as a primary antibody to mark smooth muscle. Liver paraffin sections were routinely dewaxed and rehydrated as follow:

- Xylol-2'→ Xylol-2'→ Xylol-2'→ OH 100-2'→ OH 100-2'→ OH 96-3'→ OH 70-2'→ Distilled water-2'→Distilled water-2' → Distilled water-2'

All incubations were performed at room temperature in a humid chamber. Slides were set into 90 °C target retrieval high pH bath for 1 h (High pH, Dako, Denmark). Sections were washed 5 min with a wash buffer (Dako, Denmark). Samples were left in a humid chamber with a Peroxidase Blocking Solution (Dako, Denmark) for 30 min and then washed with a wash buffer. Slides were incubated for 2 h at room temperature with a primary monoclonal rabbit anti-actin antibody (diluted 1:200; clone HHF35; Enzo Diagnostic, USA). The immunohistochemical staining was carried out using the horseradish peroxidase (HRP) anti-rabbit EnVision (Dako, Denmark) for 30 minutes and washed with acetate buffer 0.1 M, pH 5.2 for 10 min. 3-amino, 9 ethyl-carbazole diluted in 0.1 M sodium acetate-buffer containing 3 % hydrogen peroxide was used as a chromogen for 10 min. Then the slides were washed for 3 min with distilled water 3 times.

Finally, samples were counterstained with Harris haematoxylin for 3 min and washed with distilled water.

2.5. Biochemical analysis

2.5.1. Moisture

Moisture content was determined according to AOAC (2010). Approximately 0.5-2 g of sample was weighed and placed in an oven at 110 °C for 24 h. After cooling at room temperature in a desiccator, samples were weighed. Moisture was expressed as a percentage of the weight as follows:

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

A = Weight of empty flask

B = Weight of wet sample + flask

C = Weight of dry sample + flask

2.5.2. Ash

Ash content was determined according to AOAC (2010). Approximately 0.5-2 g of sample was weighed and left into an oven at 600 °C for 1 h. After 1 h of cooling at room temperature, ash was weighed. The calculation of ash percentage was performed as follows:

$$Ash\ (\%) = 100 * \frac{Ash\ (g)}{sample\ weight\ (g)}$$

2.5.3. Proteins

Protein content in tissues and feeds was determined according to the Kjeldhal method (AOAC, 2010). The protein content was measured form the nitrogen extracted from the sample. The sample was digested with a strong acid so that nitrogen is released (proteins contain approximately 16 % of nitrogen; protein factor 6.25), which can be determined by a suitable titration technique. To determine the protein content is necessary to weigh 0.200-0.400 g of homogenised sample. A catalyser tablet and 10 ml of H_2SO_4 are added. The digestion takes place at 400 °C for 1 h. Then, samples were cooled in ice for 25 min, 20 ml of H_2O added to each tube and shaken. To neutralize the excess of H_2SO_4 50 ml of NaOH are added to each tube. To quantify the nitrogen content an indicator and 30 ml of boric acid were added to each tube, turning the solution into dark colour. Finally, HCl 0.1 M is added drop by drop, waiting for colour change, what indicates that the "endpoint" has been reached and that now all the acid has been neutralized by the base. All the reactions in the process are shown below:

Digestion:

$$N^2\ organic + H_2SO_4 \rightarrow CO_2 + H_2O + (NH_4)_2SO_4$$

Distillation:

$$(NH_4)_2SO_4 + NaOH + Q \rightarrow NH_3 + HBO_2 \leftrightarrow NH_4^+ + BO_2^-$$

Titration:

$$BO_2^- + H^+ \leftrightarrow HBO_2$$

Protein content in tissues and feed were calculated using the following formula:

$$P\ (\%) = \frac{\big(HCls\ (ml) - HClw\ (ml)\big) * 0.1 * 14.007 * 6.25 * 100}{sample\ weight\ (mg)}$$

HCls = ml of HCl added to the sample until the point at which the dye changes of colour.

HClw = ml of HCl added to the white until the point at which the dye changes of colour.

0.1 = HCl normality.

14.007 = atomic weight of N_2.

100 = conversion of %.

6.25 = protein factor.

2.5.4. Lipids

Total lipids from microdiet, live preys, whole larvae (Chapters 4 and 5), kidney, heart, liver and diets (Chapters 3 and 6) were extracted with a chloroform-methanol (2:1 v/v) mixture as described by Folch et al. (1957). A sample of 50-200 mg was weighed and homogenised in an Ultra Turrax (IKA-Werke, T25 BASIC, Staufen, Germany) during 5 min with chloroform:methanol (C:M; 2:1) with 0.01 % BHT added. The Turrax was cleaned with other 5 ml of C:M (2:1). 2 ml of 0.88 % KCl were added to the solution to increase the water phase polarity. Then, the solution was centrifuged during 5 min at 2,000 rpm to separate the methanol and salt on the top and the chloroform and lipids in the lower part. The top phase was eliminated, and the lipids and chloroform filtrated through sodium sulphate anhydrous. Finally, the chloroform was evaporated under nitrogen atmosphere, dry lipids remaining in the tube. The percentage of lipids in each sample was calculated as follows:

$$Lipids = \frac{Lipids\ (g)}{Sample\ (g)} * 100$$

2.5.5. Fatty acid methyl esters

Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982). Fatty acid methyl esters (FAMES) were separated and quantified by gas–liquid chromatography following the conditions described by Izquierdo et al. (1992). To the extracted total lipids, 1 ml of toluene with BHT (50 mg L^{-1}) and 2 ml of 1 % methanol:sulphuric were added. The reaction took place under nitrogen atmosphere and dark, during 16 h at 50 °C. Once that the sample is cold, 3.5 ml of ultra pure water and 4 ml of hexane:dietil eter (1:1) with 0.01 % BHT were added. The solution was centrifuged then for 5 min at 2,000 rpm. The top layer was then transferred to a fresh tube and 4 ml of hexane:dietil eter (1:1) with 0.01 % BHT added and centrifuged. The top layer was mixed with the previous extracted top layer. Then 3 ml of 2 % $KHCO_3$ were added, shaken

and the top phase separated. Fatty acid methyl esters were filtered through NH_2 Sep-pack cartridges (Waters S.A., Massachusetts, USA) using 4-6 ml hexane. Finally, the samples were dried under nitrogen atmosphere. Fatty acid methyl esters were separated in a Supercolvax-10-fused silica capillary column (length:30 mm, internal diameter: 0.32 mm; Supelco, Bellefonte, USA) by GLC (GC-14A, Shimadzu, Tokyo, Japan) with helium as a carrier gas. The temperature of the column was 180 ºC for 10 min, then increased 2.5 ºC per min until achieving 215 ºC and held for 10 min. The quantification of the FAMES was performed by Flame ionization detector (FIED) and identified using external standards.

2.5.6. Thiobarbituric reactive substances (TBARS)

TBARS were measured in triplicate from extracted total lipids (10 mg ml^{-1}) according to Burk et al. (1980). 50 µl of 0.2 % (w/v) BHT in ethanol was added to 200 µl of lipid. Following, 0.5 ml of 1 % (w/v) TBA in MiliQ distilled water and 0.5 ml of 10 % (w/v) trichloroacetic acid in MiliQ distilled water were added to each sample, all solutions were freshly prepared. All the reagents were mixed in a stoppered test tube and heated in dark at 100 °C for 20 min. Then, samples were cooled on ice for 5 min and particulate matter was removed by centrifugation at 2,000 r.p.m. (Sigma 4K15, Osterode am Harz, Germany) for 5 min. The supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm and recorded against a blank sample. 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} using the following formula:

$$nmol\ MDA\ /g\ tissue = \frac{Abs\ (532nm)}{0.156} * \frac{50}{sample\ weight}$$

2.5.7. Vitamin E

α-TOH concentration in tissue and diet was analysed by reverse phase high performance chromatography (HPLC) with UV detection at the University of Stirling (Scotland, UK).

2.5.7.1. Vitamin E concentration in tissue

α-TOH content in tissues was analysed in triplicate according to Cowey et al. (1981). Each sample was weighed (0.5-1 g) directly in a test tube and 5 ml 2 % (w/v) ethanolic pyrogallol added as an antioxidant to avoid α-TOH degradation during the process. The sample was homogenized with the Ultra turrax (IKA-Werke, T25 BASIC, Staufen, Germany) and incubated in a water bath for 5 min at 70 ºC. Samples were cooled on ice and 1 ml 60 % KOH added. The tubes were then flushed with nitrogen, closed, shaken and put in a water bath at 70 ºC for 20 min, samples had to be manually shaken every 5 min. After the bath, samples were cooled on ice and 4 ml of distilled water and 6 ml iso-hexane + BHT added. Samples were whirl-mixed for exactly 1 min in order to extract the α-TOH into the iso-hexane layer. 4 ml of the iso-hexane layer were removed into another tube using glass pipettes. Finally, samples were evaporated on a nitrogen evaporator up to dryness. α-TOH was then re-dissolved in 1 ml ethanol and transferred to 2 ml glass vials prior to analysis by HPLC. All vials were capped with nitrogen and placed in the fridge for storage.

2.5.7.2. Vitamin E concentration in feeds

α-TOH content in feeds was analysed in triplicate according to McMurray et al. (1980). Approximately 1 g of feed was weighed directly in a test tube and 25 ml 6 % (w/v) ethanolic pyrogallol added. The sample was then homogenized with an Ultra turrax (IKA-Werke, T25 BASIC, Staufen, Germany) and incubated in a water bath for 5 min at 70 ºC. Samples were cooled on ice and 5 ml 60 % KOH added. The tubes were flushed with nitrogen, stopper added, shaken and put in a water bath at 70 ºC for further 10 min, samples had to be manually shaken every 5 min. After the bath, samples were cooled on ice and 40 ml distilled water and 30 ml diluted iso-hexane + BHT added. Samples were whirl-mixed for exactly 1 min in order to extract the α-TOH into the iso-hexane top layer. 20 ml from the iso-hexane layer were removed into a fresh tube using a glass pipette. Finally, samples were evaporated on a nitrogen evaporator to dryness. α-TOH was then re-dissolved in 5 ml of ethanol. 7 ml of the extract was transferred to a 7 ml glass vial prior to analysis by HPLC. All vials were capped with nitrogen and stored in a fridge, in the dark until injection on the HPLC.

2.5.7.3. HPLC conditions for quantification of vitamin E

HPLC analysis was performed using a 150 x 4.60 mm, 5 µm reverse-phase Luna and C18 column (Phenomenox, CA, USA). The mobile phase was methanol:ultrapure water (98:2 v/v) with a flow rate of 1.0 ml min^{-1} at room temperature, with a pressure that should not exceed 1,000 psi. The volume injection was 50 µl. A UV detection wavelength of 293 nm was used to determine the α-TOH concentrations and was achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich) as the external standard. The elution order was δ-tocopherol, β-tocopherol, γ-tocopherol and α-tocopherol and the retention time of α-tocopherol was estimated approximately at 14 min. The concentration of α-TOH in feeds or tissues was calculated as follow:

$$Vitamin\ E\ (\mu g) = std\ conc\ (\mu g) * \frac{peak\ area}{avar.std.area} * \frac{V.iso-hexane}{V.layer\ removed} * \frac{V.MeOH}{V.injected} * \frac{1}{weight}$$

2.5.8. Vitamin C

The extraction of ASA (ascorbyl-2-monophosphate) form feeds is performed by using a phosphate buffer and quantification by reversed-phase HPLC with UV detection at the University of Stirling (Scotland, UK), as described by Roche Vitamins Ltd.

2.5.8.1. Vitamin C in feeds

Before starting extraction of ASA from feeds it was necessary to prepare the extraction solution (0.4 M phosphate buffer, pH 3.0) and the standard solution. The extraction solution was prepared dissolving 54 g of KH_2PO_4 in 900 ml of filtered water and adjusting pH to 3 using o-phosphoric acid. Finally, the solution was diluted to 1,000 ml with filtered water. 5 g of each feed was weighed into a 100 ml conical flask and 50 ml of 0.4 M phosphate buffer pH 3.0 added. The samples were stirred for 15 min at room temperature and 2 aliquots of 1.5 ml transferred into an Eppendorf tube and centrifuged for 50 min at room temperature at 3,000 r.p.m. The resulting supernatant was transferred into a 7 ml glass bottle and then 1 ml removed with a disposable syringe and filtered

through a disposable 0.45 µm filter unit into a 2 ml glass bottle. Vials were kept at dark in fridge (4 ºC) until HPLC analysis.

2.5.8.2. HPLC conditions for quantification of vitamin C

The mobile phase for HPLC quantification of ASA was composed by two eluents, 30 % of eluent 1 and 70 % of eluent 2 with a flow rate of 0.8 ml min^{-1}. Eluent 1 was prepared with 21.6 g of KH_2PO_2 and 1.8 L of filtered water. 4 ml of 1.5-dimethylhexylamine were added and pH adjusted to 3.0 with phosphoric acid at room temperature. The solution was filtered through a white nylon 0.45 µm filter to degas the solvent. The final volume was adjusted to 2 L with filtered water. Eluent 2 was prepared with 900 ml of eluent 1 and 140 ml of acetonitrile/ethanol 7:3 (v/v) at room temperature.

50 µl of ASA were injected in the HPLC with UV detection. A wavelength of 254 nm and a Gemini C18 column, 5 µm particula size and 150 x 4.6 mm fitted with a Gemini pre-column of the same material were used to determine the ASA concentration and was achieved by comparison with tris (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich) as the external standard.

2.5.8.3. Standard solution and calculations

The standard solution was prepared by weighing 15 mg of 2-phospho-L-ascorbic acid tri sodium salt into a 20 ml volumetric flask. It was dissolved and adjusted to a volume with 0.4 M phosphate buffer pH 3.00. Then, 1 ml from the stock solution was dilued to 25 ml using 0.4 M phosphate buffer pH 3.0. to make approximately a 0.1 mM solution. Tris-(cyclohexylammonium) ascorbic acid-2phosphate is used as a reference substance. The concentration of ascorbyl-2-monophosphate (C_{amp}) was calculated according to the following formula:

$$\text{Camp } [\mu g/ml] = \frac{\text{mst} * 100}{500} * \frac{P}{100} * 0.463$$

mst = weight of Tris-(cyclohexylammonium) ascorbic acid-2-phosphate (mg).

1.000 = conversion from mg to µg.

500 = dilution factor.

P = purity of Tris-(cyclohexylammonium) ascorbic acid-2-phosphate (%).

100 = conversion of % purity.

0.463 = conversion from Tris-(cyclohexylammonium) ascorbic acid-2-phosphate (MW = 553.7)

Calculation of the response factor is performed according the following formula:

$$RFamp = \frac{Ast}{Camp}$$

RFamp = calculated response factor of ascorbyl-2-monophosphate (mVsml/µg).

Ast = mean peak area from injection of working standard solution (mVs).

Camp = calculate concentration of ascorbyl-2-monophosphate.

The experimental determinations were given as ascorbic equivalents (AAE) as follows:

$$Camp = \frac{Asa}{RFamp * Msa} * \frac{V1 * V3}{V2}$$

$$CAAE = Camp * \frac{176.1}{256.1}$$

CAAE = Content of ascorbic acid equivalents in sample (mg/kg).

Asa = Area obtained from the infection of the sample (mVs).

Msa = weight of the sample (g).

V1 = volume of the sample extract (ml).

V2 = volume of the aliquot taken for dilution (ml).

V3 = volume of the diluted sample solution (ml).

176.1 = MW ascorbic acid (g/mol).

256.1 = MW of ascorbyl-2-monophosphate (g/mol).

0.6876 = conversion factor of AMP to AAE.

2.5.9. Vitamin K in feeds

The concentration of vitamin K was determined in diets as described by Billedeau (1989). 1 g of sample was weighed, homogenized and dissolved in dichloromethane (10 ml) on a shaker for 30 min. The samples were centrifuged at 1,000 g for 10 min and supernatants removed to a 30 ml tube. 10 ml of 5 % sodium carbonated diluted in deionized water and 10 ml of n-pentane was added to the sample and centrifuged at 1,000 g for 1 minute. This step was repeated 2 more times and the upper n-pentane layer was transferred to a 30 ml tube and evaporated to dryness under nitrogen. Then, the sample was dissolved in 1 ml methanol, filtered through a disposable 0.45 µm filter and stored at 4° C in dark until the measurement in a gas chromatography–mass spectrometer (GC-MS).

2.5.10. Minerals

The mineral analysis of Zinc, Selenium and Manganese of the experimental diets was performed at NIFES (Bergen, Norway). Approximately 0.2 g of diet was digested in 2 mL of HNO_3 (69 % w/w) and 0.5 mL of H_2O_2 (20 % w/w) in a microwave system (Julshamn et al., 2007). The digested sample was diluted to a final volume of 25 mL with Milli-Q water. The analysis was done by inductively coupled plasma mass spectrometry (ICP-MS; iCAP-Q and FAST SC-4Q DX auto sampler, both Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) after acidic digestion of the feeds.

2.6. Molecular detection of pathogens

2.6.1. DNA extraction

Formalin fixed tissues (liver and kidney) and formalin fixed paraffin-embedded tissues (liver, heart, spleen, kidney) were sent to the University of Stirling for Polymerase chain reaction (PCR) identification of Nocardia. A Nocardia positive formalin fixed paraffin-embedded tissue block from a previous confirmed case was included. DNA was extracted from approximately 25 mg of formalin fixed tissues using Dneasy® Blood and Tissue Kit (Qiagen) following Elkesh et al. (2013). This kit lyses the samples using a proteinase K, then the lysate is loaded onto a Dneasy Mini spin column, the contaminants and enzymes inhibitors removed in two washing steps and DNA ready to elute and use.

DNA extraction was performed from 25 mg of FFBE. The FFBE tissues sections were then dewaxed in 1.5 mL xylene, vortexed and centrifuged at 9,500 r.p.m. for 5 min, before the xylene was removed and replaced with absolute ethanol (1.5 mL). The samples were then centrifuged at 9,500 r.p.m. for 5 min, the supernatant removed, and the pellets kept. The xylene/alcohol washing step was repeated before the residual alcohol could evaporate. Samples were incubated at 37 °C for 10-15 min, until ethanol was fully evaporated. The samples were then processed using the Dneasy® Blood and Tissue Kit. 20 µl proteinase K were added, the tubes mixed and incubated at 56 °C until tissues were completely lysed (1-3 h). Samples were then vortexed for 15 s and 200 µl Buffer AL (Dneasy® Blood and Tissue Kit) added and tubes vortexed again. Then, 200 µl ethanol were added and mixed. The mix was transferred into a DNeasy Mini spin column and centrifuged at 8,000 r.p.m. for 1 min. The flow-through was discarded. 50 µl Buffer AW1 (Dneasy® Blood and Tissue Kit) were added to the columns, centrifuged 1 min at 8,000 r.p.m. and the flow-through discarded. Then 500 µl of Buffer AW2 (Dneasy® Blood and Tissue Kit) were added, centrifuged for 30 min at 14,000 r.p.m. and the flow-through discarded. Finally, the column was placed in a clean 2 ml tube and 200 µl Buffer AE (Dneasy® Blood and Tissue Kit) added directly onto the DNeasy membrane, incubated at room temperature for 1 min and centrifuged at 8,000 r.p.m. for 1 min to elute.

DNA from reference Nocardia strains was used as positive control. Bacterial DNA was extracted from *N. kampachi* NCIMB 2057 following the crude boiling methods of

Seward et al. (1997). One millilitre of a bacterial suspension was grown to mid-logarithmic phase growth in Brain Heart Infusion broth (BHIB), centrifuged at 2,000 r.p.m. for 15 min, the pellet washed in 1 mL of STE buffer (0.1 M NaCl, 10 nM Tris pH 8.0, 1 mM EDTA) and re-suspended in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA). The cell suspension was heated to 95 °C for 15 min, allowed to cool on ice and centrifuged to remove cellular debris. The DNA concentration was measured using a Nanodrop spectrophotometer ND-100 (Labtech International) and then kept at -20 °C until required.

2.6.2. PCR identification

The PCR was performed using MyTaq™ HS Mix (Bioline, UK). Two sets of 16S rRNA-specific Nocardia genus primers described by Laurent et al. (1999) were used; NG1: 5′-ACCGACCACAAGGGG-3′ and the reverse primer NG2: 5′-GGTTGTAAACCTCTTTCGA-3′. The primers were expected to develop a 600 bp size band. The samples were subjected to 30 cycles, in a thermocycler (Tgradient, Biometra, Goettingen, Germany), initial denaturing for 1 min at 95 °C followed by 95 °C for 30 seconds, annealing at 55 °C for 20 seconds and 72 °C for 10 seconds. After 30 cycles of amplification, 6 µL of the PCR products was run on a 1.5 % agarose gel using Tris-acetate-EDTA (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) buffer and visualized by ethidium bromide staining.

2.7. Gene expression

Molecular analysis of kidney, liver and heart samples were analyzed at the University of Stirling (Scotland, UK) (Chapter 3) and at the ECOAQUA facilities at University of Las Palmas de Gran Canaria (ULPGC; Telde, Canary Islands, Spain) (Chapter 6). Samples stabilized in RNALater were stored at -80 °C until further analysis.

2.7.1. RNA extraction

Total RNA was extracted from, approximately, 60-100 mg using kit-RNeasy (Quiagen). Samples were weighted in a 2 ml Eppendorf and 4-6 small glass beads added

to each Eppendorf, depending on the organ, together with 1 ml of TRI Reagent® (Sigma, St Louis, MO, USA). Samples were homogenized in cold by shaking 3-5 times for 2 min with a tissuelyser (tissuelyser II, Quiagen) at a frequency of 30 Hz. Once samples were well homogenized, were centrifugated at 4 °C for 10 min at 12,000 r.p.m. and left 5 minutes at room temperature. In the lower layer was the insoluble material (extracellular membranes, polysaccharides, and high molecular mass DNA) whereas the supernatant contains RNA and protein. 200 µl of chloroform were added, the tube shaken vigorously for 15 seconds, and allowed to stand for 2–15 min at room temperature. The solution was centrifuged at 4 °C for 10 min at 12,000 r.p.m. Centrifugation separates the mixture into 3 phases: a red bottom organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA).

The aqueous phase with the RNA was transferred to a clean tube, 0.5 ml of 2-propanol 70 % added and left for 5 min at room temperature. The sample was filtered through a spin column at room temperature for 15 seconds at 8,000 r.p.m. Samples were washed with 700 µl or RWi buffer (kit-RNeasy Quiagen) and centrifuged 1 min at 10,000 r.p.m. Then 500 µl of RPE buffer (kit-RNeasy Quiagen) were added and centrifuged twice for 2 min at 10,000 r.p.m. Finally, 50 µl of RNase-free water was added to the samples, let to stand for 5 min at room temperature and the RNA precipitated will form a pellet on the side and bottom of the tube.

2.7.2. RNA quality and quantity check

RNA purity was assessed by spectrophotometry (A260/A280), using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). The ratio of absorbance at 260 nm and 280 nm is used to assess the purity of RNA, considering a ratio of ~2.0 as "pure" for RNA. A lower ratio could indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm (William et al., 1997). Moreover, to assess the integrity of total RNA, an aliquot of 2 µg of total RNA was run on a denaturing 1.4 % agarose gel stained with GelRed ™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). The 1.4 % agarose gel was prepared with 100 ml of 1 X TBE (Tris-borate-EDTA) and 1.4 g of agarose, the mix was heated at the microwave and manually shaken, until the gel was completely homogenised. Before running the gel, RNA was denaturalized at 65 °C for 10 min to separate both subunits of RNA (28s and

18s). The RNA molecules tend to form secondary structures, which run badly in the electrophoresis and do not form sharp bands, being necessary to denaturalize the RNA sample in order to determine if the sample is not degraded observing sharp ribosomal RNA bands in the gel. Samples were run at 200 V for 1 min and 60 V for 1 h using an electrophoresis cuvette (POWERPAC 300 V, Bio-Rad Laboratories S.A., California, EEUU). λ 125 was used as a marker and previously heated in a bath at 57 °C for 10 min. Finally, the gel was visualized on a Gel Doc™ EZ Imager UV-transilluminator (Bio-Rad Laboratories S.A., California, EEUU) (Figure 2.2).

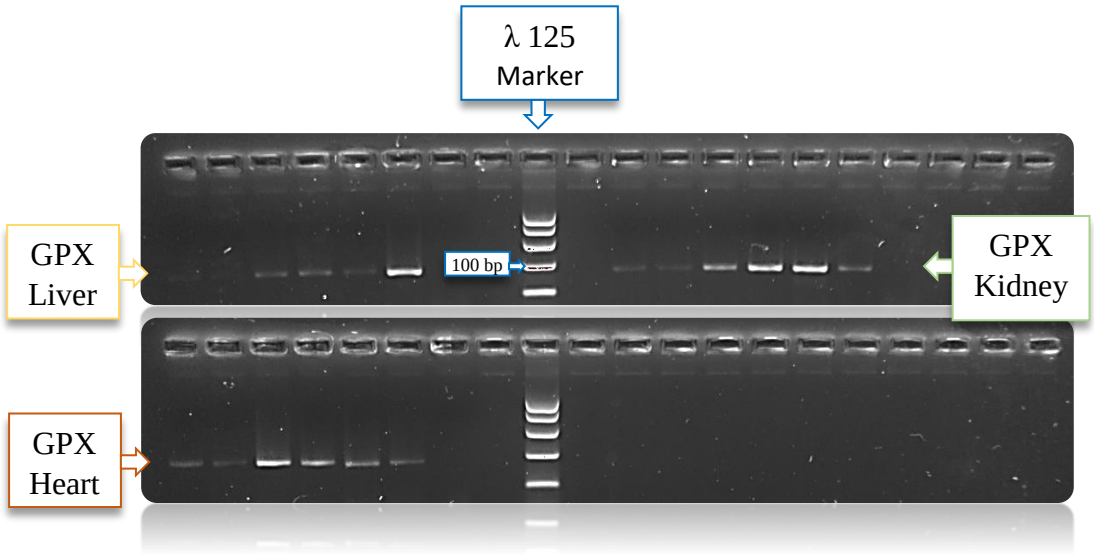

Figure 2.2. Agarose gel showing PCR products of glutathione peroxidase (103 bp) designed primers in liver, kidney and heart samples.

2.7.3. Synthesis of cDNA

The complementary DNA (cDNA) was synthetized from RNA using a reverse transcriptase. This reaction requires of primers, which can be either oligodT or random hexamers, nucleotides for DNA synthesis (dNTPs), MgC_2 and buffers required by the enzyme, all of them included in the iScript Reaction Mix (BIORAD, Hercules, California). cDNA was synthetized from 1 µg of total RNA using the iScript cDNA Synthesis Kit (BIORAD, Hercules, California) in 20 µl reactions, which included 4 µl 5× iScript Reaction Mix, 1 µl iScript Reverse Transcriptase (BIORAD, Hercules, California), 13 µl Milli-Q sterile water and 2 µl RNA (1 µg) of the sample. The reverse

transcription was done in a thermal cycler (iCycler, BIORAD, Hercules, California) at 25 °C for 5 min, 60 min at 42 °C and finally heating samples for 5 min at 85 °C.

2.7.4. Primers design and sequencing of the obtained products

Specific primers for glutathione peroxidase, superoxide dismutase and catalase were designed. The conserved regions of these genes had not been previously studied in meagre, being necessary to search conserved regions of each gene in various teleost species. The closer fish species, which have been sequenced for the genes of interest is *Larimichthys crocea*, and these were the sequences used to blast in the NCBI GeneBank in order to compare them with other teleost species. From all the results, the closer 10 sequences were aligned using MEGA 7 (Molecular Evolution Genetics Analysis) in order to identify the common conserved regions, where primers were designed using the program Primer3 (v. 0.4.0). Each set of primers was tested with a PCR, resulting products run on an agarose gel, and where a feasible band obtained sequenced (Figure 2.3). Specific RT-PCR primers were designed on the resulting sequences and tested again to ensure their specificity and efficiency (Table 2.3). Primers for gene expression of β-actin, tubulin, elongation factor 1α, tumor necrosis factor and cyclooxygenase were directly designed from a gene sequence of each of them for meagre present in the NCBI GeneBank (Table 2.3), it was not necessary to sequence these genes.

Table 2.3. Forward and reverse primers for sequencing or for real-time quantitative-PCR (5′–3′). The data include sequences and amplicon sizes.

Target	Primer 5´-3´	Fragment size (bp)
sod – Primer 1	F: TCACTTCAATCCCCACAACA	465
	R: ATTGCCTCCTTTTCCCAGAT	
sod – Primer 2	F: TCACTTCAATCCCCACAACA	203
	R: CTCCTTTTCCCAGATCGTCA	
gpx – Primer 1	F: GCCAGGTATGCTGAGAGAGG	522
	R: GCTGGTCTTTCAGCCACTTC	
gpx – Primer 2	F: CCTTGCCTTCCCTTCTAACC	161
	R: GCTGGTCTTTCAGCCACTTC	
cat- Primer 1	F: GCCAGAGACTTTGCCAGAAC	233
	R: TGGCATTTTAAGGCATCACA	
cat – Primer 2	F: AAACCACGGACCAGATGAAG	193
	R: TCTGGAATTCGTTCCCTGTC	

sod, superoxide dismutase; *gpx*, glutathione peroxidase; *cat*, catalase

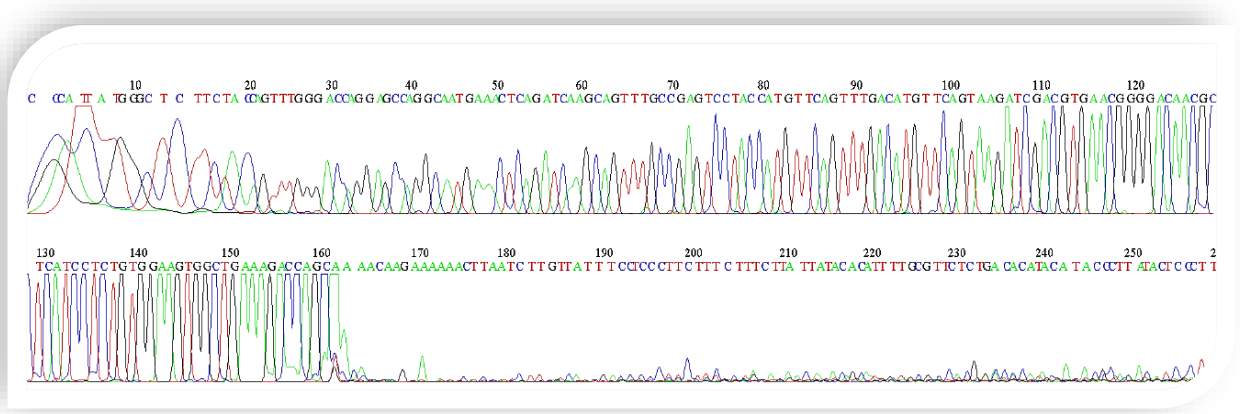

Figure 2.3. Sequenced fragment of glutathione peroxidase gene.

The sequences obtained, and the primers used for RT-PCR (underlined) were the following:

β-actin (*bact*; 455 bp; Tm 60 °C)

1- ccatcgagcacggtattgtgaccaactgggatgacatggagaagatctggcatcacaccttctacaacgagctcagagttg
> > > > > > > > > > > >
82- cccctgaggagcaccctgtcctgctcacagaggcccccctgaaccccaaagccaacagggagaagatgacccagat

160- catgttcgagaccttcaacacccctgccatgtacgttgccatccaggctgtgctgtccctgtatgcctctggtcgtaccac

244- tggtatcgtcatggactccggtgatggtgtgacccacacagtgcccatctacgagggttatgccctgccccatgccat

326- cctgcgtctggacttggccggccgcgacctcacagactacctcatgaagatcctgacagagcgcggttactccttca

406- ccaccacagccgagagggaaatcgtgcgtgacatcaaggagaagctg
< < < < < < < < < < < < < < <

Tubulin (*tub*; 161 bp, Tm 59 °C)

1- ggagtaccccgatcgtatcatgaacaccttcagtgtggtgccctctccaaaggtgtcagacacagtggtggaaccctacaa
> > > > > > > > > > > >
8- tgccaccctgtctgtccaccagctggttgagaacacagacgagacttactgcatcgacagcgaggccctgtatgacatct
< < < < < < < < < < < <

Elongation factor 1α (*ef1a*; 196 bp, Tm 59 °C)

1- ggtgctggacaaactgaaggccgagcgtgagcgtggtatcaccatcgatatcgctctgtggaagtttgagaccagcagg
> > > > > > > > > > > >
80- tactacgtgaccatcatcgatgcccctggacacagggacttcatcaagaacatgatcactggtacctctcaggctgactg

160- cgccgtgctgatcgttgctgctggtgttggtgagttc
< < < < < < < < < < <

Superoxide dismutase (*sod*; 207 bp, Tm 59 °C)

1- ggccctcacttcaatccctacagcaagaatcatgctggtcctactgatgcagacaggcatgttggagacctgggcaacgt
> > > > > > > > > > > >
81- gactgcaggggcagataatgttgccaagatcagcatcacggacagcatgctcactctcaccggccccatctccatcatt

161- ggaagaactatggtgatccacgagaaggccgacgatctgggaaaagga
< < < < < < < < < < <

Glutathione peroxidase (*gpx*; 103 bp, Tm 57 °C)

1- aagcagtttgccgagtcctaccatgttcagtttgacatgttcagtaagatcgacgtgaacggggacaacgctcatcctctgt
> > > > > > > > > > >
84- ggaagtggctgaaagaccagc
< < < < < < < < < < <

Catalase (*cat*; 205 bp, Tm 59°C)

1- gcttccaccaacccagattatgccatcggagacttgtttaacgccattgccaatgggaacttcccgtcctggactttctacat
> > > > > > > > > > >
84- ccaggttatgacctttgagcaggctgagaagttccagttcaacccattcgatcttaccaaggtttggtcacacaaagaata

167- ccctttgatccctgtgggcaaaatggtgctgaacaggaacc
< < < < < < < < < < <

Cyclooxygenase (*cox*-2; 211 bp, Tm 59 °C)

1- ggaagttggtgttgacatgcactacctcctcatgtccctgactctcaccgcttcgctgtgggtcacgaggctttcggcctg
> > > > > > > > > > > > > >
83- gtccccggtctgatgatgtacgccaccatctggctgcgggaacacaaccgggtgtgtgatgtgttgaaggaggttcacc

164- ctgactgggatgacgaaaggctcttccagaccacacggctcatcctgatt
< < < < < < < < < < < <

Tumor necrosis factor (*tnfα*; 173 bp, Tm 59 °C)

1- cacaagagcggccattcatttacaaggagaatacaaccctaacctgaccacatcagtggaatggaagaaccaggtggac
> > > > > > > > > > > > > > > >
80- cagtcctactctcaaggcgggctgaaactcgacaacaacgaaattgtgattcctcgtgatggcctctattccatctacagc
< < < < < < <
163- caagcgtctttcc
< < < < < < <

2.7.5. Primers efficiency

One of the very first steps to do when setting up a RT-PCR assay is to determine the primer efficiency. The ideal primer efficiency should be 100 %, which means that during the logarithmic phase of reaction, the PCR product is doubling with each cycle. An efficiency less than 100 % indicates that the number of replicated molecules is less than double. Poor efficiency could be due to a bad primer design or non-optimal reaction conditions. Also, secondary structures such as dimers and hairpins or inadequate melting temperatures (Tm) could affect he primer amplification. Efficiency of each interest primer, was determined from 7 dilutions of the pool of cDNA prepared with all the experimental samples (1/5, 1/10, 1/20, 1/50, 1/100, 1/200 and 1/500) using 10 µl Thermo Scientific Luminaris Color Higreen RT-PCR Master Mix (Thermo Fisher Scientific), 1 µl of forward and reverse primers (100 pmol μl^{-1}), 3 µl water nuclease-free and 5 µl of diluted cDNA, with the exception of the housekeeping genes, which were determined using 2 µL of diluted cDNA and 6 µl water nuclease-free, in a final volume of 20 µl. PCRs were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany). The PCR conditions were an uracil-DNA glycosylase pre-treatment at 50 °C for 2 min, a denaturation at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. The efficiency and sequence of each primer is shown in Table 2.4.

Table 2.4. Sequences for real-time quantitative-PCR forward and reverse primers (5′–3′). The data include sequences, efficiency, amplicon sizes and annealing temperatures (Tm).

Target	Primer 5´-3´	Fragment size (bp)	Efficiency (%)	Tm (°C)
sod	F: GGCCCTCACTTCAATCCCTA	207	96	59
	R: TCCTTTTCCCAGATCGTCGG			
gpx	F: AAGCAGTTTGCCGAGTCCTA	103	94	57
	R: GCTGGTCTTTCAGCCACTTC			
cat	F: GCTTCCACCAACCCAGATTA	205	98	59
	R: GGTTCCTGTTCAGCACCATT			
cox-2	F: GGAAGTTGGTGTTGACATGCACTAC	211	97	59
	R: AATCAGGATGAGCCGTGTGGTC			
tnfα	F: CACAAGAGCGGCCATTCATTTACAAGGAG	173	98	59
	R: GGAAAGACGCTTGGCTGTAGATGG			
bact	F: CCATCGAGCACGGTATTGT	455	95	60
	R: CAGCTTCTCCTTGATGTCACG			
tub	F: GGAGTACCCCGATCGTATCA	161	98	59
	R: AGATGTCATACAGGGCCTCG			
ef1a	F: GGTGCTGGACAAACTGAAGG	196	97	59
	R: GAACTCACCAACACCAGCAG			

sod, superoxide dismutase; *gpx*, glutathione peroxidase; *cat*, catalase; *tnfα*, tumor necrosis factor; *cox*-2, cyclooxygenase; *bact*, β-actin; *tub*, tubulin; *ef1a*, elongation factor 1α.

2.7.6. RT-PCR quantification

Real time PCR determines the changes in mRNA levels of a gene of interest (GOI) and the expression levels of each gene is normalized by the corresponding geometric average expression of a control gene (housekeeping, HK). To calculate the expression of the gene of interest in relation to the reference gene, the comparison method used was based in the cycle threshold (CT) values. CT values correspond to the number of cycles of PCR in which the detected fluorescence can be differentiated from the background noise. This value is inversely proportional to the amount of initial DNA and characteristic

of each of the reactions. This method is called Pfaffl method (Pfaffl et al., 2001) and computes an expression ratio based on RT-PCR efficiency and the crossing point deviation of the unknown sample versus a control group, with the following equation:

$$R = \frac{[(E_{target\ gene})^{\Delta CT\ target\ gene\ (mean\ control - mean\ unknown\ sample)}}{[(E_{reference\ gene})^{\Delta CT\ reference\ gene\ (mean\ control - mean\ unknown\ sample)}}$$

E = PCR efficiency

ΔCT = Crossing point deviation of an unknown sample versus a control

All RT-PCRs were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate using 10 µl Thermo Scientific Luminaris Color Higreen RT-PCR Master Mix (Thermo Fisher Scientific), 1 µl of forward and reverse primers (100 pmol μl^{-1}), 6 µl water nuclease-free and 5 µl of a 1:10 dilution of the cDNA, with the exception of the reference genes, which were determined using 2 µL of cDNA, in a final volume of 20 µl. In addition, amplifications were carried out with a systematic negative control (NTC-non template control) containing no cDNA.

The PCR conditions were an uracil-DNA glycosylase pre-treatment at 50 °C for 2 min, a denaturation at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. Expression level of each gene was normalized by the corresponding geometric average expression of β-actin, elongation factor 1α and tubulin, which were chosen as the most stable according to GeNorm.

2.8. Statistical analysis

All statistical analyses were performed with Statgraphics (Statgraphics Centurion XVI version 16.1.03 for Windows; Graphic Software Systems, Inc. USA). All data was expressed as a mean ± standard deviation (SD), unless indicated differently. A significance level of 5 % ($P<0.05$) was used in all tests performed. The normality was checked with the Kolmogorov Smirnov test. The homogeneity of variance was checked with the Levene test. For those variables satisfying the normality and homogeneity a parametric statistical analysis was carried out. The one-way analysis of variance

(ANOVA) was used to determine if there were statistically significant differences between the means of three or more groups. When statistical differences were present, a post hoc multiple comparison test (Tukey test) was used in order to determine where the differences were. A t-Student test was performed to examine the differences between two independent and small samples ($n < 30$) (This analysis was performed to study the differences in the same diets with or without vitamin K, Chapter 3). Two-way ANOVA was performed to determine if the values of a dependent variable, depended on the levels of two different factor or the interaction between both (stock density x mineral and vitamin supplementation, Chapter 6; α-TOH and ASA x vitamin K, Chapter 3; live prey x enrichment, Chapter 4).

For non-parametric variables, data which did not display a normal distribution and homogeneity of variance, a Kruskal-Wallis test was used. Dependent variable with significant differences were further tested using squared Chi *post-hoc* test.

Chapter 3

Dietary combination of vitamin E, C and K affects growth, antioxidant activity, and the incidence of systemic granulomatosis in meagre (*Argyrosomus regius*)

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Abstract

Systemic granulomatosis is a growing disease with a high morbidity, which affects to the majority of farmed meagre (*Argyrosomus regius*). The impossibility of isolating any infectious agents has hypothesized a nutritional origin of the disease. In order to try to elucidate the nutritional origin of granulomas, juvenile meagre were fed for 15 weeks with six diets containing different levels of vitamin E and C and with or without addition of vitamin K: Diet 0 (basal premix, no K, 150 mg kg^{-1} E, 20 mg kg^{-1} C), K (added 23 mg kg^{-1} K), EC (300 mg kg^{-1} E, 70 mg kg^{-1} C), KEC (23 mg kg^{-1} K, 300 mg kg^{-1} E, 70 mg kg^{-1} C), EECC (450 mg kg^{-1} E, 230 mg kg^{-1} C) and KEECC (23 mg kg^{-1} K, 450 mg kg^{-1} E, 230 mg kg^{-1} C). The diet EC significantly increased meagre growth in terms of final weight and length. Fish fed the highest levels of vitamin E and C presented lower percentage of granulomas in liver and heart than fish fed diet 0. The scored severity of granulomatosis in liver and kidney (main affected organs) tended to be lower with dietary increase of vitamin E, C and addition of vitamin K (from 1.83 diet 0 to 1.3 diet KEECC and from 0.91 diet 0 to 0.39 diet KEECC). In liver, the diet KEECC significantly increased catalase expression compared with diet 0. In kidney *tnfα* expression was significantly up-regulated in fish fed diet EECC and KEECC. In heart, low vitamin E and C levels (300 and 70 mg kg^{-1}, respectively) significantly increased superoxide dismutase and glutathione peroxidase expression and high addition increased the expression of *tnfα* and *cox*-2 (0 or 23 mg kg^{-1} K, 450 mg kg^{-1} E and 230 mg kg^{-1} C, diet EECC and KEECC). The results show that combination of high and 230 mg kg^{-1}, respectively) influenced in the incidence of the granulomatosis, which suggests that this pathology could be mediated by nutritional factors.

Keywords:

Granulomatosis, oxidative stress, meagre, juvenile, antioxidant vitamins

3.1. Introduction

Meagre, *Argyrosomus regius* (Asso, 1801), is a teleost species belonging to the family *Sciaenidae* which is found in the Mediterranean, the Black Sea and along the Atlantic coasts of Europe and the West coast of Africa (Chao, 1986; Haffray et al., 2012). The meagre is a species with great potential for the diversification of the Mediterranean aquaculture production due to its high flesh quality and good flavour (Poli et al., 2003), rapid growth and good feed conversion rates (0.9 to 1.2; Jiménez et al., 2005; Duncan et al., 2013) as well as good growth at a wide range of salinities (5-45 g L^{-1}) (Márquez, 2010). It also provides low-fat flesh even under intensive farming conditions (Piccolo et al., 2008) and has a great capacity to adapt to captivity (El-Shebly et al., 2007).

However, in the intensive culture of meagre, the main concern for commercial production is the occurrence of pathologies. Infectious diseases caused by trematodes (Hayward et al., 2007; Toksen et al., 2007; Duncan et al., 2008), nematodes (Moravec et al., 2007) and bacteria (Sorroza et al., 2012) have all been described in meagre. Furthermore, the majority of farmed populations are affected by systemic granulomatosis, which is the pathology with largest impact on meagre culture (Ghittino et al., 2004). Systemic granulomatosis is characterized by the presence of multiple granulomas in internal organs, which progressively produces a necrotic centre surrounded by a layer of epithelial cells and macrophages. This disease mostly affects the kidney and liver, where macroscopic nodules of varying diameter usually are observed. In later stages these nodules can also appear in other tissues such as spleen, heart, skin and eyes, and can lead to exophthalmia and cataracts (Ghittino et al., 2004).

Granulomas can be produced by pathogens such as *Mycobacterium* spp. and *Nocardia* sp. (Bowser, 2009; Labrie, 2008). Elkesh et al. (2013) described the first report of nocardiosis in a Mediterranean population of cultured meagre. Nevertheless, in other fish species the non-detection of any pathogens associated to granulomas has supported the hypothesis that a connection exists between systemic granulomatosis and a nutritional imbalance. Thus, older works have reported a deficiency of vitamin C in the diet (Paperna et al., 1980; Tixerant et al., 1984) or a dietary mineral imbalance (Dunbar and Herman 1971) as the most common cause associated to systemic granulomatosis.

Vitamin E and C can modulate inflammatory reactions related to nuclear factor kappaB (Han et al., 2004; Poppe et al., 2013), which is responsible of the up-regulation of inflammatory cytokines, such as tumor necrosis factor (*tnfα*) and cyclooxygenase (*cox*-2) (Fox et al., 1997). TNFα is a crucial regulator and effector in the process of mounting innate and adaptive immune responses, regulating cell death and survival (Locksley et al., 2001), while COX-2 is a prostaglandin synthesis enzyme that plays a key role in inflammation in fish (Ishikawa et al., 2007ab) and is responsible for conversion of arachidonic acid into prostaglandin, related to the fish innate immune response (Xu et al., 2008; Legler et al., 2010).

Vitamin C is a water-soluble vitamin involved in the biosynthesis of pro-collagen, growth, immune response, malformations, susceptibility to bacterial infections and reproduction among other functions (Kumari and Sahoo, 2005; Zhou et al., 2012). Vitamin C together with vitamin E (α-tocopherol) and the endogenous enzymatic antioxidant mechanisms, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) has a strong powerful antioxidant effect in tissues being able to neutralize reactive oxygen species (ROS). At low concentrations, ROS may be beneficial or even indispensable in processes such as defence against micro-organisms, contributing to phagocytic bactericidal activity. However, when an imbalance between ROS generation and ROS removal occurs, a state of oxidative stress arises (Nita, et al., 2016). This status may lead to the oxidation of various cellular constituents like lipids, proteins or DNA, causing alterations that produce a range of cellular damages which can ultimately lead to cell death (Halliwell and Gutteridge, 1995). Limited information is available on the effect of antioxidant vitamins in the formation of granulomas. In fish, it has been suggested that a deficiency of vitamin C causes an impairment of tyrosine catabolism, which leads to its precipitation in tissues and thereby cause development of the granulomas (Paperna et al., 1980; Tixerant et al., 1984). In rainbow trout (*Oncorhynchus mykiss*) granulomas of unknown aetiology have been hypothesized to be caused by a dietary mineral imbalance, which would lead to precipitation of calcium phosphate or calcium carbonate (Dunbar and Herman 1971). More recently, granulomas of unknown aetiology have also been described in Atlantic salmon (*Salmo salar*) (Good et al., 2015).

Recently, our research group has found a strong effect of vitamin K related to systemic granulomatosis occurrence as diets without vitamin K supplementation

produced higher incidence of hepatic granulomas in meagre larvae (Vidal et al., 2016). The effects of dietary inclusion of vitamin D_3 have also been studied in the development of systemic granulomatosis in juvenile meagre, and a negative correlation was found between the appearance of granulomas and levels of sterol 27-hydroxylase, a carrier of a metabolite of vitamin D_3 (Cotou et al., 2016). These findings may support a non-infectious origin of granulomatosis in this fish species. Thus, further studies are required in order to determine whether this disease can be ameliorated through nutrition.

The overarching aim of this study was to elucidate the involvement of the dietary vitamins E, C and K on the appearance and incidence of systemic granulomatosis in meagre. To reach this objective, diets containing graded levels of the three vitamins were fed to juvenile meagre to determine effects on growth, survival, histopathology as well as fish composition and gene expression of antioxidant enzymes and immune response genes.

3.2. Materials and methods

3.2.1. Fish and feeds

The experiment was carried out at the ECOAQUA facilities (Taliarte, Canary Islands, Spain). The juvenile meagre were obtained from induced spawns at the ECOAQUA facilities from brood stock adapted to farming conditions. Prior to the start of the feeding trial, fish were fed with a commercial diet (Skretting, Burgos, Spain) for 14 days to acclimatize to the experimental conditions. Fish with an initial mean weight of 79.3 ± 0.5 g were transferred to 18 fibre glass tanks of 500 L with 50 fish per tank at an initial stocking density of 7.9 kg m^{-3}. All tanks were covered with a net to prevent escapes. The temperature and dissolved oxygen concentration were measured twice a week with values ranging from 17.6 to 21.6° C and 5.8 to 6.6 mg L^{-1}, respectively.

Six isolipidic (16 % lipid) and isoproteic (50 % protein) fish meal and fish oil based diets were produced as extruded 3 mm pellets by Skretting ARC Feed Technology Plant (Stavanger, Norway) (Table 3.1). The basal diet (Diet 0) contained a vitamin premix with no vitamin K and is used in the present trial to bench-mark the experimental feeds, but not to generate granulomas due to the depletion of antioxidant vitamins. The experimental diets were obtained by supplementing the basal diet with 50 or 200 mg kg^{-}

[1] vitamin C, 150 or 300 mg kg^{-1} vitamin E and/or 23 mg kg^{-1} vitamin K. Since vitamin K is very heat labile, this component was coated on the final feed in a cement mixer and sealed with 0.5 % fish oil. Also diets without vitamin K supplementation were added 0.5 % fish oil in the cement coater. This resulted in the following combinations of vitamin supplementation: Diet K (23 mg kg^{-1} vitamin K), Diet EC (150 mg kg^{-1} and 50 mg kg^{-1} vitamin E and C, respectively), Diet KEC (23 mg kg^{-1}, 150 mg kg^{-1} and 50 mg kg^{-1} vitamin K, E and C, respectively), Diet EECC (300 mg kg^{-1} and 210 mg kg^{-1} of vitamin E and C, respectively), Diet KEECC (23 mg kg^{-1}, 300 mg kg^{-1} and 210 mg kg^{-1} vitamin K, E and C, respectively). The analysed dietary contents of vitamins K, E and C for each treatment are shown in Table 3.1.

The experimental diets were fed to fish in triplicate tanks of fish to satiation 3 times per day (8:00, 11:30, 15:00), 6 days per week for 15 weeks. All the uneaten feed was collected daily from each tank and dried in order to calculate the daily feed intake. Dead fish were recorded and removed daily and survival determined.

Table 3.1. Feed formulation in g kg^{-1}. Diet codes are according to vitamins supplemented to the basal diet (Diet 0).

	Diets					
Ingredients	0	K	EC	KEC	EECC	KEECC
Wheat[1]	15.97	15.97	15.97	15.97	15.97	15.97
Wheat gluten[1]	16.50	16.50	16.50	16.50	16.50	16.50
Soy protein concentrate[1]	16.64	16.64	16.64	16.64	16.64	16.64
Faba beans whole[1]	5.00	5.00	5.00	5.00	5.00	5.00
Fish meal, N-Atlantic[1]	35.00	35.00	35.00	35.00	35.00	35.00
Fish oil, N-Atlantic[1]	10.26	10.26	10.26	10.26	10.26	10.26
Premixes[2]	0.68	0.68	0.68	0.68	0.68	0.68
Vitamin E[3*]	-	-	0.03	0.03	0.07	0.07
Vitamin C[4*]	-	-	0.01	0.01	0.06	0.06
Vitamin K[5*]	-	0.0035	-	0.0035	-	0.0035
Proximate composition (%)						
Lipid	17.9	17.6	19.5	17.4	17.7	16.8
Protein	48.8	49.6	48.7	48.6	48.7	48.5
Ash	6.8	7.2	6.5	7.3	6.6	7.3
Dry matter	91.3	91.7	90.9	92.2	91.8	91.6
Vitamin E (mg kg^{-1})	158.7	172.5	283.6	276.5	416.4	449.1
Vitamin C (mg kg^{-1})	16.6	19	71.1	72.4	227.0	240.0
Vitamin K (mg kg^{-1})	n.d.	23.0	n.d.	22.0	n.d.	23.0
Fatty acid (%)						
Total saturated[6]	4.4	4.5	5.4	4.4	3.7	4.3
Total monosaturated[7]	5.3	5.0	5.7	4.9	4.3	4.8
20:4n-6	0.1	0.1	0.1	0.1	0.4	0.1
Total n-6 PUFA[8]	1.4	1.3	1.5	1.3	1.6	1.3
20:5n-3	1.3	1.4	1.4	1.3	2.2	1.4
22:6n-3	1.8	1.7	1.7	1.9	1.5	1.6
Total n-3 PUFA[9]	4.4	4.3	4.4	4.4	5.9	4.1
Total PUFA[10]	6.0	5.9	6.1	6.0	7.8	5.6
Total n-3 LC-PUFA[11]	3.4	3.4	3.4	3.5	5.1	3.2

[1]Skretting, Stavanger, Norway; [2]Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins, but no vitamin K and minerals. Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011); [3]Lutavit E-50, Trouw Nutrition, Boxmeer, the Netherlands; [4]Lutavit C Aquastab 35%, Trouw Nutrition, Boxmeer, the Netherlands; [5]Menadione dimethypyrimidinol bisulfite 43.7%, Trouw Nutrition, Boxmeer, the Netherlands; [6]Includes 14:0, 15:0, 16:0 17:0, 18:0 and 20:0; [7]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 16:1n-7, 18:1n-5, 18:1n-7, 18:1n-9, 20:1n-9, 20:1n-7 and 20:1n-5; [8]Includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6; [9]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3 and 22:5n-3. [10]Includes C_{16} PUFA; [11]Includes 20:3n-3 and 20:4n-3; *Amount of active vitamin. Diets 0-KEECC represent feed with increasing levels of vitamin E, C and with or without vitamin K supplemented as described in Material and Methods section. n.d., not detected; PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA.

3.2.2. Growth performance

After 15 weeks of feeding the experimental diets, the fish were killed by an overdose of anaesthetic (clove oil) before they were individually weighed, length measured and samples collected for histopathology, biochemical analysis and gene expression measurements.

Performance parameters were calculated according to the following equations: Survival (%) = 100*(final number fish – initial number fish)/ initial number fish; Growth (%) = ((final mean weight – initial mean weight)/initial mean weight)*100; Weight gain = (final mean weight- initial mean weight); SGR (specific growth rate) = 100 x (ln final mean weight – ln initial mean weight)/ number of days; FI = feed intake (g)/fish per day; FCR (feed conversion ratio) = feed intake (g)/ weight gain (g); K (condition factor (%)) = 100*(fish weight/ (fish length)3); HSI (hepatosomatic index (%)) = 100*(liver weight / fish weight); VSI (viscerosomatic index (%)) = 100* (fish weight - eviscerated fish weight)/fish weight. The growth curve was determined following the model described by Cho (1992), in which TGC (thermical growth coefficient) = (final weight$^{1/3}$ – initial weigth$^{1/3}$)/ $\sum$ effective temperature (> 12 °C). Final weight = (initial weight$^{1/3}$ + ($\sum$ effective temperature * TGC))^3.

3.2.3. Sample collection

At the beginning (n = 50 fish from the stock tank) and at the end of the experimental trial (n = 21 fish per diet) fish were sacrificed with an overdose of anaesthetic and samples of liver, kidney, heart and spleen were collected and fixed in 4 % buffered formalin for histological analysis. Additionally, 5 fish per tank (n = 15 per treatment) were sacrificed and liver, heart and kidney removed and frozen at -20° C for proximate and vitamin E analysis. Ten fish per tank were also sacrificed and the same tissues collected, pooled, stabilized in RNA later (Sigma, Poole, UK) and stored at -80° C until RNA extraction.

3.2.4. Biochemical analysis

Chemical composition of feeds and fish were analysed following standard procedures. Lipids in liver, heart, kidney and feeds were extracted with a choloroform-

methanol (2:1 v/v) mixture as described by Folch et al. (1957). In feed, protein content (Kjeldahl method), dry matter and ash were determined according to Helrich (1990).

Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982). Fatty acid methyl esters (FAMES) were separated and quantified by gas–liquid chromatography following the conditions described by Izquierdo et al. (1992).

The concentration of vitamin E was determined in diets and fish tissues (liver, heart and kidney). Samples were weighed, homogenized in ethanolic pyrogallol and saponified as described by Cowey et al., 1981 for tissue and according to McMurray et al., 1980 for diets. HPLC analysis was performed using 150 x 4.60 mm, 5 µm reverse-phase Luna and C18 column (Phenomenox, CA, USA). The mobile phase was methanol:ultrapure water (98:2 v/v) with a flow rate of 1.0 ml min^{-1} at ambient temperature. Samples were injected (50 µl) in a high performance liquid chromatograph (HPLC) with UV detection at a wavelength of 293 nm to determine the vitamin E using (+)-α-tocopherol (Sigma-Aldrich) as the external standard.

The concentration of vitamin C was determined in the experimental feeds as described by Betancor et al. (2012). Samples were weighed, homogenised and dissolved in 0.4 M phosphate buffer (adjusted to pH 3.0 with phosphoric acid). The samples were centrifuged at 3.000 rpm, supernatants removed and filtered through a disposable 0.45 µm filter and stored at 4° C until the measurement in a HPLC with UV detection. The determination of vitamin C concentration was achieved by comparison with tris (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich) as the external standard.

The concentration of vitamin K was determined in diets as described by Billedeau (1989). 1 g of sample was weighed, homogenized and dissolved in dichloromethane (10 ml) on a shaker for 30 min. The samples were centrifuged at 1,000 g for 10 min and supernatants removed to a 30 ml tube. Ten ml of 5 % sodium carbonated diluted in deionized water and 10 ml of n-pentane was added to the sample and centrifuged at 1,000 g for 1 minute. This step was repeated 2 more times and the upper n-pentane layer was transferred to a 30 ml tube and evaporated to dryness under nitrogen. Then, the sample was dissolved in 1 ml methanol and filtered through a disposable 0.45 µm filter and stored at 4° C in dark until the measurement in a gas chromatography–mass spectrometer (GC-MS).

3.2.5. Histopathology

The samples were dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 μm, fixed to the microscope slide, heated and finally stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco method (Fite et al., 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological evaluation.

Additionally, an immunohistochemistry study was performed using monoclonal anti-actin as a primary antibody to mark smooth muscle. Liver paraffin sections in which granulomas had previously been identified by H&E were routinely dewaxed and rehydrated. All incubations were performed at room temperature in a humid chamber. After antigen retrieval (High pH, Dako, Denmark), endogenous peroxidase activity was blocked by Peroxidase Blocking Solution (Dako, Denmark) for 1 h. Sections were incubated for 2 h at room temperature with a primary rabbit monoclonal antibody anti-actin (diluted 1:200; clone HHF35; Enzo Diagnostic, USA). Immunohistochemical staining was carried out using horseradish peroxidase (HRP) anti-rabbit (EnVision; Dako, Denmark) and 3-amino, 9 ethyl-carbazole diluted in 0.1 M sodium acetate-buffer containing 3 % hydrogen peroxide. The slides were counterstained with Harris haematoxylin.

3.2.6. Histopathological scoring

A quantitative method was developed to classify the severity of granulomas in each organ in four different levels (score 0-3) depending on the number of granulomas observed. The score depends on the tissue, given that the number of granulomas observed in each organ was variable. The average severity was classified in liver, kidney and heart according to the criteria shown in Supplementary Table 3.1, Appendix 3.5.

3.2.7. DNA extraction

Formalin fixed tissues (liver, kidney) and formalin fixed paraffin-embedded tissues (FFBE, liver, heart, spleen, kidney) were sent to University of Stirling for PCR identification of *Nocardia* spp. A Nocardia positive formalin fixed paraffin-embedded tissue block from previous confirmed case was included. DNA was also extracted using

Dneasy® Blood and Tissue Kit (Qiagen) following Elkesh et al. (2013). Briefly, 25 mg of paraffin-embedded tissue sections and formalin fixed tissues were cut and placed into sterile 1.5 mL Eppendorf tubes. The FFBE tissues sections were then dewaxed in 1.5 mL xylene, vortexed and centrifuged at 9500 g for 5 min, before the xylene was removed and replaced with absolute ethanol (1.5 mL). The samples were then centrifuged at 9500 g for 5 min and the supernatant removed and the pellets kept. The xylene/alcohol washing step was repeated before the residual alcohol was allowed to evaporate. The samples were then processed using the Dneasy® Blood and Tissue Kit as previously described.

DNA from reference Nocardia strains was used as positive control. Bacterial DNA was extracted *N. kampachi* NCIMB 2057 following the crude boiling methods of Seward et al. (1997). One millilitre of a bacterial suspension grown to mid-logarithmic phase growth in Brain Heart Infusion broth (BHIB) was centrifuged at 2000 g for 15 min and the pellet washed in 1 mL of STE buffer (0.1 M NaCl, 10 nM Tris pH 8.0, 1 mM EDTA) and resuspended in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA). The cell suspension was heated to 95 °C for 15 min, allowed to cool in ice and centrifuged to remove cellular debris. The DNA concentration was measured using a Nanodrop spectrophotometer ND-100 (Labtech International) and kept at -20 °C until required.

3.2.8. PCR identification

PCR was performed using MyTaq™ HS Mix (Bioline, UK) Two sets of 16S rRNA-specific Nocardia genus primers described by Laurent et al. (1999) were used; NG1: 5′-ACCGACCACAAGGGG-3′ and the reverse primer NG2: 5′-GGTTGTAAACCTCTTTCGA-3′. The primers were expected to develop 600 bp size band. The samples were subjected to 30 cycles, in a DNA thermocycler (Tgradient, Biometra) initial denaturing for 1 min at 95° C followed by 95° C for 30 s, annealing at 55° C for 20 s and 72° C for 10 seconds. After 30 cycles of amplification, 6 µL of the PCR products was run on 1.5 % agarose gel using Tris-acetate-EDTA (40 mM Tris, 20 mM acetic acid, and 1 mM EDTA, pH 8.0) buffer and visualized by ethidium bromide staining.

3.2.9. Gene expression

Total RNA was extracted from, approximately, 100 mg of sample using TRI Reagent® (Sigma). Purity was assessed by spectrophotometry (A260/A280), followed by a visual quality assessment via agarose gel electrophoresis on 2 % agarose gel stained with GelRed ™ Nucleic Acid Gel Stain (Biotium).

The cDNA was synthetized from 1 μg of total RNA using the iScript cDNA Synthesis Kit (BIORAD) in 20 μl reactions, which included 4 μl 5× iScript Reaction Mix, 1 μl iScript Reverse Transcriptase (BIORAD), 13 μl Milli-Q sterile water and 2 μl RNA (1 μg) of the sample. The reverse transcription was done in a thermal cycler (iCycler) at 25° C for 5 min, 60 min at 42° C and finally heating the samples for 5 min at 85° C.

Specific primers to each gene were designed based on the alignment of conserved coding regions of the genes of interest of other teleost fish species, using Mega 7 software (Supplementary Table 3.2; Appendix 3.5).

The relative transcript abundance of glutathione peroxidase (*gpx*), superoxide dismutase (*sod*), catalase (*cat*), tumor necrosis factor (*tnfα*) and cyclooxygenase 2 (*cox*-2) was determined by quantitative real time PCR (qPCR). Primer efficiency for each gene was previously evaluated to ensure that it was close to 100 %. All PCRs were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate using 10 μl Thermo Scientific Luminaris Color Higreen qPCR Master Mix (Bio-Rad Hercules, California), 1 μl of forward and reverse primers (100 pmol μl^{-1}), 6 μl water nuclease-free and 5 μl of a 1:10 dilution of the cDNA, with the exception of the reference genes, which were determined using 2 μl of cDNA, in a final volume of 20 μl. In addition, amplifications were carried out with a systematic negative control (NTC-non template control) containing no cDNA.

The PCR conditions were an uracil-DNA glycosylase pre-treatment at 50° C for 2 min, a denaturation at 95° C for 10 min, followed by 35 cycles: 15 s at 95° C, 30 s at the annealing Tm and 30 s at 72° C. Expression level of each gene was normalized by the corresponding geometric average expression of β actin (*bactin*), elongation factor 1α (*ef1a*) and tubulin (*tub*), which were chosen as the most stable according to GeNorm (Supplementary Table 3.2; Appendix 3.5).

3.1.10. Statistical analysis

All statistical analyses were performed on Statgraphics Centurion XVI (Version 16.1.11, StatPoint Technologies, Inc., Herndon, VA). Data were tested for normality and homogeneity of variance with Levene's test prior to one-way analysis of variance (ANOVA) with Tukey post-hoc test or two-way ANOVA (Vitamin E and C x Vitamin K). In order to compare variables from two treatments, a t-student test was used for normally distributed variables and a Mann-Whitney test for the non-parametric ones. The tissue level of severity was evaluated in a frequency distribution manner. A significance level of 0.05 was used.

3.3. Results

3.3.1. Growth performance

Inclusion of different levels of dietary vitamins E, and C with or without vitamin K affected meagre final weight and length (Table 3.2). Juvenile meagre grew from and average weight of ~ 80 g to ~ 267 g in 15 weeks, with final fish weight very similar to that estimated by the growth curve model (Supplementary Figure 3.1; Appendix 3.5). The final body weight and length were significantly increased in fish fed with low addition of vitamin E and C (Diet EC), being only higher than fish fed diet 0 (without vitamin K and no extra supplementation of vitamin E and C) and diet K with only addition of vitamin K. Total body length was significantly higher in fish fed diets EC, however, these differences were not reflected in significant differences ($p > 0.05$) in specific growth rate (SGR), survival or biometric parameters (Table 3.2). The feed conversion ratio (FCR) was good in all dietary treatments (0.83-0.87) but without significant differences.

Table 3.2. Growth performance in meagre fed the experimental diets for 104 days.

	0	K	EC	KEC	EECC	KEECC	E+C	Vit. K	Vit. E+C x Vit. K
Initial weight (g)[1]	79.1 ± 0.6	79.3 ± 1.2	80.1 ± 1.3	79.5 ± 0.8	79.5 ± 1.0	79.5 ± 1.0	n.s.	n.s.	n.s.
Final weight (g)[1]	263.5 ± 6.8^{a}	258.3 ± 7.6^{a}	278.9 ± 9.8^{b}	265.7 ± 5.6^{ab}	266.2 ± 9.1^{ab}	271.0 ± 2.6^{ab}	*	n.s.	n.s.
Weight gain (%)[1]	332.8 ± 4.1^{a}	325.5 ± 2.5^{a}	344.1 ± 6.6^{b}	333.4 ± 4.7^{ab}	338.1 ± 5.7^{ab}	337.4 ± 7.6^{ab}	*	n.s.	n.s.
Length (cm)[1]	28.1 ± 1.6^{a}	28.1 ± 1.4^{a}	28.7 ± 1.6^{b}	28.2 ± 1.5^{ab}	28.3 ± 1.4^{ab}	28.2 ± 1.6^{ab}	*	n.s.	n.s.
TGC*10^{-3}	2.86 ± 0.0	2.81 ± 0.0	3.01 ± 0.0	2.88 ± 0.0	2.91 ± 0.0	2.90 ± 0.0	n.s.	n.s.	n.s.
FI (g)[2]	1.53 ± 0.1	1.49 ± 0.0	1.57 ± 0.0	1.52 ± 0.1	1.52 ± 0.1	1.53 ± 0.0	n.s.	n.s.	n.s.
FCR[2]	0.87 ± 0.1	0.87 ± 0.0	0.83 ± 0.0	0.87 ± 0.0	0.87 ± 0.0	0.84 ± 0.0	n.s.	n.s.	n.s.
SGR[2]	1.14 ± 0.1	1.12 ± 0.0	1.19 ± 0.0	1.15 ± 0.0	1.17 ± 0.0	1.16 ± 0.0	n.s.	n.s.	n.s.
Survival[2]	97.3 ± 4.6	100.0 ± 0.0	98.0 ± 2.0	96.7 ± 4.2	96.0 ± 5.3	98.0 ± 2.0	n.s.	n.s.	n.s.
HIS[2]	1.7 ± 0.4	1.9 ± 0.6	1.8 ± 0.4	1.7 ± 0.3	1.7 ± 0.2	1.7 ± 0.3	n.s.	n.s.	n.s.
VSI[2]	3.4 ± 0.7	3.4 ± 1.0	3.4 ± 0.4	3.4 ± 0.3	3.3 ± 0.4	3.3 ± 0.4	n.s.	n.s.	n.s.
CAI[2]	96.6 ± 0.6	96.6 ± 1.0	96.6 ± 0.4	96.8 ± 0.9	96.8 ± 0.4	96.7 ± 0.4	n.s.	n.s.	n.s.
K[2]	1.2 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.0	1.1 ± 0.1	1.1 ± 0.1	n.s.	n.s.	n.s.

Data are means ± SD, where the means in each column with a different superscript are significantly different according to one-way ANOVA ($P < 0.05$). [1]n=150. [2]n=3. TGC, thermal growth coefficient; FI, feed intake; FCR, feed conversion ratio; SGR, specific growth rate; HIS, hepatosomatic index; VSI, viscerosomatic index; CAI, canal index; K, condition factor; The last three columns indicate the effect of vitamins E and C (Vit. E+C), vitamin K (Vit. K) or their interaction (Vit. E+C x Vit. K) according to two-way ANOVA.

* $p < 0.05$.

** $p < 0.01$.

3.3.2. Tissue proximate content and fatty acid profiles

The dietary treatments did not affect lipid content in the analyzed tissues amounting to 2.7 %, 4.6 % and 24.4 % in heart, kidney and liver, respectively (Table 3.3, 3.4 and 3.5). Similarly, no differences were observed in protein, ash or dry weight among fish fed the experimental dietary treatments (Supplementary Table 3.3; Appendix 3.5). The fatty acid profile of the tissues of fish reflected the dietary fatty acid content (Table 3.1). The highest levels of total monounsaturated fatty acids were observed in liver, followed by kidney and heart, whereas the total omega-3 (n-3) and total polyunsaturated fatty acid (PUFA) were higher in the heart, followed by kidney and liver (Tables 3.3, 3.4 and 3.5, respectively). All the other fatty acids were similarly distributed in the three tissues.

Table 3.3. Heart fatty acid compositions (percentage of total fatty acids) of meagre fed diets with different levels of vitamin E, C and K during 104 days.

	0	K	EC	KEC	EECC	KEECC	Vit. E+C	Vit. K	Vit. E+C x Vit. K
Lipid content (%)	2.8 ± 0.2	2.9 ± 0.3	2.7 ± 0.8	2.7 ± 0.3	2.7 ± 0.1	2.6 ± 0.2	n.s.	n.s.	n.s.
14:0	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.5	1.3 ± 0.3	1.0 ± 0.1	1.1 ± 0.1	n.s.	n.s.	n.s.
16:0	18.9 ± 1.1 ^{ab}	20.8 ± 0.2^{b}	17.5 ± 0.5^{a}	20.9 ± 1.0^{b}	18.5 ± 0.4^{a}	20.8 ± 0.8^{b}	n.s.	*	n.s.
18:0	9.0 ± 0.7^{ab}	10.0 ± 0.3^{b}	7.9 ± 0.8^{a}	9.5 ± 0.8^{ab}	8.6 ± 0.6^{ab}	9.6 ± 0.8^{ab}	n.s.	*	n.s.
Total saturated[1]	29.5 ± 1.8^{ab}	32.5 ± 0.7^{b}	27.2 ± 0.8^{a}	32.3 ± 1.7^{b}	28.6 ± 0.4^{a}	32.1 ± 1.5^{b}	n.s.	*	n.s.
16:1n-7	1.2 ± 0.4	1.2 ± 0.1	1.4 ± 0.7	1.5 ± 0.3	1.1 ± 0.2	1.2 ± 0.1	n.s.	n.s.	n.s.
18:1n-9	7.7 ± 0.6	7.7 ± 0.6	8.0 ± 1.5	8.0 ± 0.6	7.1 ± 0.3	7.4 ± 0.3	n.s.	n.s.	n.s.
18:1n-7	3.0 ± 0.2^{ab}	3.2 ± 0.1^{b}	2.9 ± 0.1^{a}	3.2 ± 0.1^{b}	2.9 ± 0.1^{ab}	3.1 ± 0.1^{b}	n.s.	*	n.s.
20:1n-7	3.4 ± 0.2	3.6 ± 0.1	3.5 ± 0.4	3.8 ± 0.1	3.3 ± 0.2	3.6 ± 0.1	n.s.	n.s.	n.s.
22:1n-11	2.0 ± 0.2	2.1 ± 0.1	2.4 ± 1.0	2.3 ± 0.3	1.9 ± 0.2	2.1 ± 0.2	n.s.	n.s.	n.s.
Total monoun-saturated[2]	18.2 ± 1.5	18.7 ± 0.5	19.1 ± 3.7	19.7 ± 1.5	17.3 ± 0.7	18.2 ± 0.5	n.s.	n.s.	n.s.
18:2n-6	6.1 ± 0.4	6.3 ± 0.2	6.3 ± 0.3	6.2 ± 0.1	5.8 ± 0.1	6.0 ± 0.2	n.s.	n.s.	n.s.
20:2n-6	0.9 ± 0.6	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	n.s.	n.s.	n.s.
20:4n-6	2.3 ± 0.2	2.4 ± 0.2	2.2 ± 0.3	2.2 ± 0.0	2.3 ± 0.3	2.3 ± 0.1	n.s.	n.s.	n.s.
Total n-6 PUFA[3]	9.7 ± 0.1	9.8 ± 0.3	9.4 ± 0.2	9.3 ± 0.1	9.1 ± 0.5	9.3 ± 0.2	n.s.	n.s.	n.s.
18:3n-3	0.6 ± 0.0	0.6 ± 0.1	0.7 ± 0.2	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	n.s.	n.s.	n.s.
18:4n-3	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.2	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	n.s.	n.s.	n.s.
20:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	n.s.	n.s.	n.s.
20:4n-3	0.4 ± 0.0^{ab}	0.4 ± 0.0^{a}	0.5 ± 0.0^{b}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{ab}	0.4 ± 0.0^{ab}	n.s.	**	n.s.
20:5n-3	8.9 ± 0.8	8.2 ± 0.4	9.4 ± 0.8	7.9 ± 0.9	9.0 ± 0.6	8.5 ± 0.4	n.s.	n.s.	n.s.
22:5n-3	1.8 ± 0.0^{abc}	1.6 ± 0.0^{a}	1.9 ± 0.1^{cb}	1.7 ± 0.2^{ab}	2.0 ± 0.2^{c}	1.7 ± 0.1^{abc}	n.s.	*	n.s.
22:6n-3	27.4 ± 2.9^{ab}	24.7 ± 1.1^{ab}	28.4 ± 2.5^{ab}	24.4 ± 1.7^{a}	29.5 ± 0.6^{b}	25.7 ± 1.0^{ab}	n.s.	*	n.s.
Total n-3 PUFA[4]	40.5 ± 2.5^{abc}	36.7 ± 1.2^{ab}	42.3 ± 3.0^{bc}	36.3 ± 2.7^{a}	42.9 ± 0.3^{c}	38.1 ± 1.0^{abc}	n.s.	*	n.s.
Total PUFA[5]	52.3 ± 2.3^{ab}	48.8 ± 1.1^{ab}	53.7 ± 3.2^{b}	48.0 ± 2.5^{a}	54.1 ± 0.6^{b}	49.7 ± 1.2^{ab}	n.s.	*	n.s.
Total n-3 LC-PUFA[5]	38.7 ± 2.6^{ab}	35.0 ± 1.3^{a}	40.3 ± 3.3^{ab}	34.5 ± 2.7^{a}	41.1 ± 0.4^{b}	36.4 ± 1.0^{ab}	n.s.	*	n.s.

Data are expressed as means ± SD (n=3). Means in each column with a different superscript are significantly different according to one-way ANOVA ($P < 0.05$). [1]Includes 15:0, 20:0 and 17:0. [2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5 and 22:1n-9. [3]Includes 22:5n-6. [4]Includes 16:3n-3, 16:4n-4, 18:3n-6, 20:3n-6 and 22:4n-6. [5]Includes C_{16} PUFA. Diets 0-KEECC represent feed with increasing levels of vitamin E. C and with or without vitamin K as described in Material and Methods section. PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA. The last three columns indicate the effect of vitamins E and C (Vit. E+C), vitamin K (Vit. K) or their interaction (Vit. E+C x Vit. K) according to two-way ANOVA. * $p < 0.05$. ** $p < 0.01$.

Table 3.4. Kidney fatty acid compositions (percentage of total fatty acids) of meagre fed diets with different levels of vitamin E, C and K during 104 days.

	0	K	EC	KEC	EECC	KEECC
Lipid content (%)	4.7 ± 0.7	4.5 ± 0.3	4.5 ± 0.5	4.7 ± 0.5	4.3 ± 0.6	4.8 ± 0.3
14:0	3.5 ± 0.3	3.5 ± 0.6	3.4 ± 0.4	3.6 ± 0.7	3.4 ± 0.3	3.6 ± 0.6
16:0	18.8 ± 0.3	19.9 ± 0.4	19.7 ± 0.8	19.6 ± 1.2	20.0 ± 0.3	19.2 ± 0.7
18:0	4.8 ± 0.4	5.4 ± 0.8	5.3 ± 0.8	5.0 ± 1.2	5.3 ± 0.3	5.0 ± 0.9
Total saturated[1]	28.1 ± 0.3	29.7 ± 0.5	29.3 ± 1.1	29.1 ± 1.6	29.5 ± 0.4	28.7 ± 0.9
16:1n-7	4.2 ± 0.5	4.1 ± 0.7	4.0 ± 0.7	4.3 ± 0.8	4.0 ± 0.5	4.2 ± 0.8
18:1n-9	13.7 ± 0.7	12.6 ± 0.8	12.8 ± 1.0	12.6 ± 1.3	12.2 ± 0.5	12.4 ± 0.8
18:1n-7	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
20:1n-7	4.9 ± 0.3	5.0 ± 0.8	4.7 ± 0.5	5.0 ± 0.8	4.7 ± 0.3	5.1 ± 0.8
22:1n-11	5.6 ± 0.7	5.5 ± 1.4	5.2 ± 1.1	5.6 ± 1.7	5.2 ± 0.7	5.7 ± 1.5
Total monounsaturated[2]	32.8 ± 2.2	31.6 ± 3.7	30.9 ± 3.2	31.9 ± 4.7	30.3 ± 2.0	31.8 ± 4.0
18:2n-6	6.8 ± 0.2	6.4 ± 0.4	6.4 ± 0.3	6.6 ± 0.6	6.3 ± 0.1	6.3 ± 0.3
20:2n-6	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.1
20:4n-6	1.2 ± 0.2	1.3 ± 0.3	1.3 ± 0.3	1.2 ± 0.4	1.3 ± 0.2	1.3 ± 0.4
Total n-6 PUFA[3]	8.7 ± 0.2	8.4 ± 0.2	8.4 ± 0.2	8.5 ± 0.3	8.4 ± 0.2	8.23 ± 0.1
18:3n-3	1.3 ± 0.0	1.0 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.1	1.1 ± 0.2
18:4n-3	1.4 ± 0.2	1.2 ± 0.3	1.2 ± 0.4	1.3 ± 0.4	1.2 ± 0.2	1.4 ± 0.4
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
20:5n-3	7.9 ± 0.3	8.1 ± 0.9	8.2 ± 0.3	8.1 ± 0.9	8.3 ± 0.4	8.4 ± 0.8
22:5n-3	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.0	1.4 ± 0.1
22:6n-3	15.7 ± 1.6	15.6 ± 2.4	16.5 ± 2.2	15.5 ± 2.7	16.8 ± 1.4	16.1 ± 2.4
Total n-3 PUFA	28.9 ± 1.8	28.8 ± 3.1	29.8 ± 2.2	29.8 ± 3.1	30.2 ± 1.7	29.7 ± 2.9
Total PUFA[4]	39.1 ± 2.0	38.7 ± 3.2	39.8 ± 2.3	39.0 ± 3.1	40.1 ± 1.9	39.5 ± 3.1
Total n-3 LC-PUFA[5]	25.6 ± 2.0	25.7 ± 3.4	26.7 ± 2.5	25.7 ± 3.5	27.2 ± 1.8	26.5 ± 3.3

Data are expressed as means ± SD (n=3). [1]Includes 15:0, 20:0 and 17:0. [2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5 and 22:1n-9. [3]Includes 22:5n-6. [4]Includes 16:3n-3, 16:4n-4, 18:3n-6, 20:3n-6 and 22:4n-6. [5]Includes C_{16} PUFA. Diets 0-KEECC represent feed with increasing levels of vitamin E. C and with or without vitamin K as described in Material and Methods section. PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA. Significant differences were not observed according to two-way ANOVA.

Table 3.5. Liver fatty acid compositions (percentage of total fatty acids) of meagre fed diets with different levels of vitamin E, C and K during 104 days.

	0	K	EC	KEC	EECC	KEECC
Lipid content (%)	25.6 ± 0.9	24.8 ± 1.3	22.7 ± 1.9	23.9 ± 1.7	24.2 ± 0.8	25.1 ± 1.1
14:0	2.8 ± 0.4	2.7 ± 0.2	2.7 ± 0.3	2.8 ± 0.2	3.0 ± 0.2	2.8 ± 0.4
16:0	21.9 ± 1.6	22.8 ± 1.3	23.0 ± 1.4	21.3 ± 1.0	21.5 ± 1.3	21.9 ± 1.9
18:0	6.2 ± 1.0	6.5 ± 0.5	6.3 ± 1.0	6.0 ± 0.6	5.8 ± 0.5	6.4 ± 1.1
Total saturated[1]	31.8 ± 2.0	32.6 ± 1.5	32.7 ± 2.1	30.9 ± 1.3	31.2 ± 1.4	31.7 ± 2.6
16:1n-7	8.7 ± 0.5	8.9 ± 0.8	9.0 ± 0.6	8.6 ± 0.4	8.6 ± 0.5	8.6 ± 0.6
18:1n-9	22.7 ± 1.5	22.6 ± 1.4	23.0 ± 1.4	21.6 ± 0.1	21.1 ± 1.3	22.0 ± 1.7
18:1n-7	2.6 ± 0.1	2.6 ± 0.1	2.6 ± 0.0	2.7 ± 0.1	2.6 ± 0.0	2.6 ± 0.1
20:1n-7	5.8 ± 0.7	5.4 ± 0.4	5.6 ± 0.5	5.9 ± 0.3	6.1 ± 0.2	5.7 ± 0.7
22:1n-11	5.5 ± 0.6	5.0 ± 0.4	5.2 ± 0.3	5.5 ± 0.2	5.8 ± 0.3	5.4 ± 0.6
Total monounsaturated[2]	48.3 ± 0.5	47.4 ± 1.4	48.3 ± 1.1	47.4 ± 0.3	47.3 ± 1.4	47.3 ± 0.7
18:2n-6	6.1 ± 1.0	5.5 ± 0.8	5.6 ± 0.7	6.2 ± 0.7	6.3 ± 0.5	5.8 ± 1.0
20:2n-6	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
Total n-6 PUFA[3]	6.9 ± 1.1	6.3 ± 0.9	6.4 ± 0.9	7.1 ± 0.7	7.2 ± 0.6	6.7 ± 1.2
18:3n-3	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.0	1.1 ± 0.0	1.1 ± 0.1	1.0 ± 0.2
18:4n-3	1.1 ± 0.2	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.0	1.2 ± 0.2	1.2 ± 0.2
20:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
20:5n-3	2.8 ± 0.3	3.0 ± 0.4	2.8 ± 0.5	3.1 ± 0.0	3.1 ± 0.5	3.2 ± 0.6
22:5n-3	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	1.1 ± 0.0	1.1 ± 0.2	1.1 ± 0.2
22:6n-3	4.8 ± 0.5	5.4 ± 1.0	4.7 ± 0.8	5.9 ± 0.4	5.5 ± 0.9	5.5 ± 0.8
Total n-3 PUFA[4]	11.9 ± 1.3	12.5 ± 1.9	11.4 ± 1.8	13.4 ± 0.4	13.1 ± 2.2	13.1 ± 2.1
Total PUFA[5]	20.0 ± 2.5	20.0 ± 2.8	19.0 ± 2.8	21.7 ± 1.1	21.5 ± 2.8	21.0 ± 3.3
Total n-3 LC-PUFA[5]	9.4 ± 1.0	10.2 ± 1.6	9.2 ± 1.6	11.0 ± 0.3	10.6 ± 1.8	10.7 ± 1.7

Data are expressed as means ± SD (=3).[1]Includes 15:0, 20:0 and 17:0. [2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, 20:1n-5 and 22:1n-9. [3]Includes 22:5n-6. [4]Includes 16:3n-3, 16:4n-4, 18:3n-6, 20:3n-6 and 22:4n-6. [5]Includes C_{16} PUFA. Diets 0-KEECC represent feed with increasing levels of vitamin E. C and with or without vitamin K as described in Material and Methods section. PUFA, polyunsaturated fatty acids; LC-PUFA, long-chain PUFA. Significant differences were not observed according to two-way ANOVA.

Significant differences were observed in the percentage of fatty acids in the heart, saturated fatty acids being lower in fish fed the diets EECC and EC compared with the rest of the diets. Significant differences were also found in the percentage of n-3 PUFA, total n-3 PUFA and total long chain PUFA (LC-PUFA) where the lowest percentage was found in fish fed the diet KEC and the highest in the diet EECC. In heart the inclusion of vitamin K in the diet increased total saturated fatty acids and decreased n-3 PUFA. In this sense, there were significant differences between fish fed diets EC and KEC in the percentage of saturated fatty acids, being higher when vitamin K was added in the diet as indicated by the two-way ANOVA. The same trend was observed in the other diets (K and KEECC). There was a significant reduction in the percentage of n-3 PUFA in heart when vitamin K was added to the diets EC and EECC observing the same tendency in fish fed the other dietary treatments.

3.3.3. Histopathology

Gross appearance of granulomas in tissues (liver, kidney and heart) was only observed in 10 fish out of 126, not being related to any particular dietary treatment. No granulomas were observed in the spleen in fish fed any of the dietary treatments. The histopathological evaluation revealed different stages of granuloma development in liver (Figure 3.1). At initial stages, granulomas were observed as isolated and irregular aggregates of macrophages and some lymphocytes (Figure 3.1a) that later formed concentric layers (Figure 3.1b). These aggregates progressively led to a necrotic centre with an external layer of fibrocytes (Figure 3.1c). In the final stages the granuloma was completely composed of laminar material, especially observed in heart (Figure 3.1d).

In order to study the origin of the granulomas, the liver immunostaining showed anti-actin reactivity (red marker) surrounding the concentric aggregates of macrophages corresponding to smooth muscle of the blood vessels (Figure 3.2). No calcification was observed at any stage or in any analyzed tissue. The specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stain), culture media (blood agar, tryptone soya agar and Lowenstein-Jensen) were negative, discarding a possible infectious origin. Additionally, none of the samples, except the positive control FFBE tissue and the NCIMB 2057 strain samples, were found to be positive to the *Nocardia* specific primers used in this study. The positive control sample produced the expected size band of approximately 600 bp.

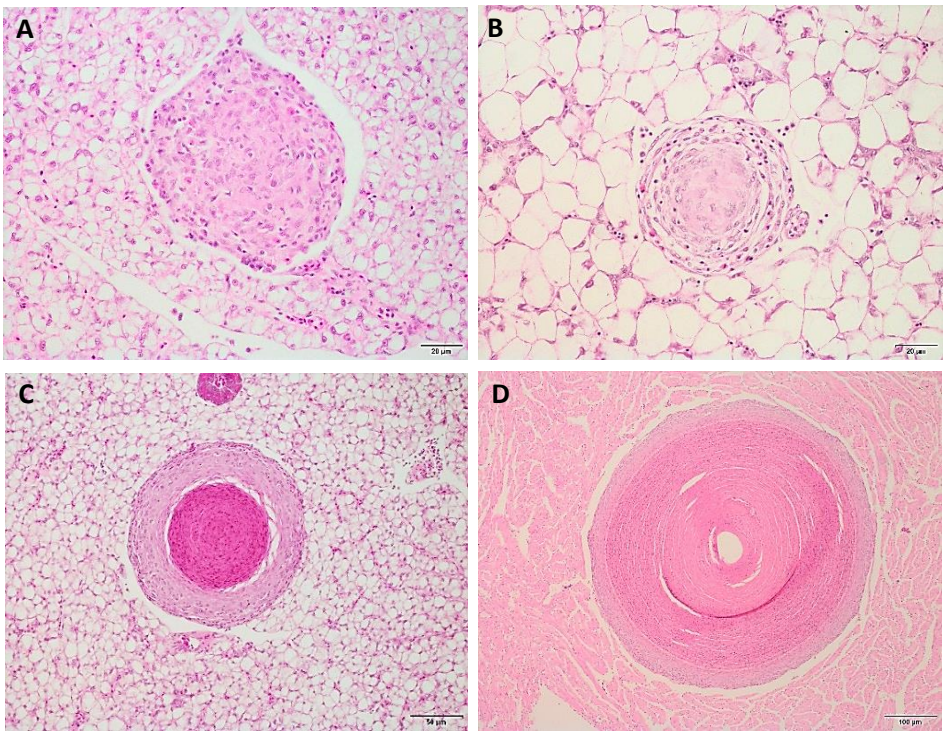


Figure 3.1. Different stages of granuloma formation. A) Irregular aggregates of macrophages and inflammatory cells. B) Concentric layers of macrophages and inflammatory cells. C) Necrotic centre with an external layer of fibrocytes. D) Granuloma composed completely of laminar material in heart.

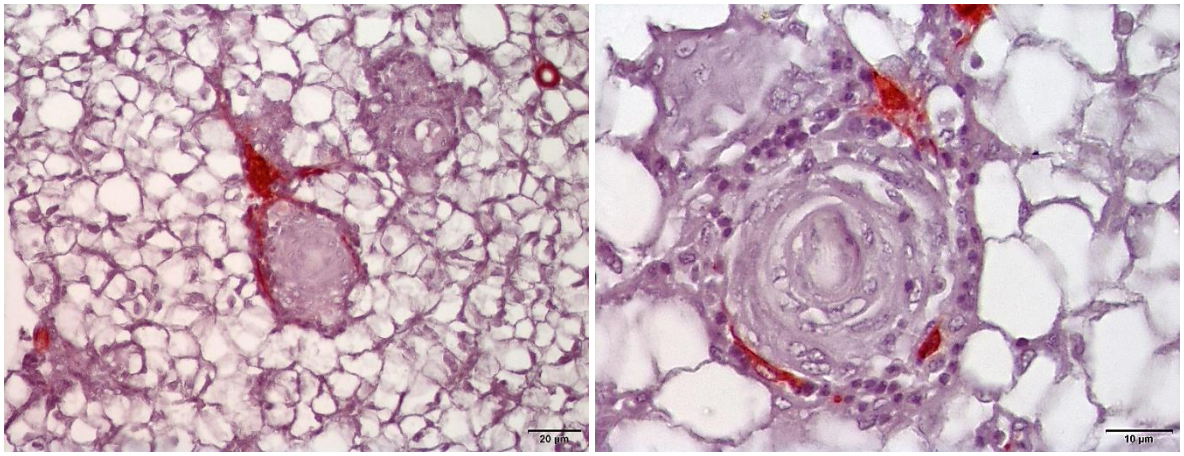


Figure 3.2. Positive immunoreactivity towards anti-actin antibody in hepatic granulomas.

At the initial sampling, the percentage of fish presenting microscopic granulomas was 45 %. At the end of the feeding period, no significant differences were found by the two-way ANOVA in the percentage of fish with granulomas among the dietary treatments. However, a tendency to a reduction in the number of fish with granulomas

when vitamins E, C and K were added to the feeds could be observed (Figure 3.3) (100 % fish with granulomas in diet 0 to 82 % fish in KEC). The most affected organ was the liver followed by kidney and heart. The lowest number of fish with hepatic granulomas were observed when high level of vitamin E, C and K were supplemented to the feeds (KEECC diet), although only significantly lower than in the fish fed diet 0 (Figure 3.4a). Similar results were observed in heart, where the highest percentage was obtained in fish fed diet 0 and the lowest in fish fed diet EECC (Figure 3.4b), as indicates the two-way ANOVA. In kidney, a tendency to a reduced presence of granulomas was observed in fish fed diets with the highest levels of vitamins E and C albeit not significant ($p = 0.085$; Figure 3.4c).

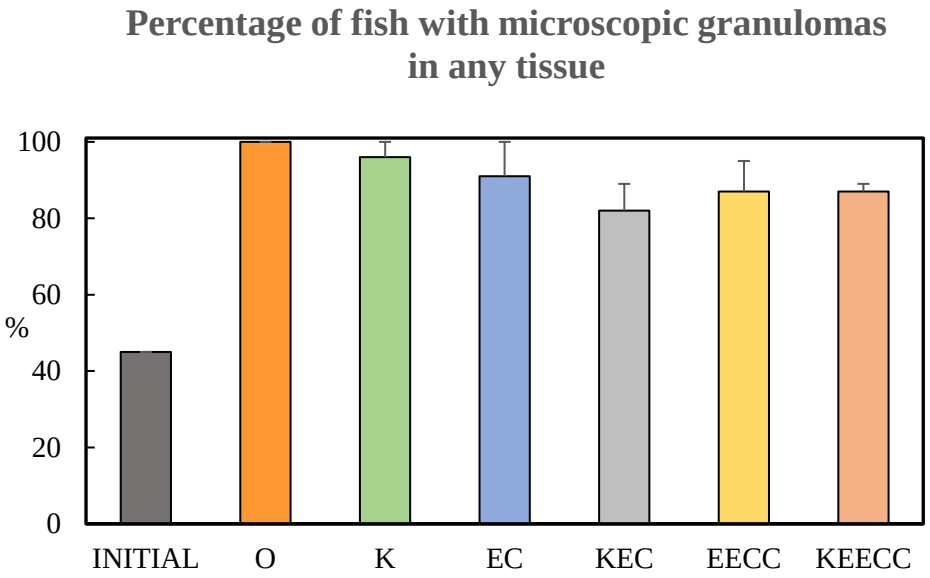
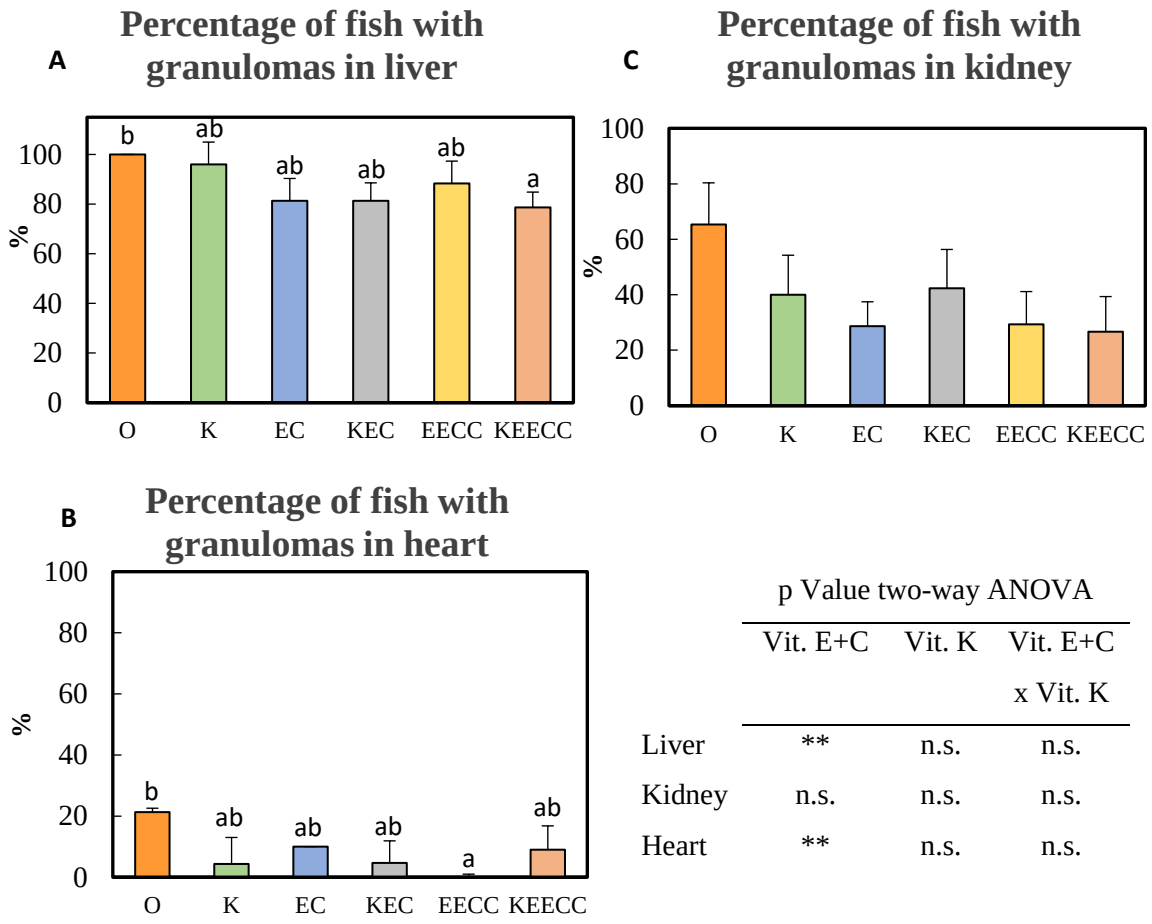


Figure 3.3. Percentage of fish affected with granulomas in any tissue after the microscopic evaluation of tissues of meagre fed the different experimental feeds containing graded levels of vitamins C, E and K ($p < 0.05$). Significant differences were not observed by the two-way ANOVA.



p Value two-way ANOVA

	Vit. E+C	Vit. K	Vit. E+C x Vit. K
Liver	**	n.s.	n.s.
Kidney	n.s.	n.s.	n.s.
Heart	**	n.s.	n.s.

Figure 3.4. Percentage of A) liver, B) kidney and C) heart with granulomas observed during the microscopic evaluation of meagre fed with different levels of vitamin C, E and K ($p < 0.05$). Vit. E+C, vitamins E and C; Vit. K, vitamin K. * $p < 0.05$; ** $p < 0.01$.

The severity score did not show significant differences among fish fed the different dietary treatments in any tissue after 15 weeks of feeding, however there was a clear tendency towards a decrease in the severity of granulomatosis in liver and kidney, dependent on diet. For instance, in liver the severity score was 1.83 in fish fed diet 0 vs 1.30 in diet KEECC and in kidney 0.91 in fish fed diet 0 vs 0.39 in diet KEECC (Table 3.6).

When the level of all three vitamins (E, C and K) were increased in the diets (KEC and KEECC diets) the number of fish scored with "0" increased and those scored with "3" in liver ($p = 0.251$) and kidney ($p = 0.125$) decreased (Figure 3.5).

Table 3.6. Average granuloma severity scored in liver, kidney and heart ($p<0.05$).

	Average granuloma severity		
	Liver	Heart	Kidney
0	1.83 ± 0.9	0.26 ± 0.5	0.91 ± 0.9
K	1.65 ± 0.8	0.04 ± 0.2	0.70 ± 1.0
EC	1.57 ± 1.0	0.17 ± 0.7	0.48 ± 0.9
KEC	1.39 ± 1.0	0.30 ± 0.9	0.65 ± 0.9
EECC	1.39 ± 1.0	0.00 ± 0.0	0.48 ± 0.9
KEECC	1.30 ± 0.9	0.17 ± 0.7	0.39 ± 0.7

Data are means ± SD (n=30). Significant differences were not observed according to two-way ANOVA.

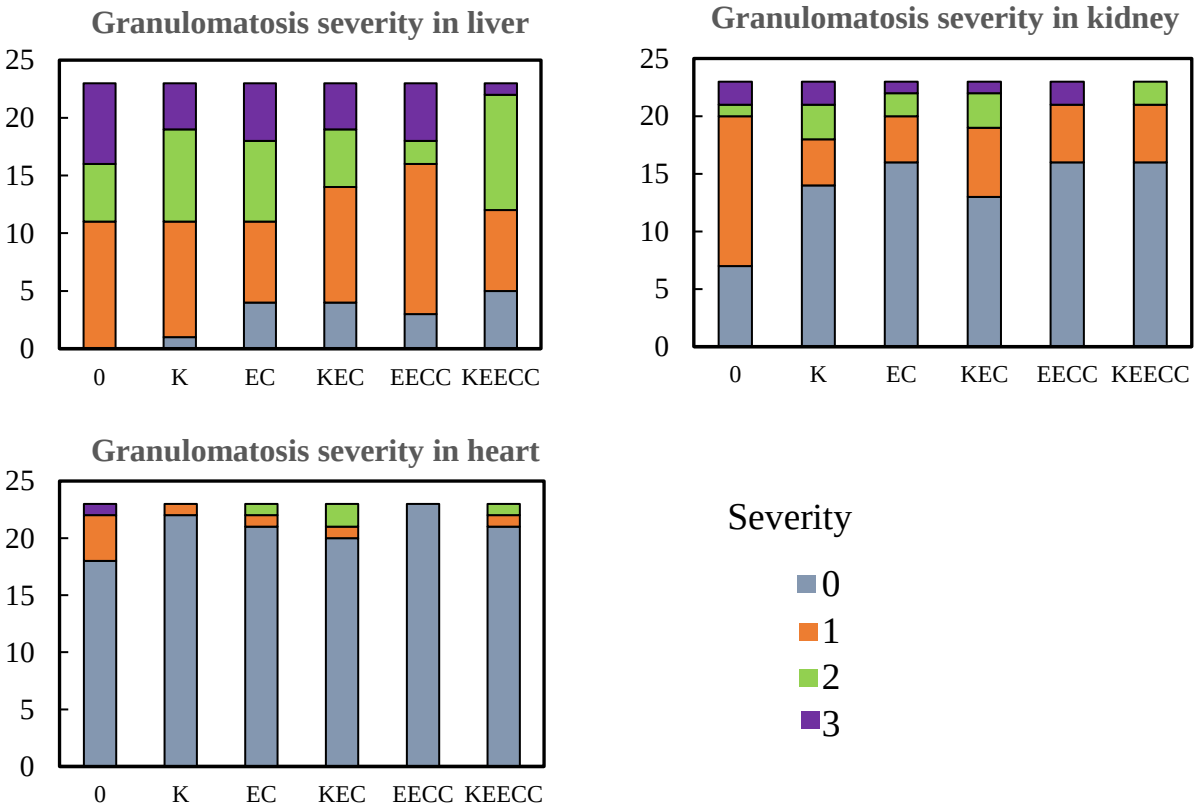


Figure 3.5. Distribution of fish in each severity stage in liver, kidney and heart after 104 days of feeding the experimental diets ($p < 0.05$). Significant differences were not observed by the two-way ANOVA.

3.3.4. Vitamin E content in fish tissues

Fish fed diet 0 and diet K (no extra supplementation of vitamin E and C) had significantly lowest concentration of vitamin E in liver, kidney and heart (Table 3.7). Increasing the dietary vitamin E concentration significantly increased vitamin E contents in liver ($y = 0.2081x – 5.4709$, $R^2 = 0.9533$), kidney ($y = 0.0529x + 17.557$, $R^2 = 0.9505$) and heart ($y = 0.0494x + 7.0763$, $R^2 = 0.9244$), showing a correlation between the amount of vitamin E in the diet and in the tissue (Table 3.7). The diet-dependent accumulation of vitamin E was most pronounced in liver whereas vitamin E levels were more stable in kidney and heart (Table 3.7).

Table 3.7. α-tocopherol content (vitamin E) in liver, kidney and heart of meagre fed experimental diets during 104 days.

	Vitamin E content (mg kg^{-1})		
	Liver	Kidney	Heart
0	27.7 ± 2.6^{a}	27.5 ± 2.2^{a}	14.6 ± 2.5^{a}
K	26.7 ± 2.8^{a}	26.4 ± 4.7^{ab}	13.8 ± 2.0^{a}
EC	64.2 ± 1.9^{bc}	30.3 ± 3.0^{ab}	21.5 ± 2.3^{ab}
KEC	48.2 ± 8.8^{b}	32.2 ± 8.0^{abc}	23.9 ± 4.0^{b}
EECC	77.8 ± 6.8^{cd}	39.0 ± 8.3^{bc}	27.1 ± 3.9^{b}
KEECC	88.2 ± 13.5^{d}	42.9 ± 8.5^{c}	28.4 ± 5.5^{b}
Two-way ANOVA			
Vit. E+C	*	**	*
Vit. K	n.s.	n.s.	n.s.
Vit. E+C x Vit. K	n.s.	n.s.	n.s.

Data are means ± SD (n=3), where the means in each column with a different superscript are significantly different according to one-way ANOVA ($P<0.05$). The last three columns indicate the effect of vitamins E and C (Vit. E+C), vitamin K (Vit. K) or their interaction (Vit. E+C x Vit. K) according to two-way ANOVA.

* $p < 0.05$.

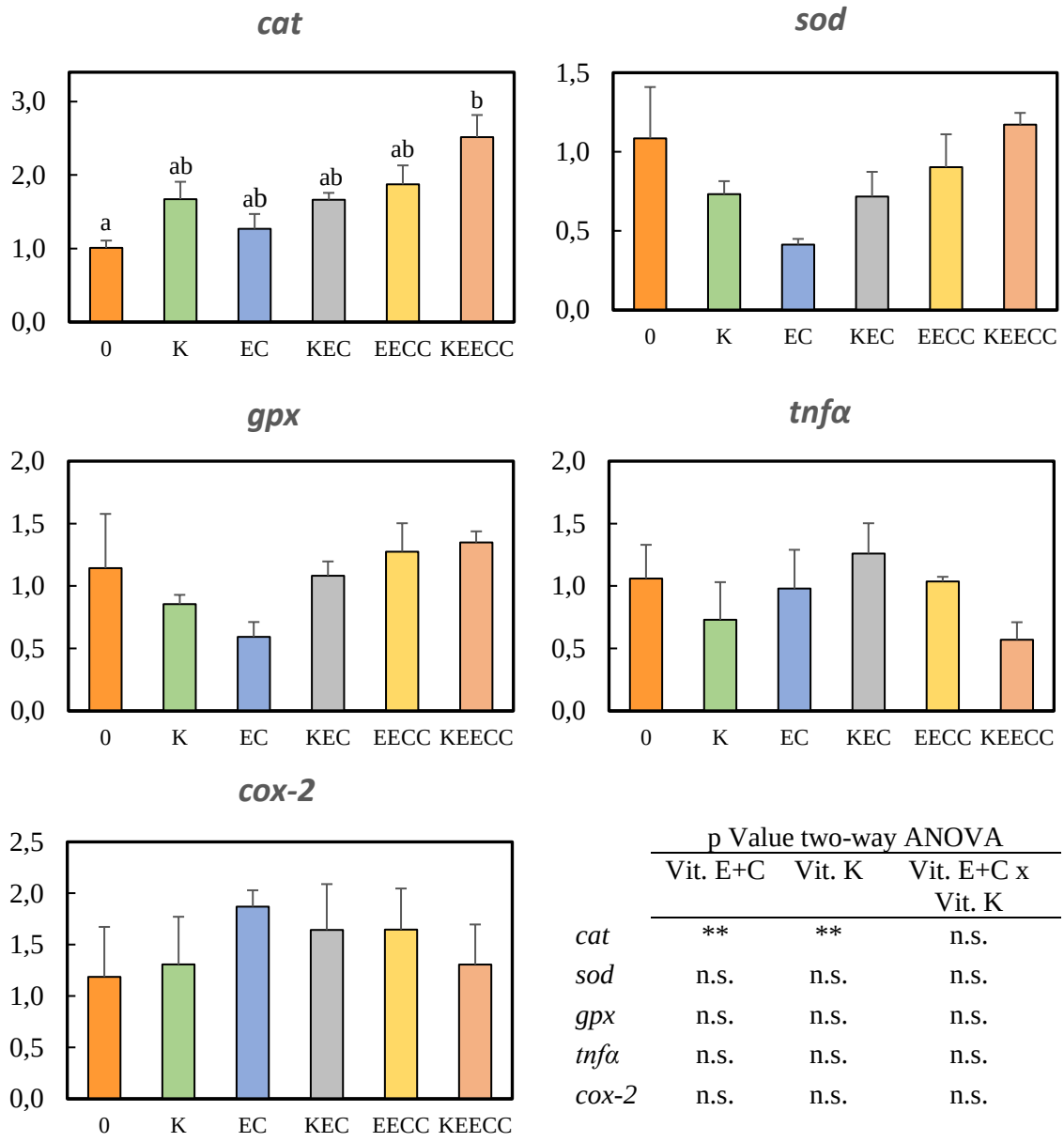
** $p < 0.01$.

3.3.5. Gene expression analysis

The hepatic expression of *cat* was significantly higher in the liver of fish fed the highest level of vitamin E, C and K than in fish fed diet 0, as denoted by the two-way ANOVA. Significant differences were not found in the gene expression of *sod* ($p = 0.09$) or *gpx* ($p = 0.201$) in liver but fish fed diets supplemented with the lowest levels of vitamin E and C, but no vitamin K (EC diet) tended to show a reduced expression of these enzymes (Figure 3.6). No differences were observed in the expression levels of *tnfα* or *cox-2*.

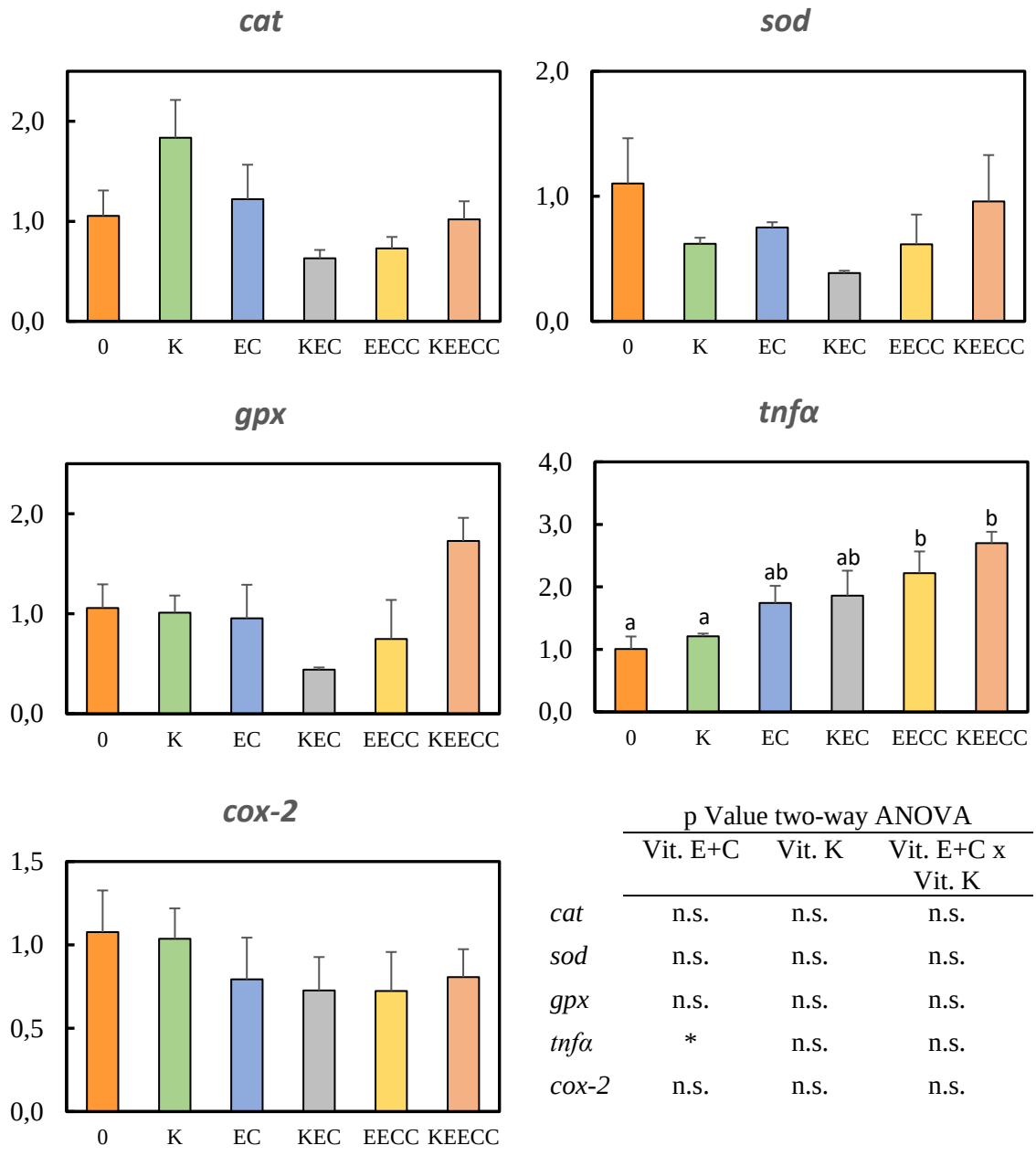
No significant differences in the gene expression of *cat*, *sod* and *gpx* were observed in kidney (Figure 3.7). There was an increase in the mRNAlevels of *gpx* in fish fed high levels of vitamins E, C and K (KEECC diet) albeit not significant ($p = 0.073$). The two-way ANOVA showed that the expression of *tnfα* was affected by the dietary level of vitamin C and E, being significantly up-regulated in fish fed diets EECC and KEECC with a mild correlation found between expression of this gene and granulomatosis severity in kidney ($R^2 = 0.8504$, $y = -0.3007x + 1.1566$). Dietary increase of vitamins E, C and K did not regulate the kidney expression of *cox-2*.

In heart, the gene expression of *sod* and *gpx* was influenced by the inclusion of the vitamin E, C and K, and the interaction of them, as denoted by the two-way ANOVA, being significantly increased in fish fed with low levels of vitamins E and C and without vitamin K (EC diet) (Figure 3.8). There were not differences in the expression of *cat* in heart. The expression of *tnfα* and *cox*-2 was significantly increased in fish fed diets EECC and KEECC.



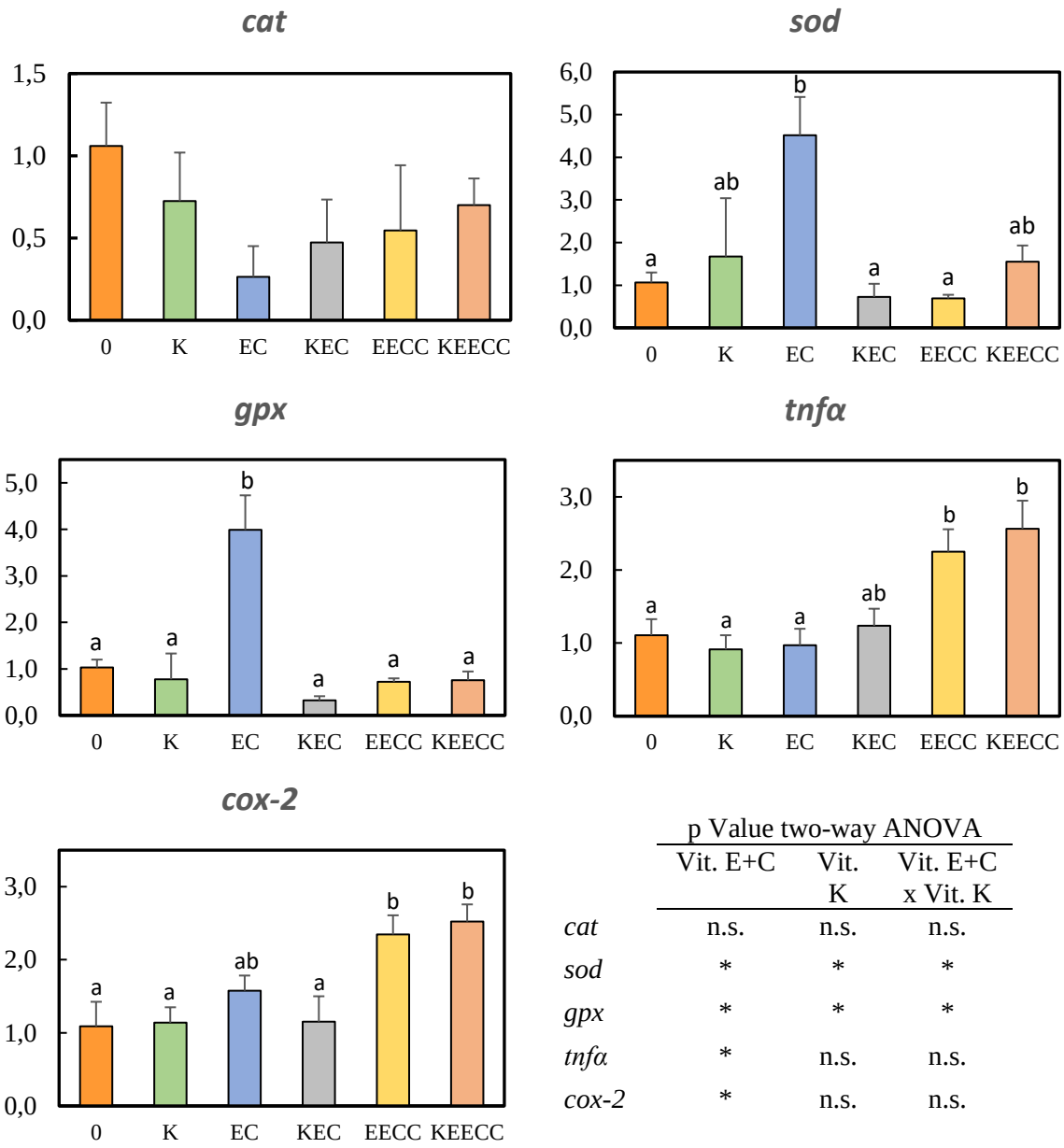
	p Value two-way ANOVA		
	Vit. E+C	Vit. K	Vit. E+C x Vit. K
cat	**	**	n.s.
sod	n.s.	n.s.	n.s.
gpx	n.s.	n.s.	n.s.
tnfα	n.s.	n.s.	n.s.
cox-2	n.s.	n.s.	n.s.

Figure 3.6. Expression levels of the antioxidant enzymes *cat*, *sod* and *gpx* measured by real-time PCR in liver of meagre ($p < 0.05$). Vit. E+C, vitamins E and C; Vit. K, vitamin K. * $p < 0.05$; ** $p < 0.01$.



	p Value two-way ANOVA		
	Vit. E+C	Vit. K	Vit. E+C x Vit. K
cat	n.s.	n.s.	n.s.
sod	n.s.	n.s.	n.s.
gpx	n.s.	n.s.	n.s.
tnfα	*	n.s.	n.s.
cox-2	n.s.	n.s.	n.s.

Figure 3.7. Expression levels of the antioxidant enzymes *cat*, *sod* and *gpx* measured by real-time PCR in kidney of meagre ($p < 0.05$). Vit. E+C, vitamins E and C; Vit. K, vitamin K. * $p < 0.05$; ** $p < 0.01$.



	p Value two-way ANOVA		
	Vit. E+C	Vit. K	Vit. E+C x Vit. K
cat	n.s.	n.s.	n.s.
sod	*	*	*
gpx	*	*	*
tnfα	*	n.s.	n.s.
cox-2	*	n.s.	n.s.

Figure 3.8. Expression levels of the antioxidant enzymes *cat*, *sod* and *gpx* measured by real-time PCR in heart of meagre ($p < 0.05$). Vit. E+C, vitamins E and C; Vit. K, vitamin K. * $p < 0.05$; ** $p < 0.01$.

3.4. Discussion

In the present study, significant differences were not found in specific growth rate (1.12-1.19), feed conversion ratio (0.83-0.87), survival, fish condition factor (K) or hepatosomatic index and viscera somatic index, all these indicators being within normal ranges for the species (Chatzifotis et al., 2010; Chatzifotis et al., 2012; Velazco-Vargas et al., 2014; Rodriguez-Lozano et al., 2017). Increasing dietary levels of vitamin E (to 300 or 450 mg kg^{-1}) and vitamin C (to 70 or 230 mg kg^{-1}) seemed to improve final weight, similar to what has been observed in other fish species (Gao et al., 2012; Gao et al., 2013; Gao et al., 2014; Chen et al., 2015; Rodriguez-Lozano et al., 2017). However, other studies reported that the addition of vitamin C and E did not affect the growth performance in large yellow croaker (*Larimichthys polyactis*) (Ai et al., 2006) or turbot (*Scophthalmus maximus*) (Tocher et al., 2002). Given the short duration of the present trial and that the differences observed among the dietary treatments were minimal (approx. a 7 % of weight increase) these differences could however, be related to small differences in initial weight among the experimental tanks.

Increasing dietary levels of vitamins E, C and K did not affect whole fish proximate or lipid composition, and the fat content of around 25 % in liver is similar to the results obtained by Rodriguez-Lozano et al. (2017). The addition of dietary vitamin K significantly seemed to increase saturated fatty acids and decrease total n-3 PUFA and total LC-PUFA in the heart. There is not a clear explanation to this observation and it must be noted that slight differences were observed among the diets fatty acid profile which could in turn explain these small differences in the fatty acid profile. On the other hand, no effects of vitamins E and C were observed on the tissue fatty acid profiles in liver or kidney, indicating that levels of antioxidant nutrients in the diet 0 were sufficient to protect against ROS. Indeed, tissue vitamin E levels were positively correlated to dietary contents indicating that only small amounts of vitamin E might have been oxidized in order to protect tissue PUFA from oxidation. Increased α-tocopherol concentration in tissues in response to dietary levels have also been reported by other authors (Peng et al., 2009; Gao et al., 2012; Betancor et al., 2012).

A high percentage of granulomas was observed among fish from all the dietary treatments. Furthermore, 45 % of affected fish with granulomas in the initial population, liver being the tissue with the highest prevalence and severity. Different stages of

development of the granulomas could be observed in all the evaluated tissues, sharing similar features to those described by Ghittino et al. (2004) in the same species. The systemic granulomatosis in meagre is similar to the pathology produced by infectious agents such as *Mycobacterium* spp. (Bowser, 2009) and *Nocardia* spp. (Labrie, 2008; Elkesh et al., 2013). The specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stains), culture media (blood agar, tryptone soya agar and Lowenstein-Jensen) and a PCR for Nocardia were negative, discarding a possible infectious origin and reinforcing the hypothesis of a nutritional origin of the disease.

In the present study, the increase in dietary levels of vitamin E, C and K to 450 mg kg^{-1}, 230 mg kg^{-1} and 23 mg kg^{-1} respectively, significantly reduced the percentage of granulomas in liver and heart compared to fish fed diet 0. Besides, increasing the dietary level of these vitamins also reduced the severity of granulomas in livers and kidney in comparison with diet 0 without extra supplementation of vitamins. Significant differences were not found, but there was a tendency to decreased severity in kidney ($p = 0.125$) and liver ($p = 0.251$) when vitamins E, C and K were supplemented to the basal diet. The severity in heart was low in all diets. Liver was the main affected organ (up to 100 % of fish), followed by the kidney (29.33 – 65.33 %) and heart (0.00 – 21.30 %). To our knowledge this is the first study to evaluate the incidence of granulomas in these tissues in meagre. Different stages of development of the granulomas were observed depending on the tissue. In liver, there were more granulomas in initial stages, suggesting that probably there is a later apparition or a continued formation of granulomas. In kidney and heart most of the granulomas were completely formed, probably because these tissues are the first where granulomas appear. A deficiency of antioxidants in the diet could cause primary lesions in the tissues and these lesions could potentially lead to the development of granulomas. α-tocopherol is the major membrane-bound lipid-soluble antioxidant (Machlin and Bendich, 1987). In addition, vitamin C can efficiently trap peroxyl radicals in the aqueous phase before they can initiate lipid peroxidation, thus protecting the biomembranes (Sies et al., 1992). Thus, the decrease in the incidence of systemic granulomatosis observed in the present study, when high levels of vitamin C and E were employed, could be due to the protective effect of these two antioxidant nutrients. It must be noted though that a longer experimental period would be necessary in order to deplete the vitamin E/C storages in the fish what explains why no deficiency symptoms were observed in fish fed diet "0".

During the microscopic evaluation, irregular aggregates of cells around the blood vessels were observed. A layer of actin was observed in some granulomas after the immunostaining for actin. This could suggest a possible origin of the granulomas in the blood vessels. The vitamins studied can be involved in the normal function of the blood vessels. For instance, vitamin C participates in the synthesis of collagen, an important protein used to generate blood vessels (Lim and Lovell, 1978; Nusgen et al., 2001). Vitamins C and E are involved in the prevention of the endothelial dysfunction in humans, the dysfunction increasing the tendency for arterial blockage due to a blood clot, or thrombosis (Riitta et al., 2003; Marguerite et al., 2003). Vitamin K is also an essential cofactor involved in blood coagulation and has a protective role against vascular injury (Butenas et al., 2002; Stafford, 2005). Therefore, it is feasible to think that these nutrients help to prevent the appearance of granulomas by exerting a role on the formation of blood vessels and other components.

Oxidative stress is the result of the imbalance between the production of ROS and antioxidant defences (Nishida, 2011). There are enzymes able to neutralize ROS, some of the most important being SOD, CAT and GPX. In the present trial, the expression of theses enzymes was affected by the addition of vitamins in the diet. Particularly, the expression of *cat* was significantly higher in liver with high levels of vitamins E, C and K. Accordingly with these results, Mahmoud et al. (2016) observed that the activity of CAT was influenced by the level of dietary vitamin C in *Pagrus major*. It has been observed in several studies a correlation between the mRNA expression levels and the activity of antioxidant enzymes (An et al., 2010; Penglase et al., 2010; Shin et al., 2010; Park et al., 2011). In the present study, *sod* and *gpx* expression showed a tendency to increase with high levels of vitamins in the diet, albeit not significantly. On the contrary, in heart these two enzymes showed a higher expression with low addition of vitamins E and C. A positive correlation has been observed between the levels of vitamin C and E in the diet and the expression of SOD and GPX by Betancor et al. (2012) The present results seem to indicate that dietary vitamins E and C may have antioxidant potential by enhancing the expression of *sod* and *gpx* in heart and *cat* in liver, being influenced by the organ where they are acting. The antioxidant effect of both vitamins in the heart may be the cause of increased expression of *sod* and *gpx*, when the diet is supplemented with vitamin E and C (EC). However, at the highest dietary levels of these vitamins (23 mg kg^{-1} vitamin K, 450 mg kg^{-1} vitamin E, 230 mg kg^{-1} vitamin C) there was no effect on the

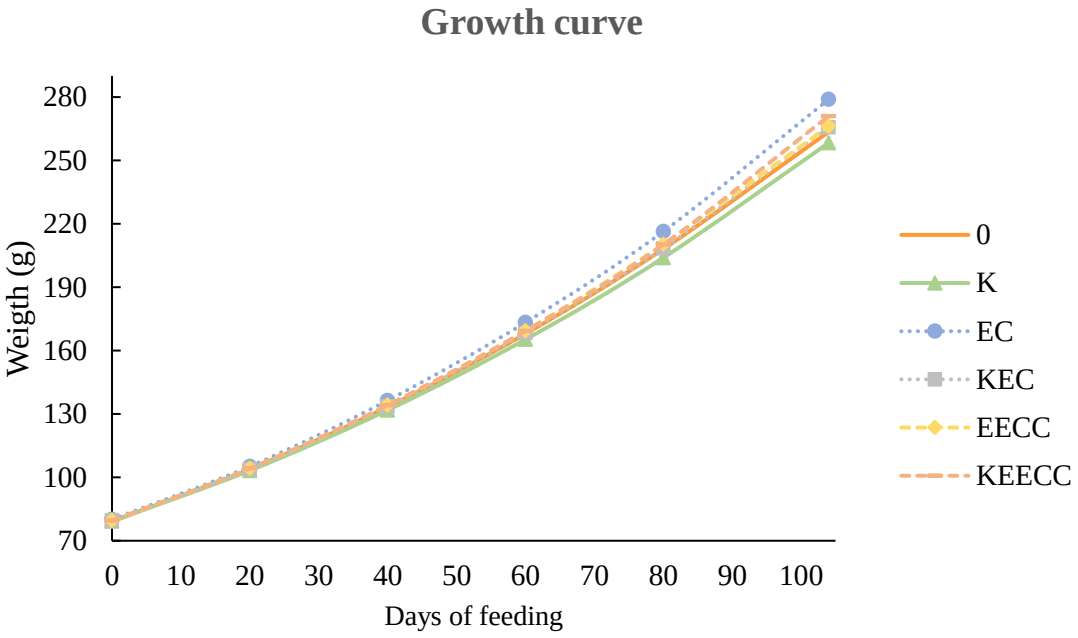
expression of antioxidants in the heart (KEECC and EECC). Therefore, high dietary vitamin E levels could have a pro-oxidant effect, as has been previously described in other teleost species (Hamre, 2011; Betancor et al., 2011).

TNFα is a crucial regulator and effector in innate and adaptive immune responses, regulating cell death and survival (Locksley et al., 2001), while *cox*-2 is a prostaglandin which plays a key role in inflammation (Ishikawa et al., 2007ab) and innate immune response (Xu et al., 2008; Legler et al., 2010) in fish. The expression of *tnfα* was significantly increased in kidney and heart of fish fed diet with the highest vitamin C and E levels (23 mg kg^{-1} vitamin K, 450 mg kg^{-1} vitamin E, 230 mg kg^{-1} vitamin C). Similar results were obtained by Niu et al. (2014) in turbot, where the addition of vitamin E from 0 to 480 mg kg^{-1} significantly increased the expression of *tnfα* in kidney and spleen and in the same species the supplementation of vitamin E increased mRNA level of *tnfα* in liver, spleen and head kidney improving immunity (Jia et al., 2017). Similarly, the expression of *cox*-2 was significantly higher in heart of fish fed with diets EECC and KEECC. Indeed, the expression of *cox*-2 has been associated with the increased pro-inflammatory cytokine *tnfα* in teleost (Wang et al., 2016). In mammals, vitamin E and C supplementation inhibit nuclear factor-kB thus reducing *tnfα* and *cox*-2 mRNA levels (Cárcamo et al., 2002; Han et al., 2004; Huey et al., 2008; Lee et al., 2008; Nakamura and Omaye, 2009), which is opposite to the regulation observed in the present study. Little is known about the effect of vitamin C and E in the expression of *tnfα* and *cox*-2 in fish, most of the studies are focused in mammalians. These divergent results could suggest a different mechanism of regulation of *tnfα* and *cox*-2 in fish and in mammals.

In summary, increasing the dietary levels of vitamins E and C (300 mg kg^{-1} vitamin E, 70 mg kg^{-1} vitamin C) significantly increased meagre growth in terms of final weight and length. Increasing dietary levels of the vitamins also affected gene expression leading to an up-regulation of *cat* in liver, *tnfα* in kidney, as well as *tnfα*, *cox*-2, *sod* and *gpx* in heart. It also reduced the percentage of granulomas in liver and heart, tending to be milder with dietary increase of vitamins E, C and K. The presence of actin around some of the granulomas and the observation of irregular aggregated of cells around the blood vessels, could suggest a possible origin of the granulomas in the blood vessels. The results show that the combination of high dietary content of vitamin K and antioxidant vitamins E and C have influence on the incidence and increases the number of fish with lower severity of the granulomatosis in meagre, which suggests that this pathology could

be mediated by nutritional factors. However, a high prevalence of granulomas was observed at the beginning of the experimental trial what prompts to evaluate the combination of vitamins at earlier life stages.

3.5. Appendix



Supplementary Figure 3.1. Growth curve of meagre fed diets with different levels of vitamin E, C and K during 104 days.

Supplementary Table 3.1. Severity score of granulomas in liver, kidney and heart.

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

Supplementary Table 3.2. Sequences for real-time quantitative-PCR forward and reverse primers (5′–3′). The data include sequences. amplicon sizes and annealing temperatures (Tm).

Target	Primer 5´-3´	Fragment size (bp)	Tm (°C)
sod	F: GGCCCTCACTTCAATCCCTA	207	59
	R: TCCTTTTCCCAGATCGTCGG		
gpx	F: AAGCAGTTTGCCGAGTCCTA	103	57
	R: GCTGGTCTTTCAGCCACTTC		
cat	F: GCTTCCACCAACCCAGATTA	205	59
	R: GGTTCCTGTTCAGCACCATT		
cox-2	F: GGAAGTTGGTGTTGACATGCACTAC	211	59
	R: AATCAGGATGAGCCGTGTGGTC		
tnfα	F: CACAAGAGCGGCCATTCATTTACAAGGAG	173	59
	R: GGAAAGACGCTTGGCTGTAGATGG		
bact	F: CCATCGAGCACGGTATTGT	455	60
	R: CAGCTTCTCCTTGATGTCACG		
tub	F: GGAGTACCCCGATCGTATCA	161	59
	R: AGATGTCATACAGGGCCTCG		
ef1a	F: GGTGCTGGACAAACTGAAGG	196	59
	R: GAACTCACCAACACCAGCAG		

sod, superoxide dismutase; *gpx*, glutathione peroxidase; *cat*, catalase; *tnfα*, tumor necrosis factor; *cox*-2, cyclooxygenase; *bact*, β-actin; *tub*, tubulin; *ef1a*, elongation factor 1α.

Supplementary Table 3.3. Proximate composition (%) of whole body, liver, kidney and heart of meagre fed diets with different levels of vitamin E, C and K during 104 days.

	Diets					
	0	K	EC	KEC	EECC	KEECC
Whole body						
Lipid (%)	4.7 ± 0.4	4.9 ± 0.5	4.7 ± 0.3	5.1 ± 0.5	5.0 ± 0.4	4.8 ± 0.4
Protein (%)	16.7 ± 0.7	15.8 ± 0.4	16.9 ± 0.7	15.8 ± 0.5	15.4 ± 0.4	16.9 ± 0.4
Ash (%)	3.8 ± 0.2	3.7 ± 0.4	3.7 ± 0.2	3.9 ± 0.3	3.8 ±0.3	4.1 ± 0.4
Moisture (%)	74.3 ± 1.2	75.1 ± 1.5	74.5 ± 1.8	75.0 ± 1.5	75.2 ± 1.4	74.1 ±1.7
Liver						
Protein (%)	8.1 ± 0.2	7.9 ± 0.5	8.2 ± 0.3	8.2 ± 0.3	8.0 ± 0.4	8.1 ± 0.2
Ash (%)	0.7 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.2	0.8 ± 0.0
Moisture (%)	65.0 ± 0.7	66.3 ± 1.6	65.7 ± 2.3	64.4 ± 2.1	66.1 ± 1.1	65.2 ± 1.8
Kidney						
Protein (%)	14.7 ± 0.7	15.1 ± 0.3	14.8 ± 0.5	14.9 ± 0.5	15.1 ± 0.6	14.8 ± 0.3
Ash (%)	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.2	0.5 ± 0.0
Moisture (%)	79.9 ± 0.3	79.8 ± 0.8	80.1 ± 1.8	79.8 ± 0.8	79.3 ± 1.0	79.6 ± 1.2
Heart						
Protein (%)	15.4 ± 0.7	15.6 ± 0.3	15.1 ± 0.5	15.2 ± 0.5	15.8 ± 0.6	15.2 ± 0.3
Ash (%)	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
Moisture (%)	80.7 ± 1.0	80.7 ± 0.6	81.6 ± 0.9	81.8 ± 1.0	79.8 ± 1.3	80.6 ± 0.7

Data are means ± SD (n=3). Significant differences were not observed according to two-way ANOVA.

Chapter 4

Incidence of systemic granulomatosis is modulated by the feeding sequence and type of enrichment in meagre (*Argyrosomus regius*) larvae

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Abstract

Systemic granulomatosis is the most frequent disease in juvenile and adult meagre, but studies regarding the first appearance of granulomas in larvae do not exist. In order to evaluate this, meagre larvae were fed four different feeding regimes as follows: RS and RO (rotifer enriched with Easy DHA Selco or Ori-Green from 3-30 dph, respectively), RAS and RAO (rotifer enriched with Easy DHA Selco or Ori-Green from 3-21 dph and *Artemia* enriched with Easy DHA Selco or Ori-Green from 12-30 dph, respectively). All treatments were also fed with commercial microdiet from 20-30 dph. At 30 dph weight, length, specific growth rate and survival was significantly higher in *Artemia*-fed larvae, regardless of the enrichment. Microscopic first appearance of granulomas was observed in 20 dph larvae fed RS and RO. At 30 dph granulomas and thiobarbituric acid reactive substances (TBARS) values were significantly higher in RS and RO-fed larvae than in RAS and RAO-fed larvae. The results showed that granulomas first appeared in meagre larvae at 20 dph when fed rotifers only. Conversely, a reduced appearance of granulomas and lipid peroxidation occurs when *Artemi*a is included in the feeding sequence reinforcing the hypothesis of a nutritional origin of the systemic granulomatosis.

Keywords: Systemic granulomatosis, meagre larvae, vitamin E, *Artemia*, Rotifer, feeding sequence.

4.1. Introduction

Meagre (*Argyrosomus regius*) is one of the fast-growing species proposed as a candidate for marine fish diversification on commercial aquaculture in Mediterranean and Eastern Atlantic coasts, mainly due to attractive attributes for the market such as large size, good processing yield (Jiménez et al., 2005; Duncan et al., 2013), low fat content, excellent taste (Poli et al., 2003), firm texture and great capacity to adapt to captivity (El-Shebly et al., 2007). Nevertheless, one of the most critical points in meagre production is the fish health considering the systemic granulomatosis as the more frequent disease in this species (Ghittino et al., 2004). The pathology is characterised by the presence of multiple granulomas in several tissues being liver, heart and kidney the most affected organs (Chapter 3). Although systemic granulomatosis is a disease of unknown aetiology, there is evidence that it could be triggered by a nutritional imbalance (Chapter 3, 5, 6; Cotou et al., 2016).

In the last years the studies on first life stages of meagre have been focused on larval density (Estevez et al., 2007; Roo et al., 2010), live prey feeding sequence (Roo et al., 2010) and light conditions (Vallés and Estévez 2013) in order to standardize the culture protocols. Additionally, El Kertaoui et al. (2017) studied the effect of vitamin E and C on growth parameters and in the fatty acids protection against oxidation in meagre larvae, while the omega 3 (n-3) polyunsaturated fatty acids (PUFA) requirement has been recently studied by Carvalho et al. (2018). However, there is not any study evaluating the relationship between nutrients and the appearance of systemic granulomatosis in meagre larvae.

The first live feed traditionally used in intensive aquaculture are rotifers (*Brachionus spp.*) due to their small body size (Theilacker and McMaster 1971; Støttrup, 2000). Rotifer are fed by filtration making it easy to enrich, furthermore they have high population growth rate at high density. In a later stage of development, when the mouth of the larvae is big enough, *Artemia* nauplii are used as a live prey. However, rotifer and *Artemia* do not cover all of the larvae nutritional requirements and for that reason it is necessary to enrich the live food in order to improve nutritional value for the marine fish larvae (Boglino et al., 2012). Presently, commercial production of meagre involves the use of both rotifers and *Artemia*, although the effect that these feeding sequences might have on the development and appearance of systemic granulomatosis is unknown.

Marine fish larvae have high requirements for long chain (LC) PUFA, namely eicosapentaenoic (20:5n-3; EPA) and docosahexaenoic acids (22:6n-3; DHA). Given that these fatty acids are highly unsaturated and thus at risk of suffering oxidation, fish larvae are more susceptible to suffering peroxidative attack than adults (Hamre et al., 2010). Reactive oxygen species (ROS) include oxygen ions, free radicals (superoxide and hydroxyl radicals), and peroxides (hydrogen peroxide) are normal products of the oxygen metabolism (Cadenas, 1989). There are enzymes capable to neutralize ROS, some of the most important are superoxide dismutase, catalase and glutathione peroxidase. Non-enzymatic compounds, such as vitamin C and E, are also powerful antioxidants (Montero et al., 1999; Ai et al., 2006; Betancor et al., 2012; Gao et al., 2014). In this sense, high concentrations of vitamin C and E have been detected in fish eggs indicating the importance of these micronutrients during fish early development (Hemre et al., 1994; Lie et al., 1994; Mukhopadhyay et al., 2003; Skřivan et al., 2013). The requirements for vitamins C and E given by NRC (2011) for fish are 30 and 25–120 mg kg^{-1}, respectively but the supplementation with extra dosage of both vitamins has proved to reduce mortality, oxidative stress as well as to improve growth in fish larvae (Stéphan et al., 1995; Merchie et al., 1997; Kolkovski et al., 2001).

On the other hand, typical visceral granulomatosis has been experimentally induced in turbot (*Scophthatmus maximus*) fed diets deficient in vitamin C (Messager et al., 1986). Also in turbot, Coustans et al. (1990) confirmed the role of ascorbic acid deficiency in the development of visceral granulomatosis and showed that the pathological condition was exacerbated by hypovitaminosis of B - complex. Vitamin C deficiency has also been responsible for the appearance of granulomatosis in the kidney of sea bream (*Sparus aurata*) (Alexis et al., 1997). In Chapter 3, it was observed that high dietary levels of vitamin C, E and K reduced the severity and incidence of systemic granulomatosis in meagre.

The objective of the present study was to evaluate the first appearance and incidence of systemic granulomatosis in meagre larvae. In order to do so, meagre larvae were fed rotifers enriched with two different commercial products with divergent levels of vitamins and with or without *Artemia* in the feeding sequence. This study will help to understand when the systemic granulomatosis first appears in the life cycle on meagre.

4.2. Material and methods

4.2.1. Fish

Meagre eggs were obtained from an induced spawning from broodstock from the ECOAQUA facilities at University of Las Palmas de Gran Canaria (ULPGC; Telde, Canary Islands, Spain) where the experiment was carried out. Eggs were volumetrically counted and set into 12 light grey colour cylindrical fibreglass tanks (4 triplicate treatments) of 170 l capacity at a density of 50 egg l^{-1}. All tanks were equipped with continuous aeration and supplied with filtered UV-sterilized seawater at an increasing rate from 5% h^{-1} to a 100% h^{-1}, to guarantee good water quality during the trial. Water entered the tanks at the bottom and exited at the surface. Oxygen (4.5-6.5 g l^{-1}), salinity (0.034 mg l^{-1}) and temperature (24.6 to 25.3 ºC) was daily measured. Photoperiod was kept at 12 h light: 12 h dark by fluorescent lights. The microalgae (*Nannochloropsis* sp.) was added to tanks at a density of 3 x 10^6 as green water, providing nutrients directly to the larvae, contributing to the preservation of live prey nutritional quality, promoting changes in visual contrast of the medium and minimizing the exposure to light, improving water quality reducing ammonium ion concentrations as well as increasing dissolved oxygen concentrations (Øie et al., 1997; Reitan et al., 1997; Huervana et al., 2006; Nakase et al., 2007; Stuart and Drawbridge, 2011).

All procedures were conducted in accordance with the regulations set forward by the Spanish RD 53/2013 (BOE 8th February 2013) and the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The experiment was subjected to ethical review by the Animal Welfare and Bioethical Committee at the University of Las Palmas de Gran Canaria (OEBA-ULPGC 06/2018).

4.2.2. Dietary trial

The feeding trial was performed in triplicate tanks and rotifers (*Brachionus* sp.) or *Artemia* sp. nauplii (Type AF, INVE, Dendermonde, Belgium) were enriched with different products. Rotifers (R) and *Artemia* (A) were enriched with either Easy DHA Selco (S; INVE, Dendermonde, Belgium) or Ori-Green (O; Skretting, Norway), giving place to four dietary treatments: RAS, RAO, RS and RO. Additionally inert microdiets

were used (Gemma Micro 150 and 300 µm; Skretting, France) at the end of the feeding experiment (from 20 to 30 dph; Table 4.1, Supplementary Figure 4.1; Appendix 4.5).

Larvae were fed with enriched rotifers twice daily (9:00 and 14:00) from 3 to 21 (RAS and RAO treatments) or 3 to 30 (RS and RO treatments) days post hatching (dph). Larvae fed diets RAS and RAO were fed enriched *Artemia* twice daily (11:00 and 15:00) from 12 to 30 dph. Additionally, larvae from all the dietary treatments were co-fed with inert microdiets from 20 to 30 dph at a feeding rate of 10-15 % of the biomass.

Table 4.1. Meagre (*Argyrosomus regius*) larvae feed sequence from 3 dph to 30 dph.

TREATMENT	RAS	RAO	RS	RO	Feed added
		Day post hatch (dph)			
Phytoplankton	3-18	3-18	3-30	3-30	300.000 cells ml^{-1}
Enriched Rotifer	3-21	3-21	3-30	3-30	10 ind ml^{-1}
Enriched *Artemia*	12-13	12-13	-	-	0.5 ind ml^{-1}
Enriched *Artemia*	14-30	14-30	-	-	1.3-1.5 ind ml^{-1}
Microdiet 150-300 µm (50 %)	20-30	20-30	20-30	20-30	1.5-2 g

4.2.3. Live prey culture and enrichment protocol

Rotifers and *Artemia* were cultured at the ECOAQUA facilities at the University of Las Palmas de Gran Canaria (ULPGC; Telde, Canary Islands, Spain). Rotifers were cultured at a density of 400 rotifers ml^{-1} in 500 L enrichment troncoconical-tanks. Enrichment tanks were well aerated (5 mg l^{-1} O_2) with filtered and UV-treated seawater (0.037 mg l^{-1}Rotifer were enriched with Ori-Green (0.15 - 0.25 g $million^{-1}$ rotifers, RO dietary treatment) for two hours before being fed to the meagre larvae or with Easy DHA Selco (0.6 g l^{-1}, RS dietary treatment) for 24 hours.

Artemia cysts were hatched at 27° C and 0.030 mg l^{-1}salinity until 100 % hatch was achieved. Then they were rinsed with sea water and transferred to a culture tank at 24° C. *Artemia* was enriched with Ori-Green (0.8 g $million^{-1}$ *Artemia*, RAO dietary treatment) for 12 hours or with Easy DHA Selco (0.6 g $million^{-1}$ *Artemia*, RAS dietary treatment) for 24 hours before being fed to the larvae. During the co-feeding period micro-diets Gemma Micro 150 and 300 (Skretting, France) were used (15 % biomass d^{-1}). Composition and fatty acid profile of all dietary treatments is shown at Tables 4.2 and 4.3

Table 4.2. Ingredients, fatty acid composition and gross composition of the enrichment and inert microdiets fed to meagre (*Argyrosomus regius*) larvae.

	Easy DHA Selco	**Ori-green**	**Micro-diet**
Proteins (%)	27	43	59
Lipids (%)	67	30	14
Vitamin A (IU kg^{-1})	1,500	350	23,000
Vitamin D_3 (IU kg^{-1})	150,000	50	2,800
Vitamin E (mg kg^{-1})	3,600	4,000	400
Vitamin C (mg kg^{-1})	800	-	1,000
Fatty acids (%)			
14:0	3.66	1.87	1.89
16:0	15.89	20.14	17.79
18:0	4.09	5.26	3.59
20:0	0.29	0.46	0.21
Σ Saturated[†]	24.76	28.37	23.9
16:1n-7	5.07	2.81	2.41
18:1n-9	23.75	16.44	13.83
18:1n-7	3.10	2.05	2.23
20:1n-7	3.48	1.26	2.22
22:1n-11	2.74	0.64	1.89
Σ Monosaturated[‡]	39.97	24.39	23.8
18:2n-6	8.68	14.28	28.14
18:3n-6	0.21	0.21	0.08
20:2n-6	0.42	0.25	0.23
20:3n-6	0.12	0.10	0.05
20:4n-6	0.93	1.38	0.71
Σ n-6PUFA[§]	10.60	16.50	29.3
18:3n-3	1.98	2.55	3.18
18:4n-3	0,91	0,41	0.69
20:3n-3	0,19	0,16	0.13
20:4n-3	0,76	0,36	0.27
20:5n-3	5,13	4,21	4.79
22:5n-3	1,78	1,16	0.68
22:6n-3	12,08	20,77	12.28
Σ n-3PUFA[¶]	22,84	29,59	22.0
(n-3+n-6) PUFA	10,60	16,50	51.3
Total n-3 LC-PUFA[††]	19.75	26.5	18.02

[†]Includes 15:0 and 17:0. [‡]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9 and 20:1n-5. [§]Includes. 22:5n-6 and 22:4n-6. [¶]Includes 16:3n-3 and 16:4n-3. [††]Includes. 22:1n-9, 20:3n-9, 20:2n-9, 20:1n-9, 18:2n-9 and 18:1n-9. [‡‡]LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Table 4.3. Fatty acid composition (percentage of total fatty acids) and vitamin E content of *Artemia* (A) and rotifers (R) enriched with either Easy DHA Selco (S) or Ori green (O) used to feed meagre (*Argyrosomus regius*) larvae as well as the commercial microdiet used (Gemma Micro 150 and 300 µm; Skretting).

Fatty acids (%)	**AS**	**AO**	**RS**	**RO**
14:0	0.90	0.61	2.05	1.66
16:0	11.32	11.23	12.83	10.14
18:0	6.53	6.07	5.55	3.70
20:0	0.22	0.19	0.19	0.14
Σ Saturated[†]	20.1	19.2	22.1	17.0
16:1n-7	2.33	1.80	12.35	15.76
18:1n-9	21.77	18.29	22.95	18.71
18:1n-7	6.09	5.66	5.47	4.05
20:1n-7	1.49	0.72	2.63	2.90
22:1n-11	0.60	0.04	0.96	0.56
Σ Monosaturated[‡]	34.0	28.4	49.0	46.4
18:2n-6	6.12	6.38	8.89	12.85
18:3n-6	0.50	0.68	0.19	0.11
20:2n-6	0.37	0.28	0.32	0.52
20:3n-6	0.16	0.13	0.40	0.83
20:4n-6	0.79	0.42	1.35	1.30
Σ n-6PUFA[§]	8.0	7.9	11.4	15.8
18:3n-3	22.55	31.22	1.58	2.22
18:4n-3	4.15	6.03	0.59	0.21
20:3n-3	1.19	1.46	0.11	0.14
20:4n-3	0.97	1.11	0.66	0.56
20:5n-3	3.29	1.11	4.05	3.98
22:5n-3	0.59	0.09	1.40	1.20
22:6n-3	3.54	1.31	5.92	8.48
Σ n-3PUFA[¶]	36.3	42.3	14.3	16.8
(n-3+n-6) PUFA	44.3	50.2	25.7	32.6
Total n-3 LC-PUFA[††]	8.39	3.62	12.03	14.22
Vitamins				
Vitamin E (mg kg^{-1})	410.7^{d}	368^{c}	310.1^{b}	189.9^{a}
Vitamin C (mg kg^{-1})	1037.4^{b}	1033^{b}	279.8^{a}	287.5^{a}
Proximate composition				
Protein	52.2	53.5	48.3	49.3
Lipid	22.4	20.3	18.3	17.1
Ash	11.5	12.7	10.2	11.9
Moisture	10.1	9.8	9.4	9.7

Data expressed as means of three technical replicates per batch of diet. [†]Includes 15:0 and 17:0. [‡]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. [§]Includes. 22:5n-6 and 22:4n-6. [¶]Includes 16:3n-3 and 16:4n-3. [††] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

4.2.4. Sample collection

Samplings were performed at day 10, 20 and 30 dph. On each sampling 50 larvae per tank (150 per diet) were sacrified with clove oil and measured for total length. After measurement, that the 50 larvae were fixed in 4 % buffered formalin for histological analysis. Another 30 larvae were collected to determine dry weight at each sampling point. Additional 30 larvae were collected for biochemical and TBARS analysis and stored at -80 °C until analysis.

4.2.5. Growth and survival

Every 10 days, 50 larvae from each tank were sampled and measured for dry weight (100 °C for 24 hours) and total length (TL) using a profile projector (Mitutoyo PJ-3000A, Kanagawa, Japan). Final survival was determined at 30 dph by counting remaining alive larvae in experimental tanks.

4.2.6. Biochemical analysis

Larvae and feeds biochemical composition analysis were conducted following standard procedures (AOAC, 2010). Lipids of larvae and feeds were extracted with a choloroform:methanol (2:1 v/v) mixture as described by Folch et al. (1957).

Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982). Fatty acid methyl esters (FAMES) were separated and quantified by gas–liquid chromatography following the conditions described by Izquierdo et al. (1992).

TBARS were measured in triplicate from extracted total fatty acids (10 mg ml^{-1}) according to Burk et al. (1980). 50 µl of 0.2 % (w/v) BHT in ethanol was added to 200 µl of lipid sampled. Following 0.5 ml of 1 % (w/v) TBA in MiliQ distillate water and 0.5 ml of 10 % (w/v) trichloroacetic acid in MiliQ distillate water were added to the sample, all solutions were freshly prepared. All mixed reagents were mixed in a stoppered test tube and heated in dark at 100 °C for 20 min. Then, samples were cooled in ice 5 min and particulate matter was removed by centrifugation at 2000 g (Sigma 4K15, Osterode am Harz, Germany) 5 min. Supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm and recorded against a blank sample. 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}.

The concentration of vitamin E was determined in the dietary treatments (Table 4.3). The α-TOH was injected (50 μl) in a high performance liquid chromatograph (HPLC) with UV detection at the University of Stirling (Scotland, UK). Samples were weighed, homogenized in ethanolic pyrogallol and saponified as described by McMurray et al. (1980). HPLC analysis was performed using 150 x 4.60 mm, 5 μm reverse-phase Luna and C18 column (Phenomenox, CA, USA). The mobile phase was methanol:ultrapure water (98:2 v/v) with a flow rate of 1.0 ml min^{-1} in ambient temperature. It was used a wavelength of 293 nm to determine the vitamin E concentrations and was achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich) as the external standard.

The concentration of vitamin C was determined in the dietary treatments (Table 4.3) as described by Betancor et al. (2012). Samples were weighed, homogenised and dissolved in 0.4 M phosphate buffer (adjusted to pH 3.0 with phosphoric acid). The samples were centrifuged at 3.000 rpm, supernatants removed and filtered through a disposable 0.45 μm filter and stored at 4° C until the measurement in a HPLC with UV detection. The determination of vitamin C concentration was achieved by comparison with tris (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich) as the external standard.

4.2.7. Histopathology

In order to estimate the first appearance of granulomas, every 10 days (10, 20, 30 dph) 50 larvae per tank (150 larvae per treatment) were sacrificed with clove oil and fixed in 4 % buffered formalin for histological analysis. The samples were dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 μm, fixed to the microscope slide, heated and finally stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco method (Fite, Cambre, and Turner, 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological evaluation.

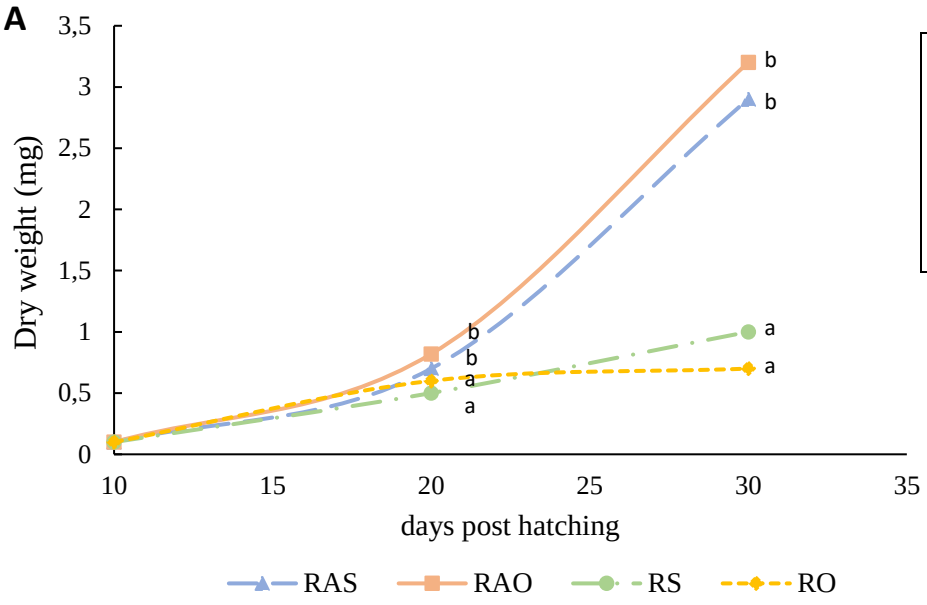
4.2.8. Statistical analysis

All statistical analyses were done with Statgraphics (Statgraphics Centurion XVI version 16.1.03 for Windows; Graphic Software Systems, Inc. USA). Prior top statistical analysis percentage data were arsin transformed. Survival, growth, percentage of larvae with granulomas and biochemical analysis were tested for normality with the Kolmogorov Smirnov test and homogeneity of variance was performed with the Levene test. Survival, growth, percentage of larvae with granulomas and biochemical analysis data were treated by one-way ANOVA followed by Tukey post-hoc test ($P<0.05$). Growth, survival, fatty acid composition and percentage of granulomas were analysed by two-way analysis of variance (ANOVA). A significance level of 0.05 was used.

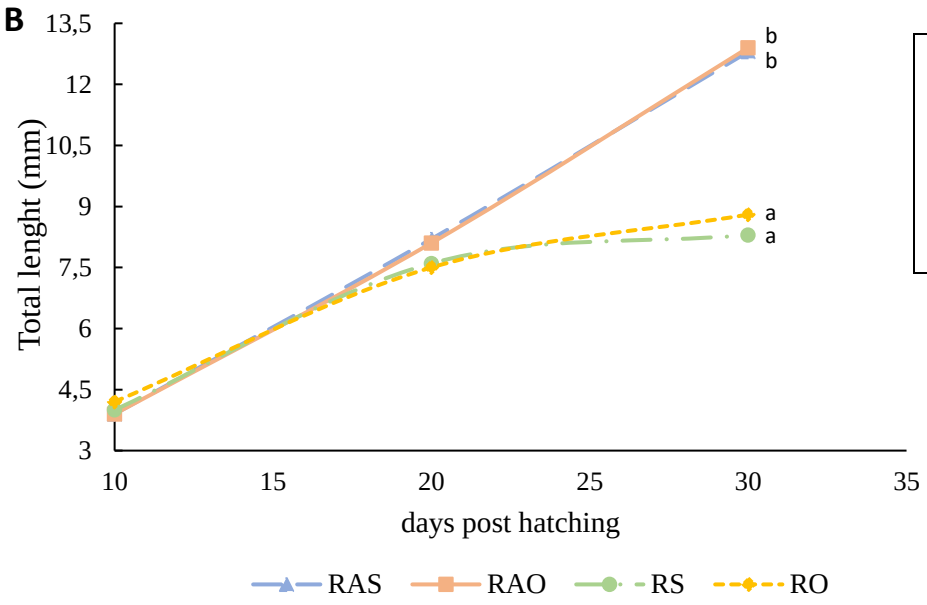
4.3. Results

4.3.1. Growth and survival

Significant differences in larvae dry weight and total length were found among the dietary treatments at 20 dph, being higher in larvae fed with *Artemia* regardless of the enrichment (Figure 4.1). This superior growth in *Artemia*-fed larvae was maintained at 30 dph. Specific growth rate (SGR) was significantly higher in larvae fed dietary treatments RAS and RAO (15.7 and 16.6 %, respectively) compared with those fed RS and RO (11.6 and 8.3 %, respectively) (Table 4.4). Survival was significantly higher in larvae fed with *Artemia* (Table 4.4). All these differences could be explained by the factor "live prey" as indicated by the two-way ANOVA, with "enrichment media" also affecting dry weight at 20 dph. No interactive effect between "live prey" and "enrichment media" was observed for any of the performance parameters.



	p Value two-way ANOVA		
	LP	EN	LP*EN
10 dph	n.s.	n.s.	n.s.
20 dph	**	*	n.s.
30 dph	**	n.s.	n.s.



	p Value two-way ANOVA		
	LP	EN	LP*EN
10 dph	**	n.s.	n.s.
20 dph	**	n.s.	n.s.
30 dph	**	n.s.	n.s.

Figure 4.1. Meagre (*Argyrosomus regius*) larvae A) dry weight (mg) and B) total length (mm) after 10, 20 and 30 dph of experimental trial fed with *Artemia* (A) or rotifers (R) enriched with either Easy DHA Selco (S) or Ori-green (O). LP, live prey; EN, enrichment; LP*EN, interaction between live prey and enrichment. * $p < 0.05$; ** $p < 0.01$.

4.3.2. Histopathology

First observation of granulomas under the microscope was at 20 dph in liver of fish fed rotifer enriched with Selco (RS; 1.3 %) and with Ori-Green (RO; 2.0 %), without significant differences (Figure 4.2).

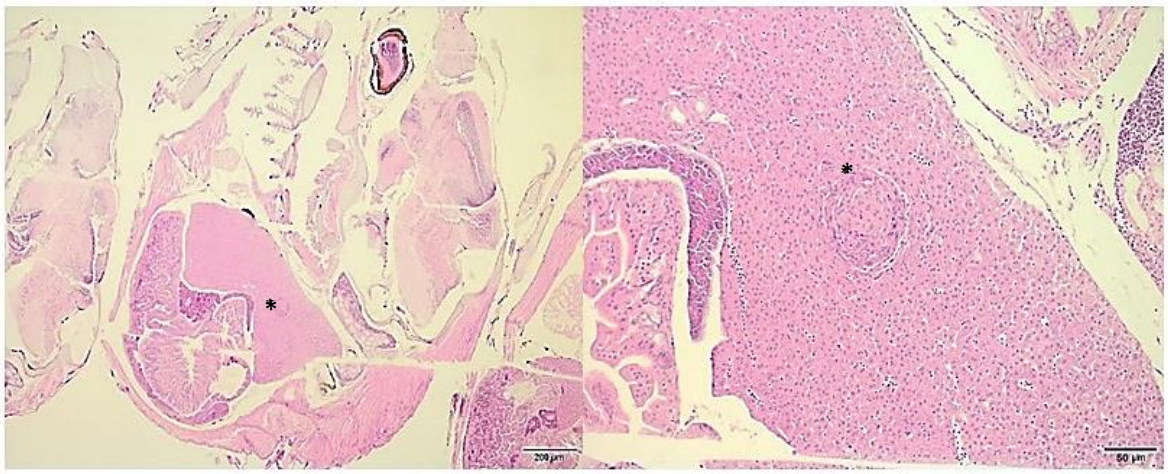


Figure 4.2. Granuloma in the liver of meagre larvae fed with experimental diets at 20 dph. *Microscopic granuloma.

The incidence of granulomas in meagre larvae increased after 30 dph, mainly in larvae fed rotifer-only together with microdiet. The percentage of affected larvae with granulomas was significantly higher in RS and RO-fed larvae (10.7 and 15.3 %, respectively) compared with larvae fed RAS (0 %) and RAO (0.7%) (Figure 4.3). “Live prey” was the only factor driving the differences in the appearance of granulomas at 30 dph.

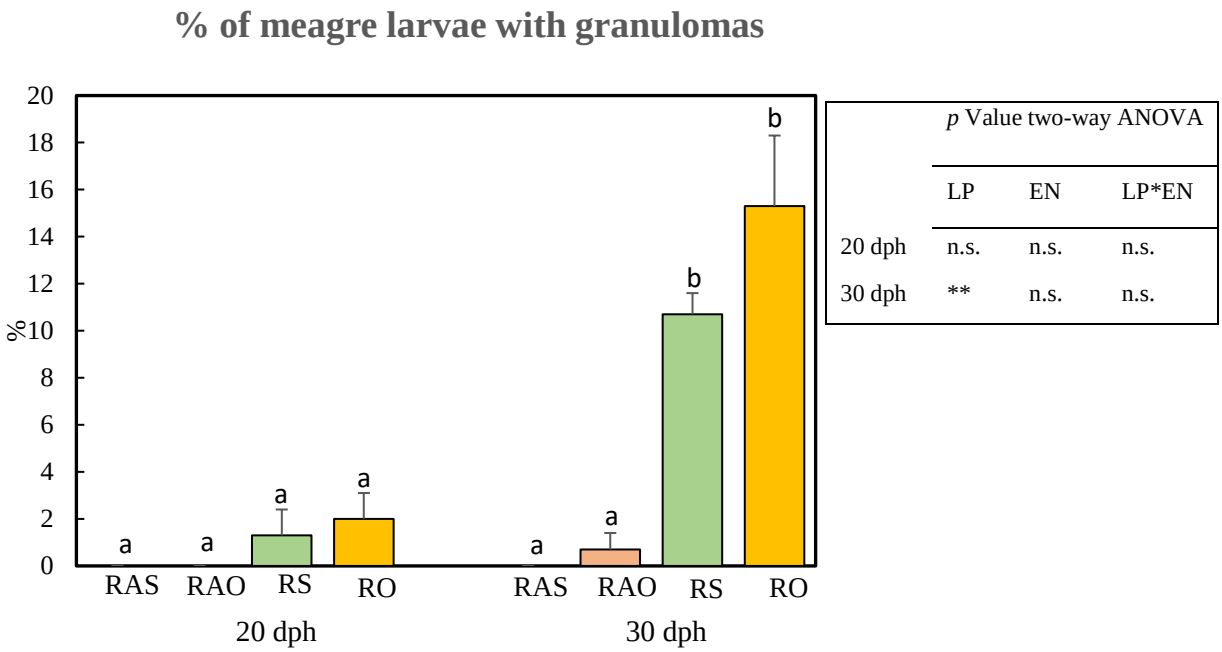


Figure 4.3. Incidence of granulomas (%) in meagre larvae after feeding with *Artemia* (A) or rotifers (R) enriched with either Easy DHA Selco (S) or Ori-green (O) for 20 or 30 dph. Each value represents mean ± SD (n=600). Different superscript letters denote differences among treatments identified by one-way ANOVA. The inset table presents p values for the effect of live prey, enrichment and their interaction on both factors on presence of granulomas.

Regarding the morphology of the granulomas, all were at initial stages of development and could be observed as isolated and irregular aggregated of macrophages with scattered lymphocytes (Figure 4.4A) that later were forming concentric layers with a necrotic centre (Figure 4.4B). The main affected organ with granulomas was liver, followed by kidney. The specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stain) were all negative, discarding a possible infectious origin (Figure 4.5A, 4.5B and 4.5C).

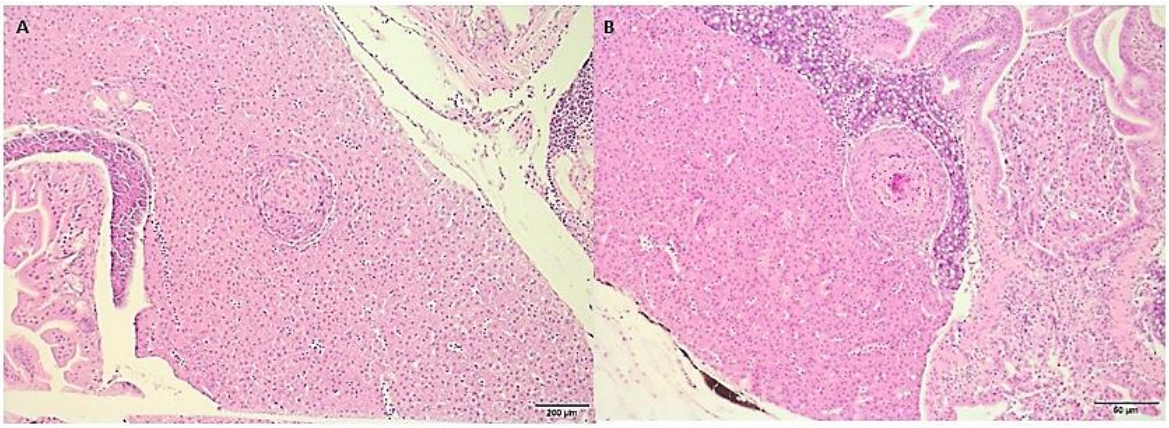


Figure 4.4. Different stages of granuloma development in meagre larvae of 20 and 30 dph fed with experimental diets. **A)** Irregular aggregated of macrophages and inflammatory cells. **B)** Concentric layers of macrophages and necrotic centre.

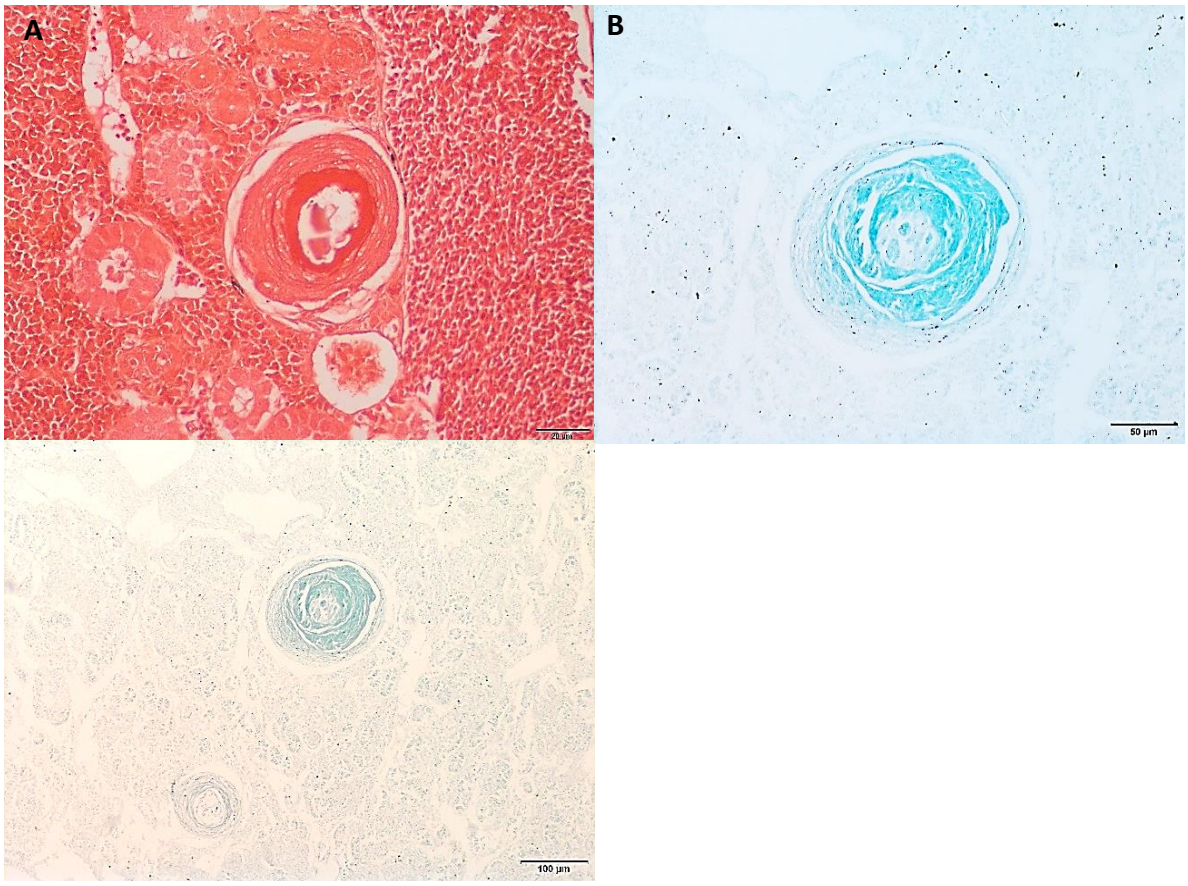


Figure 4.5. Negative staining in granulomas with A) Gram stain in kidney, B) Ziehl-Neelsen stain in liver and C) Fite-Faraco stain in liver.

4.3.3. Whole larvae composition, TBARS content and fatty acid profiles

There were not differences in the tissue lipid, protein or ash content among larvae fed the different experimental diets (Table 4.4). The level of lipid peroxides, as indicated by TBARS content (μmol g^{-1} larval tissues), was significantly higher in those larvae fed rotifer only together with microdiet (diets RO and RS) (Table 4.4). The factor "enrichment media" did not drive the differences observed according to the two-way ANOVA, indeed only live prey factor influenced TBARS content.

Table 4.4. Proximate composition and TBARS content in meagre (*Argyrosomus regius*) larvae after 30 dph of experimental trial fed with *Artemia* (A) or rotifers (R) enriched with either Easy DHA Selco (S) or Ori-green (O).

					two-way Anova p		
Proximate composition (%)	RAS	RAO	RS	RO	LP	EN	LP*EN
Lipid	2.1 ± 0.2	2.0 ± 0.2	1.9 ± 0.1	1.9 ± 0.2	n.s.	n.s.	n.s.
Protein	10.1 ± 2.1	10.7 ± 1.5	10.9 ± 2.0	10.6 ± 1.3	n.s.	n.s.	n.s.
Ash	2.7 ± 0.1	2.8 ± 0.2	2.7 ± 0.8	2.9 ± 0.3	n.s.	n.s.	n.s.
Moisture	82.1 ± 2.8	82.3 ± 2.1	82.7 ± 2.2	82.3 ± 2.7	n.s.	n.s.	n.s.
TBARS content (μmol g^{-1})	67.4 ± 1.9^{a}	56.8 ± 7.1^{a}	288 ± 15.9^{b}	282.9 ± 11^{b}	**	n.s.	n.s.
SGR	15.7 ± 1.3^{b}	16.6 ± 2.3^{b}	11.6 ± 2.5^{a}	8.3 ± 1.1^{a}	**	n.s.	n.s.
Survival	19.5 ± 3.4^{b}	17.2 ± 1.7^{b}	14.8 ± 0.8^{a}	12.8 ± 1.4^{a}	**	n.s.	n.s.

Data expressed as means of three technical replicates per batch of larvae. SGR, specific growth rate; LP, live prey; EN, enrichment; LP*EN, interaction between live prey and enrichment. Different superscript letters denote differences among treatments identified by one-way ANOVA.

Fatty acid composition of the larvae (Table 4.5) generally reflected the fatty acid composition of the diet (Table 4.2 and 4.3). Meagre larvae fed RAS showed significantly higher monounsaturated fatty acids and n-9 PUFA. Larvae fed RAS and RAO had significantly higher concentration of linoleic acid (18:2n-6) and n-6. Larvae fed with rotifers only had significantly higher concentration of saturated fatty acids, arachidonic acid (ARA) and DHA. The factor "live prey" explained most of the differences in the fatty acid profile. However, an interaction between "live prey" and "enrichment media" lead to differences in the percentages of certain fatty acids such as 16:1n-7, 22:1n-11, 18:3n-3, 20:3n-3 and 20:4n-3.

Table 4.5. Fatty acid composition (percentage of fatty acids) of meagre (*Argyrosomus regius*) larvae fed with experimental diets.

					two-way Anova p		
Fatty acids (%)	**RAS**	**RAO**	**RS**	**RO**	**LP**	**EN**	**LP*EN**
14:0	0.8 ± 0.2^{b}	0.6 ± 0.0^{b}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	*	n.s.	n.s.
16:0	16.8 ± 0.9	17.0 ± 1.7	19.5 ± 3.4	18.0 ± 1.7	n.s.	n.s.	n.s.
18:0	8.0 ± 0.3^{a}	9.8 ± 1.3^{a}	14.1 ± 1.9^{b}	13.5 ± 1.2^{b}	**	n.s.	n.s.
20:0	0.3 ± 0.0^{a}	0.4 ± 0.0^{a}	0.5 ± 0.1^{b}	0.5 ± 0.0^{b}	**	n.s.	n.s.
Σ Saturated†	26.9 ± 1.5^{a}	28.8 ± 3.1^{a}	35.3 ± 5.6^{b}	33.2 ± 3.0^{b}	*	n.s.	n.s.
16:1n-7	2.3 ± 0.3^{b}	1.6 ± 0.1^{a}	2.1 ± 0.1^{b}	2.3 ± 0.3^{b}	n.s.	n.s.	*
18:1n-9	18.4 ± 1.8^{d}	15.7 ± 0.9^{c}	14.1 ± 1.0^{ab}	13.2 ± 0.8^{a}	**	n.s.	n.s.
18:1n-7	4.4 ± 0.3^{b}	4.7 ± 0.3^{b}	3.5 ± 0.0^{a}	3.4 ± 0.3^{a}	**	n.s.	n.s.
20:1n-7	1.6 ± 0.1^{b}	1.2 ± 0.0^{a}	1.4 ± 0.2^{ab}	1.3 ± 0.1^{a}	n.s.	**	n.s.
22:1n-11	0.8 ± 0.1^{c}	0.5 ± 0.0^{b}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	**	**	*
Σ Monosaturated‡	28.9 ± 2.8^{b}	25.2 ± 1.5^{a}	23.6 ± 1.7^{a}	22.6 ± 1.8^{a}	*	n.s.	n.s.
18:2n-6	12.2 ± 0.7^{b}	11.7 ± 0.3^{b}	8.1 ± 1.1^{a}	9.0 ± 0.6^{a}	**	n.s.	n.s.
18:3n-6	0.2 ± 0.0^{a}	0.3 ± 0.0^{b}	0.1 ± 0.1^{a}	0.1 ± 0.1^{a}	**	n.s.	n.s.
20:2n-6	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	n.s.	n.s.	n.s.
20:3n-6	0.2 ± 0.0^{a}	0.3 ± 0.0^{ab}	0.3 ± 0.1^{b}	0.5 ± 0.1^{c}	**	*	n.s.
20:4n-6	1.9 ± 0.4^{a}	2.1 ± 0.2^{a}	3.6 ± 0.5^{b}	4.1 ± 0.4^{b}	**	n.s.	n.s.
Σ n-6PUFA§	15.2 ± 1.2^{b}	15.4 ± 1.1^{b}	12.9 ± 1.8^{a}	14.7 ± 1.3^{b}	**	*	n.s.
18:3n-3	7.5 ± 0.7^{b}	11.1 ± 1.9^{c}	0.5 ± 0.1^{a}	0.5 ± 0.0^{a}	**	*	*
18:4n-3	0.9 ± 0.1^{b}	1.4 ± 0.4^{c}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	**	n.s.	n.s.
20:3n-3	0.6 ± 0.0^{b}	0.9 ± 0.1^{c}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	**	**	**
20:4n-3	0.6 ± 0.0^{b}	0.7 ± 0.1^{c}	0.2 ± 0.1^{a}	0.2 ± 0.0^{a}	**	n.s.	**
20:5n-3	3.9 ± 0.6	3.0 ± 0.5	3.1 ± 1.0	3.1 ± 0.5	n.s.	n.s.	n.s.
22:5n-3	1.3 ± 0.3^{b}	0.7 ± 0.0^{a}	1.7 ± 0.5^{b}	1.3 ± 0.2^{b}	*	*	n.s.
22:6n-3	11.6 ± 2.8^{a}	9.7 ± 1.9^{a}	17.2 ± 4.5^{b}	19.6 ± 4.1^{b}	*	n.s.	n.s.
Σ n-3PUFA¶	26.4 ± 5.7	27.7 ± 4.9	22.9 ± 7.2	24.8 ± 4.9	*	n.s.	n.s.
Σ n-9PUFA††	19.2 1.9^{b}	16.5 0.9^{a}	15.2 1.2^{a}	14.4 1.1^{a}	**	n.s.	n.s.
(n-3+n-6) PUFA	41.7 ± 6.9	43.1 ± 6.1	35.9 ± 9.0	39.8 ± 6.2	**	*	n.s.
Total n-3 LC-PUFA‡‡	17.4 ± 4.9^{ab}	14.2 ± 2.5^{a}	22.2 ± 5.1^{ab}	24.2 ± 4.8^{b}	*	n.s.	n.s.

Data expressed as means of three technical replicates per batch of larvae. †Includes 15:0 and 17:0. ‡Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5. §Includes. 22:5n-6 and 22:4n-6. ¶Includes 16:3n-3 and 16:4n-3. ††Includes. 22:1n-9. 20:3n-9. 20:2n-9. 20:1n-9. 18:2n-9. 18:1n-9. ‡‡LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3). LP, live prey; EN, enrichment; LP*EN, interaction between live prey and enrichment. Different superscript letters denote differences among treatments identified by one-way ANOVA.

* $p < 0.05$.

** $p < 0.01$.

4.4. Discussion

Significant differences in terms of dry weight, SGR and total length were observed at 30 dph being higher in meagre larvae fed with the combination of rotifers, *Artemia* and microdiets (diets RS and RO) regardless of the enrichment media used. Survival was also significantly higher in those larvae fed with *Artemia* (19.5 % RAS and 17.8 % RAO) compared with larvae fed rotifers only (14.8 % RS and 12.8 % RO). Similar results were obtained by Fernández-Palacios et al. (2009b) who showed that meagre larvae were significantly larger, and the survival was higher when co-fed with *Artemia* instead of rotifers only. The energy that is required to breakdown, absorb, transport and assimilate nutrients from a meal adds up to 25 % of the daily energy expenditures in fish (Secor, 2011). As larvae grow, the range of accessible particle size increase, however, the ingested prey also needs to supply mass and energy. The growing larvae try to ingest the maximum quantity of nutrients required for growth, while maintaining a favourable ratio between the energy gained by the ingestion of the prey and the energy spent in the capture (Herbing et al. 2001; Puvanendran et al., 2004). The lack of *Artemia* in the feeding sequence lead to poor survival and growth probably because of a high energy cost/benefit related to the small size of the prey (rotifers), indicating that it is necessary an intermediate feeding between rotifers and microdiet. The present study suggests that it is possible to wean meagre larvae directly from rotifer to an inert feed from 20 dph (survival 12.8-14.8 %), but a co-feeding period with *Artemia* implies higher survival and growth of the larvae. Survival and growth results were in the same range as those reported by Estevez et al. (2007) and Rodríguez-Rúa et al. (2007) for the same teleost species.

Apart from larvae performance, one of the aims of the present study was to assess the first appearance of granulomas in meagre larvae (10 to 30 dph). To the authors knowledge this is the first report of systemic granulomatosis in meagre larvae. Specifically, the first observation of microscopic granulomas was at 20 dph on fish fed with rotifer only either enriched with Selco (RS; 1.3 %) or with Ori-Green (RO; 2.0 %). On the contrary, fish fed with *Artemia* did not show any granuloma until 30 dph (only 0.7 % incidence; RAO-fed larvae). However, after 30 dph significant differences were observed in the percentage of granulomas among the dietary treatments, being higher in fish fed diet RO and RS (15.7 and 10.7 %, respectively) compared with fish fed RAO and RAS (0.7 and 0.0 %, respectively). In the present study, the concentration of vitamin E in rotifers (310.1-179.9 mg kg^{-1}) was lower than in *Artemia* (410.7 and 368.0 mg kg^{-1})

and was also influenced by the enrichment media, being higher the concentration in the diets RAS and RS compared with RAO and RO. The vitamin content of rotifer and *Artemia* has been previously studied by Meeren et al. (2008) being the level of vitamin E slightly lower in rotifer but those of vitamin C clearly lower (220.1 μg g^{-1}) compared with *Artemia* (530.6 μg g^{-1}). Vitamin C is a powerful antioxidant and its deficiency has been related with the appearance of granulomas in turbot (Messager et al., 1986; Coustans et al., 1990), sea bream (Alexis et al., 1997) and meagre (Chapter 3). The lack of *Artemia* in the feeding sequence increased the appearance of granulomas in meagre larvae after 30 dph (diets RO and RS) related likely with the differences in antioxidant vitamins of the live prey. Nevertheless, feeding with enriched rotifer, followed by enriched *Artemia* and microdiet, could prevent the occurrence of granulomas in meagre larvae after 30 dph (diet RAS), being the period before feeding with microdiet, a critical point to the development of systemic granulomatosis in meagre.

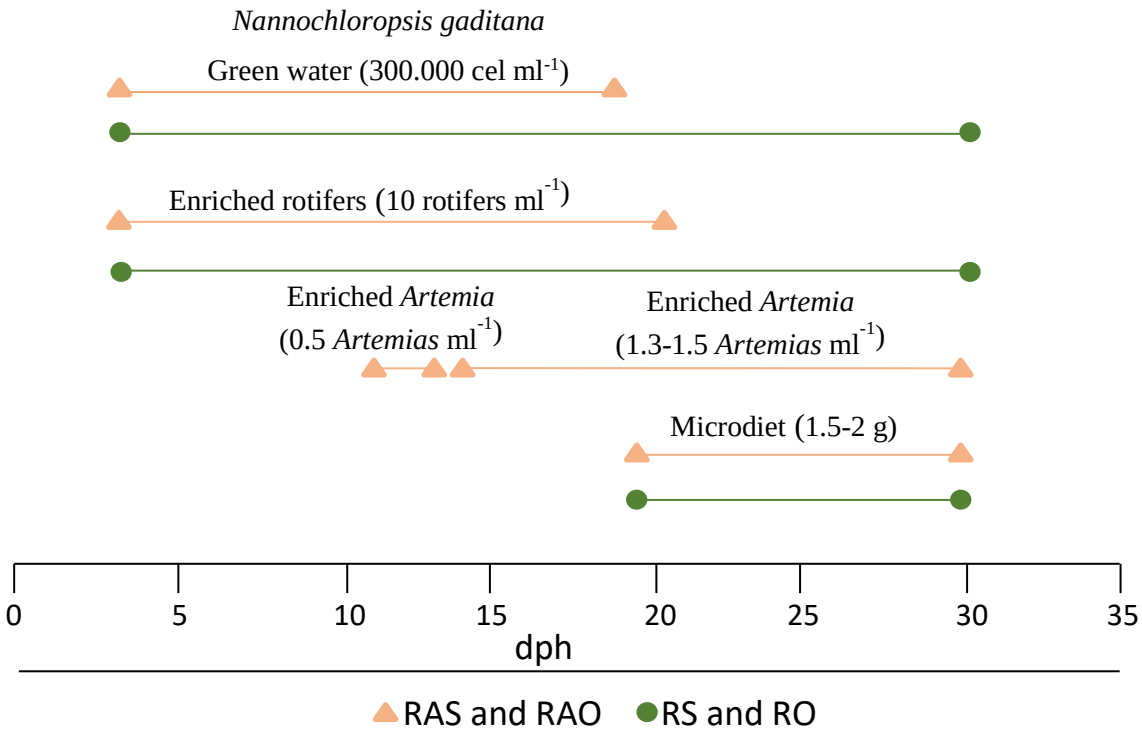
Besides, in the present study the appearance of systemic granulomatosis was highly correlated with TBARS content in meagre larvae (R^2=0.948, y=0.084x-4.3924), corroborating a possible relationship between lipid peroxidation and the appearance of granulomas. In this sense, TBARS levels were significantly higher in meagre larvae fed with rotifers only and microdiet (282.9-288.0 μmol g $tissue^{-1}$) compared with larvae fed with rotifer, *Artemia* and microdiet (56.8-67.4 μmol g $tissue^{-1}$). Moreover, a higher incidence of granulomas was observed at 30 dph in meagre larvae fed diets RS (10.7 %) and RO (15.7 %) with less analysed vitamin E contents in the diet (310.1 and 179.9 mg kg^{-1}, respectively) than in diets RAS (0 %) and RAO (0.7 %) with higher concentration of vitamin E (410.7 and 368.0 mg kg^{-1}, respectively). Betancor et al. (2011) observed that in sea bass (*Dicentrarchus labrax*) larvae the supplementation of vitamin E up to 3,000 mg kg^{-1} diet had an antioxidant effect reducing TBARS values, decreasing lipid peroxidation and reducing the incidence of muscular lesions. In juveniles of meagre, the dietary increase of vitamin E and C lead to a reduction in the percentage and severity of granulomas in liver and heart together with a decrease in TBARS content (Chapter 3, 5, 6), suggesting that these vitamins have an important role in the development of systemic granulomatosis in this species.

El Kertaoui et al. (2017) determined that the requirements of n-3 PUFA for meagre larvae were a 3 % (1.5-1.7 % DHA) in order to improve lipid absorption, fatty acid profile and growth. The levels of n-3 PUFA in all diets in the present study probably fulfilled the

requirements However, these fatty acids, specially DHA, are very prone to oxidation (Izquierdo et al., 2013) being necessary an adequate level of antioxidant nutrients to avoid lipid peroxidation. The combination of a high concentration of DHA together with low vitamin E and C levels can lead to an imbalance between prooxidant and antioxidant nutrients, as observed in a higher TBARS values in meagre larvae fed diets RO and RS. When an imbalance between the generation and removal of ROS by cellular defences occurs a status of oxidative stress takes place. The oxidative stress has been related with some diseases such as haemolysis (Kawatsu, 1969), anaemia, liver degeneration (Cowey et al., 1984), jaundice (Sakai et al., 1989), skeletal alterations (Watanabe et al., 1989; Lewis-McCrea and Lall, 2007) or muscular dystrophy (Betancor et al., 2012). In agreement, the results of this study suggest that the systemic granulomatosis could be related to this imbalance. Vitamin E also seems to be related with the development of granulomas, as larvae fed with RAO and RO (enrichment media with the lowest vitamin E content) had a higher incidence of granulomas compared with larvae fed RAS and RS. On the other hand, the systemic granulomatosis in meagre is similar to the pathology produced by infectious agents such as *Mycobacterium* spp. (Gauthier and Martha, 2009) and *Nocardia* spp. (Labrie et al., 2008; Elkesh et al., 2013). The inability to determinate an infectious origin and the relation observed between the appearance of the systemic granulomatosis and the feeding sequence in meagre larvae reinforce the hypothesis of a nutritional origin of the disease.

Concluding, the inclusion of *Artemia* in the feeding sequence of meagre larvae significantly increased dry weight, total length, SGR and survival and reduced the incidence of systemic granulomatosis and TBARS content after 30 dph. Furthermore, larvae fed with diet RO and RS, with a higher concentration of DHA and lower vitamin E, had high TBARS values what suggests an imbalance between prooxidants and antioxidants. Feeding larvae with a combination of enriched rotifers and *Artemia* greatly decreased the incidence of granulomas, not finding any at 20 dph and just a 0.7 % at 30 dph in larvae fed RAO. This is the first study to report the appearance of granulomas in meagre larvae and to determine the implication of antioxidant nutrients in their development. Further investigation is required to understand the pathogenesis of the granulomas as consequence of an imbalance nutritional supply.

4.5. Appendix



Supplementary Figure 4.1. Meagre (*Argyrosomus regius*) larvae feed sequence from 3 dph to 30 dph.

Chapter 5

Appearance of systemic granulomatosis is modulated by the dietary supplementation of vitamin E and C in meagre (*Argyrosomus regius*) larvae fed inert microdiets

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Abstract

Systemic granulomatosis has already been reported in meagre larvae with an adequate feeding protocol and enrichment media preventing its appearance in the first weeks of life. Afterwards, the control of this disease could be prevented through nutritional components of the inert food, being the antioxidants the key to success. For this reason, in the present study, meagre larvae were reared from 30 days post hatching (dph) with five isonitrogenous and isolipidic experimental microdiets with different levels of vitamin E and C: C- (40 mg kg^{-1} E, 100 mg kg^{-1} C), C+ (400 mg kg^{-1} E, 1,000 mg kg^{-1} C), Krill (400 mg kg^{-1} E, 1,000 mg kg^{-1} C and substitution of fish oil by krill oil), EC (200 mg kg^{-1} E, 500 mg kg^{-1} C) and EECC (800 mg kg^{-1} E, 2,000 mg kg^{-1} C). Prior to this, larvae were co-fed with rotifers and *Artemia* following a protocol which prevented the appearance of granulomas, as previously demonstrated. The substitution of fish oil by krill oil significantly increased levels of eicosapentaenoic acid (EPA, 16.6 %) and docosahexaenoic acid (DHA, 17.6 %) in meagre, consequently increasing the peroxidation index, which in turn translated into a higher incidence of granulomas. Although even low levels of vitamin E and C (40 mg kg^{-1} E, 100 mg kg^{-1} C; C-) allowed the adequate growth of larvae, these levels were not enough to prevent the appearance of granulomas, requiring superior levels of both antioxidant vitamins (800 mg kg^{-1} E and 2,000 mg kg^{-1} C) to mitigate systemic granulomatosis. This mitigation was simultaneous with the reduction of thiobarbituric acid reactive substances TBARs content in larvae, which were highly correlated with the appearance of granulomas (R^2=0.892, y=0.0446x+0.0756). A strong negative correlation was observed between the dietary levels of vitamin E (y = -0.0098x + 11.174, R^2 = 0.8766, p value = 0.019, r = -0.93) and vitamin C (y = -0.0022x + 6.4777, R^2 = 0.9278, p value = 0.003, r = -0.96) and the percentage of larvae with granulomas. The results showed that the occurrence of systemic granulomatosis seems to be associated to the larvae peroxidation status, so that high dietary levels of vitamin E and C (800 and 2,000 mg kg^{-1}, respectively; Diet EECC), reduced lipid peroxidation and completely prevented the appearance of granulomas in meagre larvae at 44 dph.

Keywords: meagre larvae, antioxidant vitamins, granulomatosis.

5.1. Introduction

The whole life cycle of meagre (*Argyrosomus regius*) has been successfully closed, however there are still some challenges in meagre farming, being one of the more predominant ones the systemic granulomatosis. Systemic granulomatosis is a disease of unknown aetiology, although it has recently been evidenced that nutritional imbalances can promote its appearance (Chapter 3, 4, 6; Cotou et al., 2016). It is a non-infectious disease that affects internal organs, mainly liver, kidney and heart, where granulomas composed by a necrotic centre and surrounded by a layer of epithelial cells and macrophages are observed in the final stages (Chapter 3). It must be noted that the prevalence of systemic granulomatosis is so high in adult meagre that it can affect almost 100 % of population (Ghittino et al., 2004), being this stage too late to try to avoid the appearance of the disease. Nevertheless, granulomas have not only been detected in adult fish, but meagre larvae have also been found to show this histological alteration at very early stages (Chapter 4). In the afore mentioned study, granulomas were first described in liver and kidney at 20 days post hatching (dph) albeit differences were found among larvae fed the different dietary treatments/feeding sequences. In this sense, a co-feeding with rotifers (*Brachionus plicatilis*) and *Artemia* prior to weaning on an inert commercial microdiet proved to prevent the appearance of granulomas. On the other hand, when *Artemia* was not included in the feeding sequence, granulomas were detected from 20 dph although the incidence varied depending on the enrichment media used what again strengthens the hypothesis of a nutritional origin of the pathology. Therefore, a balanced nutrition during the first life stages of meagre could potentially prevent the development of systemic granulomatosis.

Imbalances in vitamins, particularly antioxidant vitamins such as vitamin E and C, have long been speculated to play a pivotal role in the appearance of systemic granulomatosis. Appearance of granulomas in gilthead sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) has been associated to a dietary deficiency of vitamin C (Paperna et a., 1980; Baudin-Laurencin et al., 1989, Coustans et al., 1990; Alexis et al., 1997).A deficiency in this nutrient causes an impairment of tyrosine catabolism, which leads to its precipitation in tissues and thereby the development of the granulomas (Goldsmith, 1978). In previous studies, the combination of high dietary content of antioxidant vitamin E, C and K (15, 450 and 230 mg kg^{-1}, respectively) reduced the incidence of granulomas in juvenile meagre (Chapter 3). However, a high prevalence of granulomas was observed at the beginning of the

experimental trial what prompted to evaluate the combination of vitamins at earlier life stages. If vitamins are to be blamed for the appearance of systemic granulomatosis, meagre larvae might be then at a higher risk of suffering the pathology as their higher growth and metabolic rates mean that vitamin requirements might be higher for larvae than juveniles or adult fish (Dabrowski, 1992). Additionally, limited information is available about the requirements of vitamin E and C in meagre larvae almost of the studies have been mainly focused on adults or juvenile fish. A recent study by El Kertaoui et al. (2017) showed that high levels of both vitamin E and C (1,500 and 1,800 mg kg^{-1}, respectively) improved growth and protection against oxidative stress in meagre larvae, but the effect of these antioxidant vitamins on the appearance of granulomas was not evaluated. Recently, the appearance of systemic granulomatosis has been observed to be affected by the fatty acid profile of the diet in meagre larvae, where the lowest supplementation of n-3 LC-PUFA (0.8 %) lead to a higher incidence of granulomas in liver (Carvalho et al., 2018). Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are considered essential fatty acids in marine fish and are involved in the maintenance of structural and functional integrity of cell membranes (Izquierdo and Koven, 2011), normal growth (Rodríguez *et al*., 1994; Salhi et al., 1997) and immune-deficiency (Izquierdo, 1996).The different fatty acid profile between fish oil and krill oil could have an impact on the appearance of granulomas, moreover, an absence of adequate levels of antioxidants, may lead to lipid oxidation as long as PUFA are available for oxidation (Hamre, 2011). Krill oil is higher in some fatty acids, such as EPA and DHA, compared to fish oil (Tou et al., 2007). Moreover, the phospholipid composition is different in both oils, in fish oil fatty acids are mainly stored as triglycerides, whereas in krill 30–65 % of the fatty acids are incorporated into phospholipids (Tou et al., 2007), which have higher bioavailability and are involved in regulation of more metabolic pathways (Ulven and Holven, 2015).

The overall aim of the present study was to evaluate the role of vitamin E and C in the appearance of systemic granulomatosis in weaned larvae (30 dph). Prior to the start of the study larvae were co-fed with rotifer and *Artemia* enriched with Easy DHA Selco as larvae fed this dietary regime did not show any granulomas at 30 dph in a previous trial (Chapter 4). Following this feeding sequence meagre will be fed four microdiets formulated to contain graded levels of inclusion of vitamin E and C. Additionally a fifth diet was formulated to contain krill oil as the single lipid source. Fish larvae growth and survival, histopathological evaluation and biochemical analysis were determined.

5.2. Materials and methods

5.2.1. Fish

Meagre eggs were obtained from an induced spawning from broodstock from the ECOAQUA facilities at University of Las Palmas de Gran Canaria (ULPGC; Telde, Canary Islands, Spain) where the experiment was carried out.

Rotifers were cultured at a density of 400 rotifers mL^{-1} in 500 L enrichment troncoconical-tanks, with 80 % seawater and 20 % freshwater. Rotifers were enriched with Easy DHA Selco (INVE, Dendermonde, Belgium) (0.6 g L^{-1}) for 24 h. Meagre larvae were fed with enriched rotifers twice daily from 3 to 21 dph, before each feeding, rotifers were counted and added to maintained at a density of 10 rotifers L^{-1} in the experimental tanks. *Artemia* cyst were hatched at 27 °C and 0.030 mg L^{-1} salinity until 100 % hatch was achieved. Then, they were rinsed with seawater and transferred to a culture tank at 24 °C. *Artemia* was enriched with Easy DHA Selco (0.6 g million *Artemia*$^{-1}$) for 24 h before being fed to the larvae. Meagre larvae were fed with enriched *Artemia* from 12 to 30 dph following the protocol established in Chapter 4. Before each feeding, *Artemia* were counted and added to maintained at a density of 1.3-1.5 *Artemia* L^{-1} in the experimental tanks. From 20 to 30 dph larvae were co-fed with *Artemia* and microdiet and fed microdiet only from 30 to 44 dph.

Larvae of 30 dph (total length 8.83± 0.65 mm, dry body weight 1.1 ± 0.01 mg) were randomly distributed in light grey colour cylindrical fibreglass experimental tanks (15 tanks; triplicate treatment) of 170 L capacity at a density of 3000 larvae $tank^{-1}$ and fed one of the five experimental diets for 14 days. All tanks were equipped with continuous aeration and supplied with filtered UV-sterilized seawater at an increasing rate from 35% h^{-1} to a 100% h^{-1}, to guarantee good water quality during the trial. Water entered the tanks at the bottom and exited at the surface. Oxygen (4.5-6.5 g L^{-1}), salinity (34 g L^{-1}) salinity and temperature (21.8 to 22.3º C) was daily measured. Photoperiod was kept at 12 h light: 12 h dark by fluorescent lights.

All procedures were conducted in accordance with the regulations set forward by the Spanish RD 53/2013 (BOE 8th February 2013) and the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The experiment was subjected to ethical review by

the Animal Welfare and Bioethical Committee at the University of Las Palmas de Gran Canaria (Ref 06/2018 OEBA ULPGC).

5.2.2. Diets

Five isonitrogenous and isolipidic experimental microdiets (pellet size 120-250 & 250-500 µm) were formulated (Tables 5.1 and 5.2). Krill meal was the source of protein whereas fish oil was the source of lipid, excepting for the diet labelled "Krill" in which krill oil was the single lipid source. Prior to preparing the feeds, the krill meal was defatted (three consecutive times with a chloroform: krill meal ratio of 3:1) to allow a better control of the fatty acid profile of the microdiet. A positive control diet (C+) was formulated based on the vitamin E (400 mg kg^{-1}) and C (1,000 mg kg^{-1}) levels found in a commercial microdiet (Gemma Micro 150 and 300 µm; Skretting, France). Based on this level of vitamins, other three diets with higher and lower levels of vitamin E and C was formulated, diet C-, Krill, EC and EECC (40/100, 400/1,000, 200/500 and 800/2,000 mg kg^{-1} vitamin E and C, respectively). Krill oil diet was formulated using krill oil as the only lipid source. Soy lecithin was used as a source of phospholipids, excepting in diet "krill" were phospholipid were provided from the krill oil used.

The microdiet was prepared according to Liu et al. (2002) as follows: the krill meal was mixed with the water-soluble ingredients (attractants, minerals and water-soluble vitamins). Oil and fat-soluble vitamins were mixed and blended with the dry ingredients. Finally, gelatine dissolved in warm water was added to the mix. The paste was pelleted and dried at 38° C for 24 h. The final pellets were ground and sieved in two different particle sizes (120-250 and 250-500 µm). Diets were kept at 4° C during the feeding period. Proximate composition and fatty acids levels were analysed for each diet prior to the start of the trial (Table 5.1 and 5.2). Fatty acid profile was similar in all the experimental diets excepting for diet "KRILL", which showed higher amounts of EPA (16.0 %) and DHA (8 %) than the other diets, what in turn increased total n-3 PUFA (Table 5.2). On the other hand, total n-6 PUFA was lower in KRILL, mainly due to the higher amount of linoleic acid in the diets with fish oil (7.2 %versus 4.1 %). Fish larvae were fed each 45 min daily from 8:00 to 20:00 with 3, 3.5 and 4 g $tank^{-1}$, during the first, second and third week respectively.

Table 5.1. Formulation and analysed proximate composition of diets fed to meagre larvae from 30 to 44 dph, containing different levels of vitamin E and C and either fish or krill oil as the lipid source.

	Diets				
Ingredient (%)	C+	C-	EC	EECC	Krill
Krill meal	74.47	74.60	74.54	74.33	75.47
Krill oil	-	-	-	-	6.00
Gelatin[1]	3.00	3.00	3.00	3.00	3.00
Fish oil	7.00	7.00	7.00	7.00	-
Soy lecithin[2]	2.00	2.00	2.00	2.00	2.00
Vitamin E[3]	0.04	0.004	0.02	0.08	0.04
Vitamin C[3]	0.10	0.01	0.05	0.20	0.10
Mineral Premix[4]	4.70	4.70	4.70	4.70	4.70
Vitamin Premix[5]	5.69	5.69	5.69	5.69	5.69
Attractant[6]	3.00	3.00	3.00	3.00	3.00
Proximate composition					
Vitamin E (mg kg^{-1})	844.3	497.1	632.7	1082.3	859.8
Vitamin C (mg kg^{-1})	1460.8	153.1	758.5	2910.5	1450.2
Protein (%)	48.5	48.9	48.9	48.0	49.6
Lipid (%)	30.1	30.6	29.9	30.8	29.7
Moisture (%)	3.7	3.7	3.6	4.0	4.1
Ash (%)	11.8	11.7	11.9	11.9	11.9

[1]Panreac, Barcelona, Spain. [2]Acrofarma, Barcelona, Spain. [3] $g \cdot 100^{-1}$, Vitamin E: α-tocopheryl acetate (Sigma-Aldrich, Madrid, Spain), Ascorbyl monophosphate ROVIMIX Stay-C-35 (Roche, Paris, France). [4]Mineral premix supplied g per 100 g diet: NaCl 215.133 mg, $MgSO_4$ $7H_2O$ 677.545 mg, NaH_2PO_4 H_2O 381.453 mg, $Ca(H_2PO_4)$ $2H_2O$ 671.610 mg, $FeC_6H_5O_7$ 146.884 mg, $C_3H_5O_3$ 1/2Ca 1,617.210 mg, $Al_2(SO_4)_3$ $6H_2O$ 0.693 mg, $ZnSO_4$ $7H_20$ 14.837 mg, $CuSO_4$ $5H_2O$ 1.247 mg, $MnSO_4$ H_2O 2.998 mg, $CoSO_4$ $7H_2O$ 10.706 mg. [5]Vitamin premix supplied per 100 g diet: cyanocobalamine 0.03 mg, astaxanthin 5.0 mg, folic acid 5.4 mg, pyridoxine-HCI 17.3 mg, thiamine 21.7 mg, riboflavin 72.5 mg, calcium-pantothenate 101.5 mg, p-aminobenzoic acid 145.0 mg, nicotinic acid 290.1 mg, myo-inositol 1450.9 mg, menadione 17.3 mg. [6]Attractant premix supplied per 100 g diet: inosine-5-monophosphate 500.0 mg, betaine 660.0 mg, L-serine 170.0 mg, L-phenylalanine 250.0 mg, DL-alanine 500.0 mg, L-sodium aspartate 330.0 mg, L-valine 250.0 mg, glycine 170.0 mg. Proximate composition (%).

Table 5.2. Diets fatty acid composition (percentage of fatty acids) used for feeding meagre larvae fed from 30 to 44 days post hatching (dph) in the present trial.

	Diets				
Fatty acids (%)	C+	C-	EC	EECC	KRILL
14:0	7.1	7.1	7.2	7.2	9.7
16:0	19.4	19.4	19.6	19.7	22.6
18:0	2.1	2.1	2.1	2.1	1.7
20:0	0.1	0.1	0.1	0.1	0.1
Σ Saturated[1]	29.3	29.3	29.6	29.7	34.8
16:1n-7	5.4	5.3	5.4	5.4	6.5
18:1n-9	20.4	20.5	20.5	20.3	13.3
18:1n-7	5.5	5.5	5.6	5.6	6.6
20:1n-7	1.8	1.8	1.8	1.8	1.0
22:1n-11	0.7	0.8	0.7	0.7	0.0
Σ Monosaturated[2]	35.6	35.8	35.8	35.5	29.5
18:2n-6	7.2	7.2	7.2	7.2	4.1
18:3n-6	0.1	0.1	0.1	0.1	0.1
20:2n-6	0.3	0.3	0.3	0.3	0.0
20:3n-6	0.1	0.1	0.1	0.1	0.0
20:4n-6	0.3	0.3	0.3	0.3	0.3
Σ n-6PUFA[3]	8.1	8.1	8.1	8.1	4.6
18:3n-3	1.9	1.9	1.9	1.9	1.0
18:4n-3	2.4	2.4	2.3	2.4	3.1
20:3n-3	0.1	0.1	0.1	0.1	0.1
20:4n-3	0.4	0.4	0.4	0.4	0.3
20:5n-3	12.1	12.0	11.9	12.1	16.0
22:5n-3	0.6	0.6	0.6	0.6	0.7
22:6n-3	7.4	7.4	7.3	7.2	8.0
Σ n-3PUFA[4]	25.0	24.8	24.6	24.7	28.8
(n-3+n-6) PUFA	33.2	32.9	32.7	32.8	33.4
Total n-3 LC-PUFA[5]	20.5	20.4	20.2	20.3	24.7
PIn	166.4	165.1	163.6	163.9	190.3

Data expressed as means of three technical replicates per batch of diet.[1]Includes 15:0 and 17:0.[2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5.[3]Includes. 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5]LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

5.2.3. Sample collection

Samplings were performed at 30 and 44 dph. At the beginning of the experiment (30 dph) 100 larvae were sacrificed with an overdose of anaesthetic (clove oil; Guinama, Valencia, Spain) and fixed in 4 % buffered formalin for histological analysis. After two weeks (44 dph) 70 larvae per tank were sacrified with clove oil and kept in ice during the sampling. 40 larvae were measured for total length (TL) using a profile projector (Mitutoyo PJ- 3000A, Kanagawa, Japan) and fixed in 4 % buffered formalin for histological analysis (120 larvae per diet). The remaining 30 larvae were collected to determine dry weight at each sampling point. At 44 dph all remaining larvae were collected for biochemical and TBARs analysis and stored at -80 °C until analysis.

5.2.4. Growth and survival

Larvae were sampled and measured for dry weight (100 °C for 24 h) and total length at the end of the experiment (44 dph). Final survival was determined at 44 dph by counting the remaining alive larvae in experimental tanks. Performance parameters were calculated according to the following equations: Survival (%) = 100*(final number fish − initial number fish)/initial number fish; SGR (specific growth rate) = 100*(ln final mean weight - ln initial mean weight)/number of days.

5.2.5. Histopathology

Formalin fixed samples were dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 μm on a microtome, fixed to the microscope slide and finally stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco method (Fite et al., 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological evaluation, analysing all tissues and focusing especially, in liver, kidney and heart, given that these organs are the main affected by granulomas (Chapter 3).

5.2.6. Biochemical analysis

Larvae and diet biochemical composition analysis were conducted following standard procedures. Lipids of larvae and feeds were extracted with a choloroform-methanol (2:1 v/v) mixture as described by Folch et al. (1957). Protein content (Kjeldahl method), dry matter and ash were determined in feeds according to AOAC (2010).

Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982). Fatty acid methyl esters (FAMES) were separated and quantified by gas-liquid chromatography following the conditions described by Izquierdo et al. (1992). Lipid susceptibility to oxidation was estimated using the peroxidation index (PIn) with following formula: PIn =0.025 × (percentage of monoenoics) + 1 × (percentage of dienoics) + 2 × (percentage of trienoics) + 4 × (percentage of tetraenoics) + 6 × (percentage of pentaenoics) + 8 × (percentage of hexaenoics) (Witting and Horwitt, 1964).

Thiobarbituric acid reactive substances (TBARs) were measured in triplicate from extracted total lipids (10 mg/ml) according to Burk et al. (1980). Firstly, 50 µl of 0.2 % (w/v) BHT in ethanol were added to 2 mg of lipid followed by 0.5 ml of 1 % (w/v) TBA and 0.5 ml of 10 % (w/v) trichloroacetic acid, all solutions freshly prepared. Samples were vortexed in stoppered test tubes and heated in darkness at 100 °C for 20 min. Then, samples were cooled in ice for 5 min and particulate matter was removed by centrifugation at 2,000 g (Sigma 4K15, Osterode am Harz, Germany) for 5 min. The supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm and recorded against a blank sample. The concentration of TBA-malondialdehyde (MDA) was expressed as µmol MDA per g of tissue and was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

The concentration of vitamin E was determined in diets. Samples were weighed, homogenized in ethanolic pyrogallol and saponified as described McMurray et al., 1980. HPLC analysis was performed using 150 x 4.60 mm, 5 µm reverse-phase Luna and C18 column (Phenomenox, CA, USA). The mobile phase was methanol:ultrapure water (98:2 v/v) with a flow rate of 1.0 ml min^{-1} at ambient temperature. Samples were injected (50 µl) in a high performance liquid chromatograph (HPLC) with UV detection at a wavelength of 293 nm to determine the vitamin E using (+)-α-tocopherol (Sigma-Aldrich) as the external standard.

The concentration of vitamin C was determined in the experimental feeds as described by Betancor et al. (2012). Samples were weighed, homogenised and dissolved in 0.4 M phosphate buffer (adjusted to pH 3.0 with phosphoric acid). The samples were centrifuged at 3.000 rpm, supernatants removed and filtered through a disposable 0.45 µm filter and stored at 4° C until the measurement in a HPLC with UV detection. The determination of vitamin C concentration was achieved by comparison with tris (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich) as the external standard.

5.2.7. Statistical analysis

All statistical analyses were done with Statgraphics (Statgraphics Centurion XVI version 16.1.03 for Windows; Graphic Software Systems, Inc. USA). Survival, growth, percentage of larvae with granulomas and biochemical analysis were tested for normality with the Kolmogorov Smirnov test and homogeneity of variance was performed with the Levene test. With the variables that satisfied the normality and homogeneity was carried out a parametric one-way (ANOVA) and Tukey test post-hoc test. A significance level of 0.05 was used.

5.3. Results

5.3.1. Growth and survival

All experimental diets were well accepted by larvae. Final total length, dry weight and survival were not significant different among larvae fed the different experimental feeds at the end of the feeding trial (44 dph). The average final total length was 25.8 ± 0.4 mm, dry weight 17.5 ± 1.3 mg, survival 20.1 ± 0.5 % and daily growth of 17.1 ± 1.2 % (Table 5.3).

Table 5.3. Growth performance of meagre larvae fed the experimental feeds at 30 (initial) and 44 days post hatching (dph).

		Diets				
	Initial	C+	C-	EC	EECC	KRILL
Total length (mm)	8.8 ± 1.4	26.3 ± 4.2	25.6 ± 3.9	24.9 ± 4.3	26.0 ± 4.1	26.2 ± 4.3
Dry weight (mg)	1.1 ± 0.2	16.2 ± 4.0	16.6 ± 1.8	16.4 ± 4.7	19.2 ± 3.5	17.3 ± 3.7
SGR (%)	-	16.8 ± 4.0	17.5 ± 1.8	16.9 ± 4.7	18.5 ± 3.5	17.2 ± 3.7
Survival (%)	-	19.5 ± 1.3	20.1 ± 1.2	20.9 ± 2.3	20.3 ± 1.0	19.7 ± 0.9

Data are means ± SD. dph, days post hatching; SGR, specific growth rate.

5.3.2. Histopathology

At the beginning of the experiment (30 dph) no granulomas were observed at the microscopic evaluation. Nevertheless, after 14 days (44 dph) significant differences were found in the percentage of larvae with granulomas among diets, being higher in larvae fed diets C-, Krill and EC (40/100, 400/1,000 and 200/500 mg kg^{-1} of vitamin E and C, respectively) followed by diet C+ (400/1,000 mg kg^{-1} of vitamin E and C, respectively) (Figure 5.1). No granulomas were observed in any larvae fed with the highest levels of vitamin E and C (800/2,000 mg kg^{-1}). Kidney was the main affected tissue with granulomas (86.7 % of fish with granulomas), followed by liver (13.3 % of fish with granulomas) (Figure 5.2).

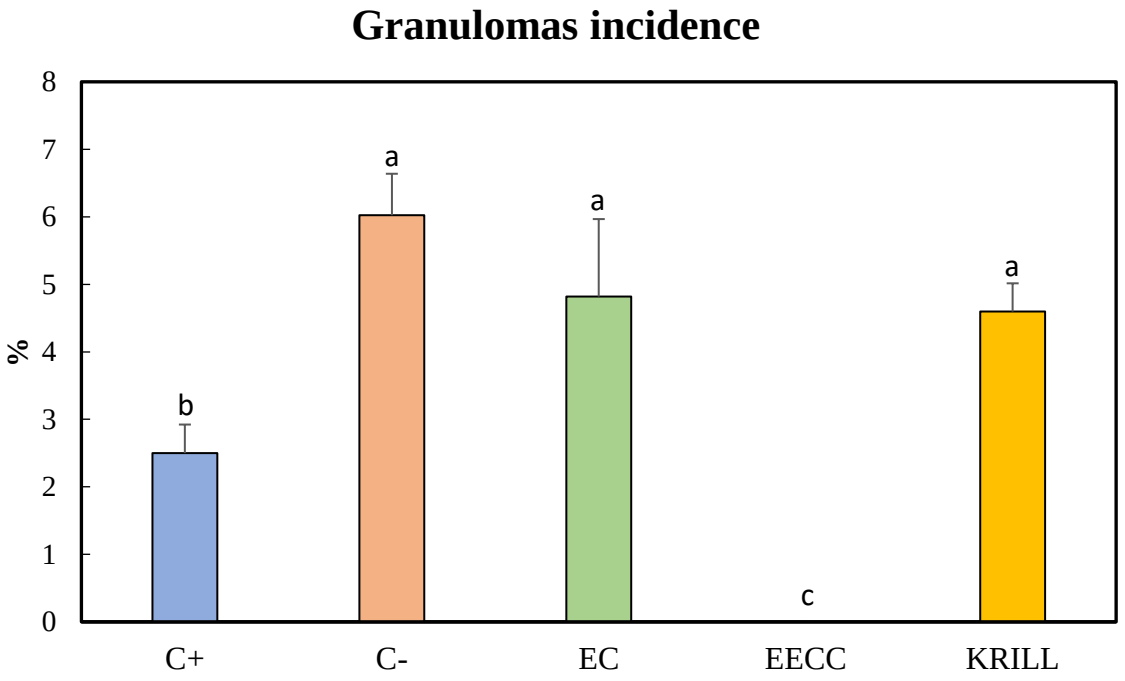


Figure 5.1. Incidence of granulomas (%) in meagre larvae at the end of the dietary trial (44 dph). Each value represents mean ± SD (n= 120).

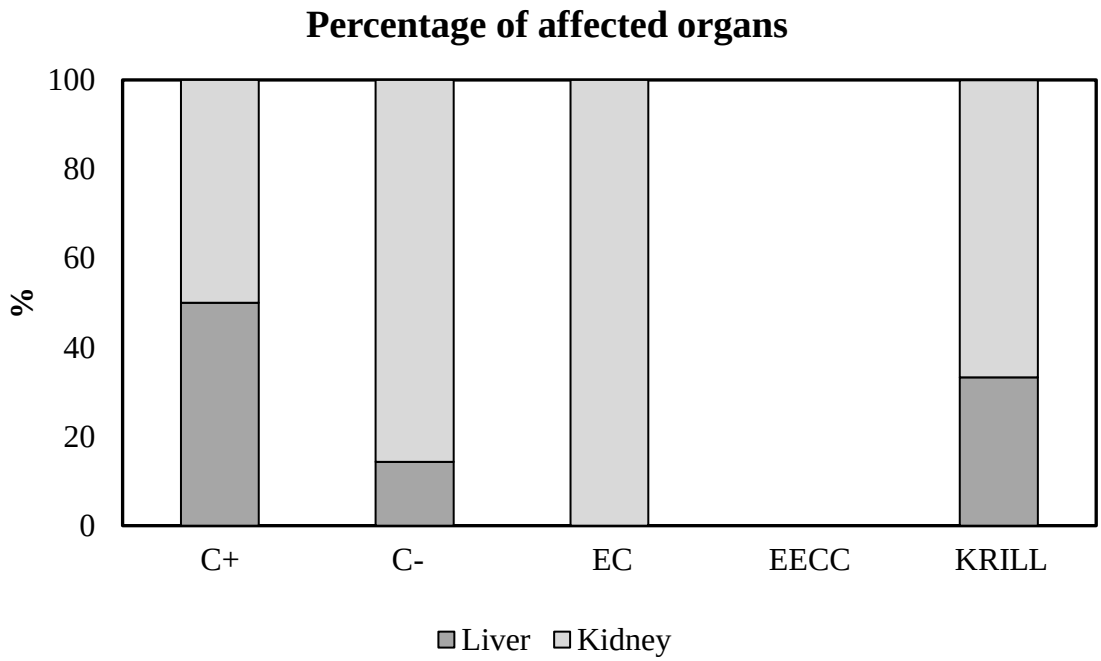


Figure 5.2. Percentage of affected organs with granulomas in meagre larvae of 44 dph fed different levels of vitamin E and C.

There was a strong and significant negative correlation between the percentage of larvae with granulomas and dietary concentration of vitamin E (y = -0.0098x + 11.174, R^2 = 0.8766; Pearson's correlation coefficient (r) = -0.93) and vitamin C (y = -0.0022x + 6.4777, R^2 = 0.9278; Pearson's correlation coefficient (r) = -0.96) (Figure 5.3). The TBARs content was highly correlated with the appearance of granulomas (R^2=0.892, y=0.0446x+0.0756). All the specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stain) were negative, discarding a possible infectious origin of the granulomas (Supplementary Figure 5.1; Appendix 5.5).

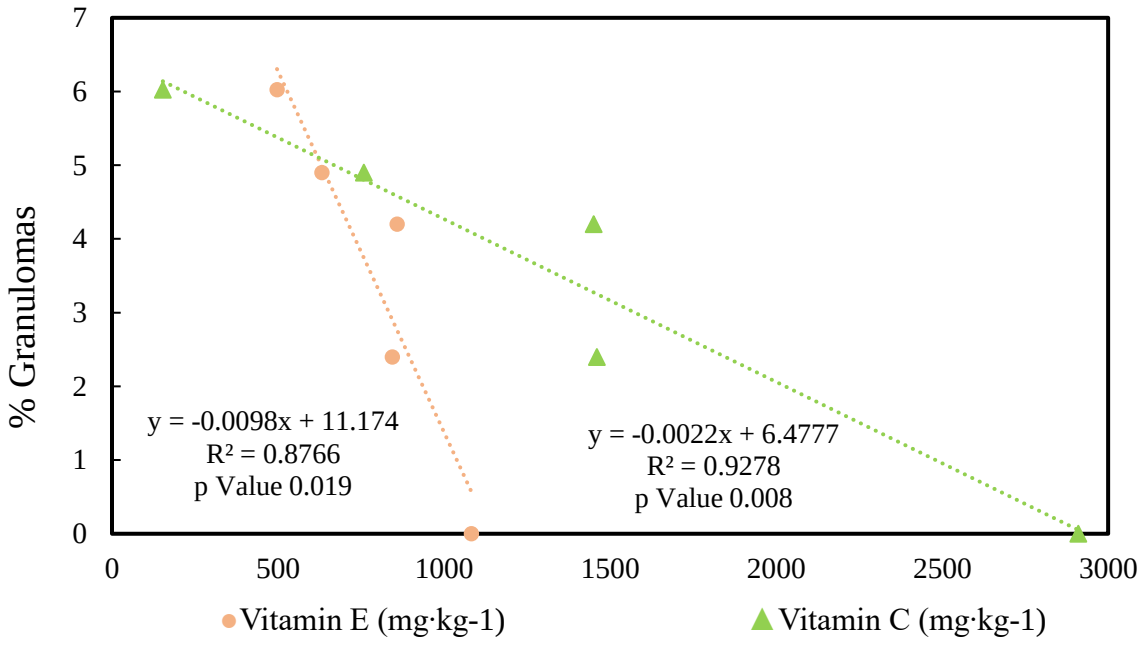


Figure 5.3. Effect of dietary vitamin E and C on percentage of affected meagre larvae with granulomas at 44 dph.

The histopathological evaluation revealed granulomas in different stages of development (Supplementary Figure 5.2; Appendix 5.5) as described in Chapter 3 in ongrowing meagre. At initial stages, granulomas were observed as isolated and irregular aggregated of macrophages (Supplementary Figure 5.2A; Appendix 5.5) that later were forming concentric layers (Supplementary Figure 5.2B; Appendix 5.5). These aggregated progressively lead to a necrotic centre with external layers of fibrocytes (Supplementary Figure 5.2C; Appendix 5.5). However, final stages of development, in which the granuloma is completely composed of laminar material, were not observed.

5.3.3. Biochemical analysis

5.3.3.1. Whole larvae proximal composition and fatty acid profile

Dietary treatment did not affect larvae whole body proximate composition after 14 days of feeding, with a protein content averaging 11 % and lipid exceeding 2 % among larvae fed the different dietary treatments (Table 5.4). The substitution of krill oil by fish oil significantly increased de levels of eicosapentaenoic acid (EPA, 16.6 vs 13.7 %) and docosahexaenoic acid (DHA, 17.63 vs 16.05 %) in meagre larvae at the end of the feeding trial, compared with the larvae fed with the other diets (EPA ~ 13.7 % and DHA ~ 16.1 %) (Table 5.5). Furthermore, the addition of krill oil significantly increased the peroxidation index in the larvae (275.3 vs 245.7) (Table 5.5) and the concentration of saturated fatty acids (31.2 vs 29.0 %), n-3 PUFA (38.2 vs 34.3 %) and n-3 LC-PUFA (35.6 vs 31.6 %) (Table 5.5). Larvae fed fish oil diets showed significant higher concentration of oleic acid, linoleic acid, monosaturated fatty acids, n-6 and n-9 PUFA regardless dietary levels of vitamin E and C (Table 5.5).

Table 5.4. Proximate composition and TBARs content in meagre larvae (44 dph) fed with the experimental diets.

	Diets				
	C+	C-	EC	EECC	KRILL
Proximate composition					
Protein	11.0 ± 1.1	10.4 ± 2.1	10.6 ± 1.4	10.9 ± 1.8	11.8 ± 2.0
Lipid	2.8 ± 0.4	2.5 ± 0.2	2.3 ± 0.3	2.4 ± 0.4	2.4 ± 0.1
Moisture	82.9 ± 2.8	83.9 ± 2.1	83.7 ± 2.1	82.7 ± 0.9	82.9 ± 2.2
Ash	2.5 ± 0.6	2.8 ± 0.1	2.9 ± 0.2	3.1 ± 0.2	2.3 ± 0.1
TBARs content (μmol g^{-1} dry mass)	769.2 ± 110.5^{a}	1028.8 ± 159.3^{a}	862.2 ± 136.3^{a}	138.5 ± 45.7^{b}	974.6 ± 118.9^{a}

Data expressed as means of three technical replicates per batch of larvae (n = 3). Different superscript letters denote differences among treatments identified by one-way ANOVA.

Table 5.5. Fatty acid composition (percentage of fatty acids) of meagre larvae fed with experimental diets at the end of the dietary trial (44 days post hatching).

Fatty acids (%)	C+	C-	EC	EECC	KRILL
14:0	2.6 ± 0.1	2.6 ± 0.2	2.5 ± 0.2	2.5 ± 0.2	2.9 ± 0.2
16:0	20.9 ± 0.3^{a}	20.9 ± 0.3^{a}	21.3 ± 0.6^{a}	21.0 ± 0.2^{a}	22.5 ± 0.2^{b}
18:0	4.8 ± 0.3	4.8 ± 0.2	5.0 ± 0.2	5.1 ± 0.6	5.1 ± 0.2
20:0	0.1 ± 0.0^{ab}	0.2 ± 0.0^{b}	0.1 ± 0.0^{a}	0.0 ± 0.0^{ab}	0.1 ± 0.0^{a}
Σ Saturated[1]	28.8 ± 0.5^{a}	28.9 ± 0.2^{a}	29.3 ± 0.7^{a}	29.1 ± 0.3^{a}	31.2 ± 0.3^{b}
16:1n-7	3.6 ± 0.1	3.6 ± 0.2	3.4 ± 0.1	3.4 ± 0.1	3.5 ± 0.3
18:1n-9	15.2 ± 0.1^{b}	15.5 ± 0.3^{b}	15.1 ± 1.0^{b}	14.7 ± 0.2^{b}	11.0 ± 0.7^{a}
18:1n-7	5.3 ± 0.1^{a}	5.5 ± 0.0^{a}	5.4 ± 0.0^{a}	5.3 ± 0.1^{a}	5.9 ± 0.1^{b}
20:1n-7	1.0 ± 0.0^{b}	1.1 ± 0.0^{b}	1.0 ± 0.1^{b}	1.0 ± 0.0^{b}	0.7 ± 0.0^{a}
22:1n-11	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.1^{b}	0.2 ± 0.1^{b}	0.0 ± 0.0^{a}
Σ Monosaturated[2]	26.6 ± 0.31^{b}	27.1 ± 0.6^{b}	26.6 ± 1.5^{b}	26.0 ± 0.3^{b}	22.8 ± 1.1^{a}
18:2n-6	6.6 ± 0.1^{b}	6.5 ± 0.2^{b}	6.6 ± 0.0^{b}	6.6 ± 0.1^{b}	4.5 ± 0.1^{a}
18:3n-6	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{a}
20:2n-6	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.1 ± 0.0^{a}
20:3n-6	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{a}
20:4n-6	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.3	0.9 ± 0.1
Σ n-6 PUFA[3]	8.2 ± 0.2^{b}	8.0 ± 0.1^{b}	8.1 ± 0.1^{b}	8.3 ± 0.4^{b}	5.8 ± 0.2^{a}
18:3n-3	1.4 ± 0.0^{b}	1.4 ± 0.0^{b}	1.3 ± 0.1^{b}	1.4 ± 0.2^{b}	0.9 ± 0.0^{a}
18:4n-3	1.4 ± 0.1^{a}	1.4 ± 0.0^{a}	1.3 ± 0.0^{a}	1.3 ± 0.1^{a}	1.6 ± 0.1^{b}
20:3n-3	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.1 ± 0.0^{a}
20:4n-3	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{a}
20:5n-3	13.7 ± 0.8^{a}	13.7 ± 0.3^{a}	13.7 ± 0.6^{a}	13.7 ± 1.2^{a}	16.6 ± 1.4^{b}
22:5n-3	1.3 ± 0.0	1.3 ± 0.1	1.3 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
22:6n-3	16.0 ± 0.6^{a}	15.9 ± 0.8^{a}	16.0 ± 0.6^{a}	16.3 ± 0.4^{a}	17.6 ± 0.2^{b}
Σ n-3PUFA[4]	34.4 ± 0.4^{a}	34.1 ± 0.9^{a}	34.1 ± 1.1^{a}	34.6 ± 0.8^{a}	38.3 ± 1.5^{b}
Σ n-9PUFA[5]	15.9 ± 0.1^{b}	16.3 ± 0.3^{b}	15.9 ± 1.1^{b}	15.5 ± 0.2^{b}	11.8 ± 0.6^{a}
(n-3+n-6) PUFA	8.2 ± 0.2^{b}	8.0 ± 0.1^{b}	8.1 ± 0.1^{b}	8.3 ± 0.4^{b}	5.8 ± 0.2^{a}
Total n-3 LC-PUFA[6]	31.5 ± 0.3^{a}	31.2 ± 1.0^{a}	31.3 ± 1.2^{a}	32.5 ± 2.1^{a}	35.7 ± 1.5^{b}
PIn	246.3 ± 2.1^{a}	243.7 ± 7.7^{a}	244.4 ± 8.2^{a}	248.5 ± 3.9^{a}	275.3 ± 3.0^{b}

Data expressed as means of three technical replicates per batch of larvae (n = 3). Different superscript letters denote differences among treatments identified by one-way ANOVA ($P<0.05$). [1]Includes 15:0 and 17:0.[2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9 and 20:1n-5.[3]Includes 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5]Includes. 22:1n-9. 20:3n-9. 20:2n-9. 20:1n-9. 18:2n-9. 18:1n-9. [6] LC-PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3. 20:5n-3 22:5n-3 and 22:6n-3). PIn, peroxidation index.

5.3.3.2. TBARs content

The level of lipid peroxides, as indicated by TBARs content (μmol g^{-1} larval tissues), was significantly lower in those larvae fed diets with the highest levels of vitamin E and C (Table 5.4).

5.4. Discussion

It has previously been shown that the co-feeding with rotifer and *Artemia* enriched with Easy DHA Selco prior to eating an inert commercial microdiet prevented the appearance of granulomas in meagre larvae (Chapter 4). Consistently, no granulomas were observed at 30 dph in the present trial after following the same feeding sequencing and enrichment protocol what seems to reinforce the role of nutrition as the main trigger in the appearance of systemic granulomatosis. The results of the present trial showed that the dietary addition of different levels of vitamin E (40, 200, 400 and 800 mg kg^{-1}) and C (100, 500, 1,000 and 2,000 mg kg^{-1}) did not affect meagre larvae performance in terms of growth, length, survival and SGR at 44 dph. However, granulomas were observed in larvae fed with low levels of vitamin E and C (from 40/100 to 400/1,000 mg kg^{-1}, vitamin E/C). The results suggest that low levels of vitamin E and C (40 and 100 mg kg^{-1}, respectively) probably fulfilled the requirement for normal growth what explains the lack of differences in terms of fish performance among larvae fed the different dietary treatments but were not enough to prevent systemic granulomatosis. On this matter, a strong negative correlation was observed between the dietary levels of vitamin E ($y = -0.0098x + 11.174$, $R^2 = 0.8766$) and vitamin C ($y = -0.0022x + 6.4777$, $R^2 = 0.9278$) and the incidence of granulomas. Little is known about requirements of vitamin E and C in meagre larvae. Only El Kertaoui et al. (2017) observed that high levels (1,500 and 1,800 mg kg^{-1} of vitamin E and C, respectively) were required to improve growth and antioxidant defenses in meagre larvae at 28 dph. It is well known that the requirement for antioxidant vitamins is conditioned by the dietary fatty acids content. In this regard, all the experimental microdiets contained a sufficient amount of essential fatty acids for most marine fish species, which require at least 2 % EPA and DHA (NRC, 2011). Nevertheless, those larvae fed with higher amounts of DHA and EPA together with low dietary vitamin E and C (diet Krill) presented high incidence of granulomas, suggesting an imbalance between prooxidant and antioxidant nutrients. Accordingly, TBARs content, an indicator

of lipid oxidation, was affected by the dietary inclusion of vitamin E and C, with the high supplementation of vitamin E (800 mg kg^{-1}) and C (2,000 mg kg^{-1}) significantly reducing TBARs values. Indeed, TBARs contents were highly correlated with the appearance of granulomas (R^2=0.892, y=0.0446x+0.0756). Therefore, adequate dietary levels of vitamins E and C seem to mitigate the appearance of systemic granulomatosis in meagre larvae, probably due to the decrease of the oxidation rate.

Vitamin E together with vitamin C are strong antioxidants in tissues, being able to neutralize reactive oxygen species (ROS) (Montero et al., 1999; Ai et al., 2006; Betancor et al., 2012; Gao et al., 2014) and increase the protection against lipid peroxidation (Lee and Dabrowski, 2003). The oxidative stress has been related with some diseases (Kawatsu, 1969; Cowey et al., 1984; Sakai et al., 1989; Watanabe et al., 1989; Sies et al., 1992; Padayatty and Levine, 2001; Lewis-McCrea and Lall, 2007), therefore it is feasible to think that granulomas could also be originated by an oxidative imbalance. Lipid peroxidation contributes to the inflammatory response (Morita el al., 2016). Granuloma formation is an inflammatory response, and is composed basically by macrophages, lymphocytes and fibrocytes, being its appearance not necessarily associated with infectious diseases. This inflammation can occur in blood vessels (Petersen and Smith, 2013; Hilhorst et al., 2014). In this sense, in the present and previous studies (Chapter 3) irregular aggregates of cells and granulomas have been observed surrounding blood vessels, which suggests that granulomas could have a vascular origin. Vitamin C has been related with the synthesis of collagen, an important protein involved in the generation of blood vessels (Lim and Lovell, 1978; Nusgen et al., 2001). Besides, vitamins C and E are involved in the prevention of endothelial dysfunction and the prevention of oxidative stress (Riitta et al., 2003; Engler et al., 2003). In this sense, an imbalance between ROS and antioxidants could be happening in larvae fed with low addition of vitamin E and C, as indicated by TBARs values, which could lead to inflammatory response in blood vessel with the subsequent macrophages infiltration and formation of granulomas. Limited information is available on the effect of antioxidant vitamins in the formation of granulomas. In other fish species vitamin C deficiency has been related to precipitation of tyrosine in tissues, being the origin of granulomas, in species such as sea bream and turbot (Baudin-Laurencin et al., 1989; Coustans et al., 1990; Alexis et al., 1997). In agreement, a previous study showed that the dietary increase of vitamins E and C lead to a reduction in the percentage of granulomas in liver and heart

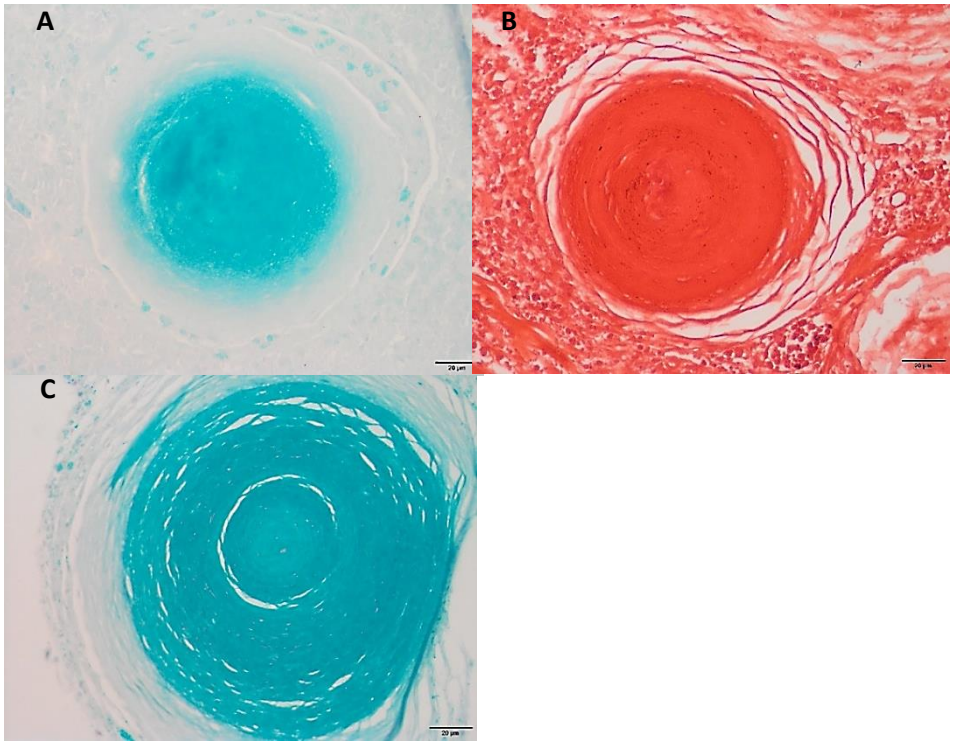
of juvenile meagre together with a decrease in TBARs contents (Chapter 3, 4, 6), what indicates less lipid peroxidation.

The substitution of fish oil by krill oil significantly increased de levels of eicosapentaenoic acid (EPA, 16.6 %) and docosahexaenoic acid (DHA, 17.6 %) in meagre larvae with 44 dph, compared with the larvae fed the other diets (EPA ~ 13.7 and DHA ~ 16.1 %). This difference in the levels of n-3 LC-PUFA seemed to have an impact on the TBARs content which in turn translated into a higher incidence of granulomas compared to larvae fed fish oil in combination with the same dietary levels of antioxidant vitamins (Diet C+, 400 and 1,000 mg kg^{-1} vitamin E and C, respectively). Apart from being an excellent source of EPA and DHA, krill oil is rich in phospholipids and particularly phosphatidylcholine (Winther et al., 2011). Phospholipids have been described to have a stronger biological effect than triglycerides, because they can be more rapidly digested and are more effectively incorporated to the tissues than triglycerides (Ackman and Ratnayake 1989), can act as ligands for nuclear receptor (Li et al., 2005; Chakravarthy et al., 2009), are involved in the steroidogenesis and cholesterol metabolism, and have been shown to augment the bioavailability of DHA and EPA (Amate et al., 2001; Cansell et al., 2003; Cansell et al., 2009). Despite of the high phospholipid level provided by the krill oil, it could not prevent the appearance of granulomas, needing supplementation with higher levels of vitamin E and C (over 400 and 1,000 mg kg^{-1}, respectively) in order to inhibit its appearance. However, the percentage of granulomas was significantly higher in larvae fed diet "krill" than those larvae fed diet "C+", although both diets contained the same levels of vitamin E (844 and 859 mg kg^{-1}, respectively) and C (1,460 and 1,450 mg kg^{-1}, respectively) were roughly the same. This could be related to the higher EPA and DHA contents (therefore, higher peroxidation index) found in larvae fed diet "krill", what suggests that the balance between prooxidant and antioxidant nutrients is disturbed in favour of prooxidants. In this point, it should be noted that the higher peroxidation index should be correlated to higher TBARs values. Nevertheless, larvae fed diet "krill" were not different to those of fish fed fish oil (C+). This could be due the fact that EPA and DHA are in phospholipid forms and were more protected in the krill diet, while in the diet with fish oil they were in triglycerides, being more susceptible to oxidation. Moreover, although krill oil contains antioxidants, mainly astaxanthin (Tou et al., 2007), these were no able to prevent the appearance of granulomas. These results suggest that the appearance of granulomas is

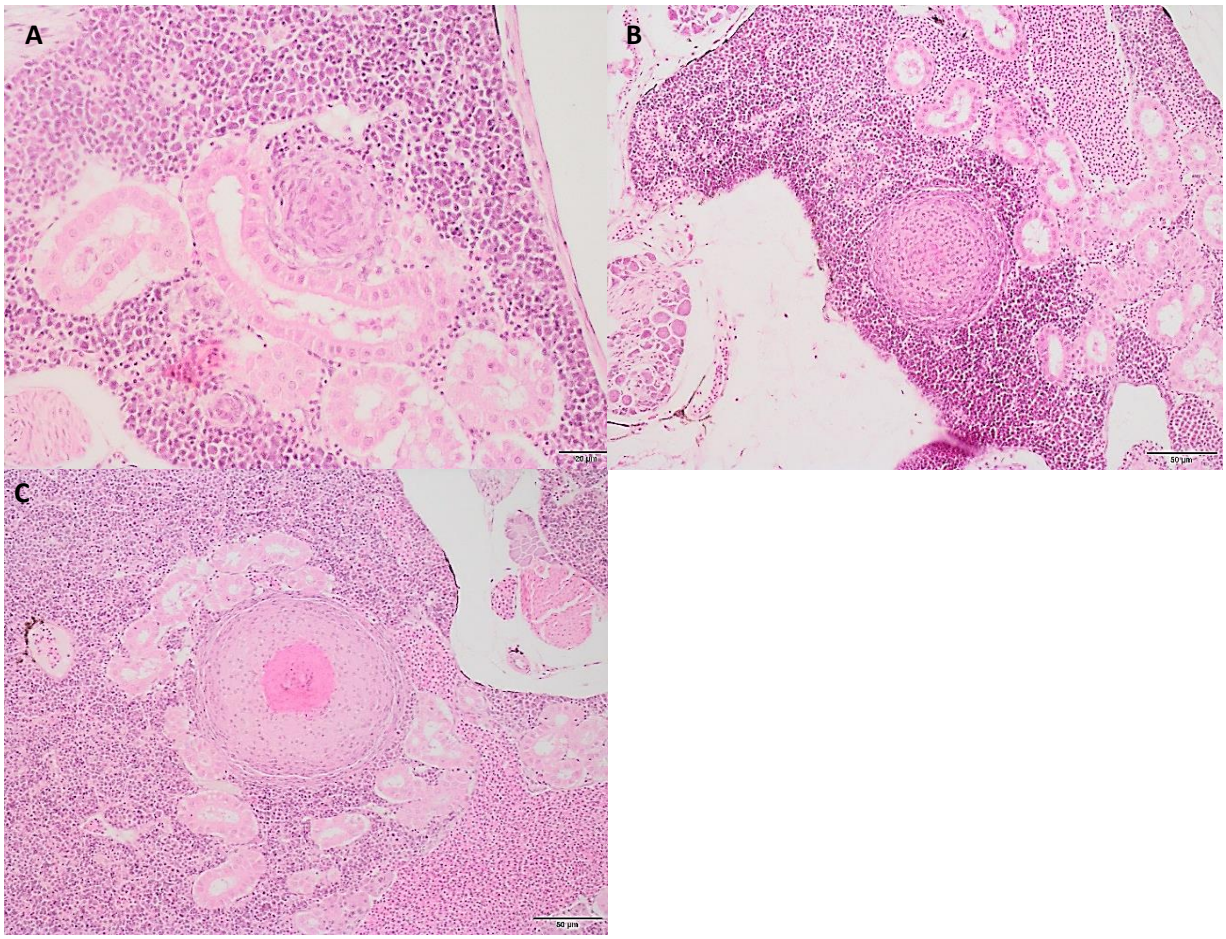
more related to the supplementation of different levels of vitamin E and C more than to the source of dietary fatty acids. In fact, in a previous study the appearance of granulomas in juvenile meagre was modulated by the inclusion of different levels of the antioxidants vitamins E and C (Chapter 3).

Concluding, the supplementation of vitamin E and C at 40 and 100 mg kg^{-1} respectively is adequate to ensure good meagre larvae performance. However, these vitamin levels might not be enough to prevent the appearance of systemic granulomatosis, as indicated by the strong negative correlation between dietary vitamin E and C contents and the prevalence of granulomas and TBARs values. Levels of dietary vitamin E and C of 1,082 and 2,910 mg kg^{-1} (Diet EECC) completely prevented the appearance of granulomas. The substitution of fish oil by krill oil was enough to the correct growth of meagre larvae but increased the percentage of granulomas and the peroxidation index. Therefore, it has been demonstrated in the present and previous studies (Chapter 4) that systemic granulomatosis can be completely mitigated in meagre larvae by controlling feeding sequence as well as levels of antioxidant nutrients.

5.5. Appendix



Supplementary Figure 5.1. Negative results in granulomas for specific stains. A) Ziehl-Neelsen, B) Gram stain and C) Fite-Faraco stain in kidney.



Supplementary Figure 5.2. Granulomas at different stages of development in kidney of meagre larvae (44 dph) at the end of the experimental trial. **A)** Irregular aggregated of macrophages. **B)** Concentric layers of macrophages and some lymphocytes. **C)** Necrotic center surrounded by layers of macrophages and an outer layer of fibrocytes.

Chapter 6

The effect of fish stocking density and dietary supplementation of vitamin C and micronutrients (Mn, Zn and Se) on the development of systemic granulomatosis in juvenile meagre (*Argyrosomus regius*)

This manuscript has been submitted to *Aquaculture*.

Abstract

Systematic granulomatosis is a chronic disease affecting the majority of farmed meagre (*Argyrosomus regius*). Recently, oxidative stress has been associated to the appearance of granulomas. In addition, in the intensive aquaculture fish are farmed at high stock density and the overcrowding can be a stressful. Nutritional imbalances and overcrowding can increase the risk to suffer oxidative stress in fish, and consequently affect to the incidence of granulomatosis. In order to try to know better the nutritional origin of granulomas and to study the effect of the stock density on the granulomatosis incidence, juvenile meagre were fed five isolipidic (16.7 %) and isoproteic (49.6 %) fish meal and fish oil-based feeds prepared by adding different levels of vitamin C, minerals (Mn, Zn, Se) and vitamin E and K (100 and 35 mg kg^{-1}, respectively): Diet KEC (100 mg kg^{-1}C), Diet KEC+Mn/Zn/Se (100 mg kg^{-1}C, 40 mg kg^{-1}Mn, 200 mg kg^{-1}Zn, 1.5 mg kg^{-1}Se), Diet KECC (600 mg kg^{-1}C), Diet KECCC (1.200 mg kg^{-1}C), Diet KECCCC (3,200 mg kg^{-1}C). All diets were tested with 100 fish/tank (3.20 kg m^{-3}), but diet KECC and KECCCC was also tested at a higher density (175 fish $tank^{-1}$; 6.20 kg m^{-3}). Growth performance (final weight, SGR, FCR and K) was only affected by stocking density, being lower at high density. Percentage of fish with granulomas in any tissue was significantly lower in fish fed with the highest dietary vitamin C contents (KECCC and KECCCC) at low density (80 and 76 %, respectively), compared with fish fed all the other diets (87-90 %). A lower number of fish with hepatic granulomas was found in fish fed KECCC and KECCCC at low density as well as fish fed KECCCC in high density. TBARS content was correlated with the percentage of granulomas in liver (R^2=0.9439, y=0.003x-0.1242) denoting the involvement of an imbalance oxidative status in the appearance of granulomas. In addition, fish fed KECCCC showed the highest expression of *cat* in liver and kidney as well as the highest expression of *sod* and *gpx* in liver and heart. The present results show that high levels of vitamin C (1,200-3,200 mg kg^{-1} C) and low stock density (3.20 kg m^{-3}) favours the growth of juvenile meagre, reducing the lipid peroxidation indicators and decreasing the incidence of granulomas, which confirms that this pathology is mostly triggered by deficiency of antioxidant nutrients, particularly vitamin C.

Keywords: Granulomatosis, oxidative stress, vitamin E, vitamin C, Mn, Zn, Se, stocking density

6.1. Introduction

The Mediterranean production of gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) is consolidated, and their production is saturating the fish market. In the last years there has been an increase in the relevance of meagre, *Argyrosomus regius* (Asso, 1801), as a farmed species in the Mediterranean aquaculture. However, the intensive farming of meagre is affected by systemic granulomatosis. This disease has a chronic course, causing low mortalities but high prevalence and intensity, being this pathology the one with largest impact on meagre culture (Ghittino et al., 2004). Although some infectious agents like Nocardia can produce granulomatosis in meagre (Elkesh et al., 2013) there are evidences that this pathology can be caused by a nutritional imbalance, given that an infectious agent could not be associated with the appearance of granulomas (Katharios et al., 2011; Cotou et al., 2016; Carvalho et al., 2019; Chapter 3, 4, 5).

Granulomas can firstly appear at very early stages in meagre larvae (20 days post hatching; dph), although its development could be avoided by the modulation of the feeding sequence (Chapter 4) and by using a microdiet with high enough levels of the antioxidant vitamins E and C (Chapter 5). Inadequate levels of these two vitamins could cause a nutritional imbalance between pro and antioxidant nutrients, which could in turn lead to a status of oxidative stress. Indeed, a previous study demonstrated that the dietary supplementation with vitamin E and C (300 mg kg^{-1} vitamin E, 100 mg kg^{-1} vitamin C) in feeds for on-growing meagre reduced the incidence and severity of granulomas in liver together with a promotion of fish growth (Chapter 3). The effects of dietary inclusion of vitamin D_3 have also been studied in the development of systemic granulomatosis in juvenile meagre (Cotou et al., 2016). Additionally, there are other micronutrients that can act as antioxidants. Zinc (Zn) has antioxidant properties and protects tissue from oxidative damage (Ho and Ames, 2002). Selenium (Se) is an exogenous antioxidant involved in the prevention of oxidative stress (Felton et al., 1996; Biller-Takahashi et al., 2015; Silva-Brito et al., 2016) being one of the components of glutathione peroxidase (GPX) reducing hydroperoxides at the expense of reduced glutathione (Arteel and Sies, 2001). Manganese (Mn) is another mineral involved in many cellular processes including lipid, protein and carbohydrate metabolism and acts as a cofactor or activator for many enzymes systems, such as Mn superoxide dismutase (Mn-SOD) (De Rosa et al., 1980; Andreini et al., 2008).

One strategy to increase the benefits in intensive aquaculture is to optimize the space with high stock densities. However, overcrowding can be a stressful factor (Wendelaar Bonga, 1997; Ashley, 2007) and negatively affect fish growth, reproduction and immune system (Barton and Iwama, 1991; Pickering, 1998; Di Marco et al., 2008; Shubha and Reddy 2011). Dietary supplementation of vitamin C and E has been shown to modulate the stress response (Montero et al., 1998; Kolkovski et al., 2001) including in fish reared under different stocking density (Montero et al., 1999; Belo et al., 2005), but little is known about its effects on meagre reared different stocking densities. The effect of the stock density has been studied in meagre larvae, observing that high rearing density could reduce growth and survival, and also decrease resistance to an activity test (Estévez et al., 2007; Roo et al., 2007). Nevertheless, negative effects have been associated to high stock density such as reduced growth and food conversion in juvenile rainbow trout (*Oncorhynchus mykiss*) (Procarione et al., 1999), or increased plasma cortisol and free amino acids in Senegalese sole (*Solea senegalensis*) (Costas et al., 2008). In this sense, high densities can activate the fish stress response affecting negatively different metabolic pathways (Costas et al., 2008; Laiz-Carrión et al., 2012). Therefore, non-optimal culture conditions could affect normal growth of meagre and even be a source of stress, which in turn could potentially facilitate the appearance of disease.

The objective of the present study was to elucidate the involvement of the dietary vitamins E and C and the addition of Mn, Zn and Se on the appearance and incidence of systemic granulomatosis in meagre. Additionally, the effect of the stock density on granulomas appearance was tested in fish fed either low or high levels of vitamin C levels (600 and 3,200 mg kg^{-1}, respectively). To reach this objective, diets containing several graded levels of these vitamins and minerals were fed to juvenile meagre and growth, survival, biochemical composition, histopathological evaluation, and gene expression of antioxidant enzymes were determined.

6.2. Materials and methods

6.2.1. Fish and feeding

The experiment was carried out at the ECOAQUA facilities (Taliarte, Canary Islands, Spain). The juvenile meagre were obtained from induced spawns at the ECOAQUA facilities. Juveniles were acclimated to the experimental conditions and the basal diet for 2 weeks. Five isolipidic (16.7 % lipid) and isoproteic (49.6 % protein) fish meal and fish oil based feeds were prepared by adding different levels of vitamin C, Mn, Zn and Se and adittion of vitamin E and K (100 and 35 mg kg^{-1}, respectively) by Skretting ARC (Stavanger, Norway). Diet KEC (100 mg kg^{-1} C), Diet KEC+Mn/Zn/Se (100 mg kg^{-1} C, 40 mg kg^{-1} Mn, 200 mg kg^{-1} Zn, 1.5 mg kg^{-1} Se), Diet KECC (600 mg kg^{-1} C), Diet KECCC (1200 mg kg^{-1} C), Diet KECCCC (3200 mg kg^{-1} C). The formulation, proximate composition and fatty acid content of each feeds is shown in Table 6.1 and Supplementary Table 6.1, Appendix 6.5. The juvenile meagre were fed 3 times per day (8:00, 11:30, 15:00), 6 days per week during 90 days with the different experimental diets. All the uneaten feed was daily collected from each tank and dryed in order to calculate the daily feed intake. Dead fish were recorded daily and survival was determined. The experiment was carried out in 21 fibre glass tanks of 500 L with 100 fish $tank^{-1}$ (3.20 kg m^{-3}) for all the diets, and for diet KECC and KECCCC was tested at higher density (175 fish $tank^{-1}$, 6.20 kg m^{-3}, diets KECC*2 and KECCCC*2). The initial mean weight was 15.75 ± 0.56 g. All tanks were covered with a net to prevent escapes. During the feeding trial the temperature and dissolved oxygen concentration were measured twice a week with values ranging from 18.2-21.5 °C and 5.5-6.5 mg l^{-1}, respectively. Fish were reared under the natural light conditions throughout the feeding trial.

Table 6.1. Feed formulation. Diet codes are according to vitamins and minerals supplemented to the basal diet (Diet KEC).

	Diets				
	KEC	KEC+Mn, Zn, Se	KECC	KECCC	KECCCC
Ingredients (%)					
Wheat[1]	17.1	17.0	16.9	16.7	16.1
Corn gluten[1]	5.0	5.0	5.0	5.0	5.0
Wheat gluten[1]	6.8	6.8	6.9	6.9	7.2
Soya concentrate[1]	25.1	25.1	25.0	25.0	24.7
Fish meal[1]	35.0	35.0	35.0	35.0	35.0
Fish oil[1]	10.4	10.4	10.4	10.4	10.4
Mineral Vitamin premix[2]	0.1	0.1	0.1	0.1	0.1
Vitamin C[3]	-	-	0.2	0.3	0.9
Zn+Se+Mn Mix[4]	-	0.015	-	-	-
Proximate composition (%)					
Lipid	16.8	16.8	16.4	16.8	16.5
Protein	49.6	49.3	49.6	48.9	49.5
Ash	7.1	6.9	7.0	7.0	7.4
Moisture	7.2	8.0	7.9	8.2	7.6
Vitamin C (mg kg^{-1})	98.0	96.0	586.0	1180.0	2835.0
Vitamin E (mg kg^{-1})	228.0	242.0	243.0	241.0	255.0
Vitamin K (mg kg^{-1})	23.0	23.0	23.0	22.0	23.0
Se (mg kg^{-1})	1.1	1.6	1.1	1.1	1.2
Mn (mg kg^{-1})	37	49	34	34	35
Zn (mg kg^{-1})	130	180	130	140	140

[1]Skretting, Stavanger, Norway; [2]Trouw Nutrition, Boxmeer, the Netherlands. Proprietary composition Skretting ARC, including vitamins, but no vitamin K and minerals. Vitamin and mineral supplementation as estimated to cover requirements according NRC (2011); [3]Lutavit C Aquastab 35%, Trouw Nutrition, Boxmeer, the Netherlands. [4]$ZnSO_4$ (0.011), Na_2SeO_3 (0.001) and $MnSO_4$ H_2O (0.003).

6.2.2. Sample collection

At the beginning (n = 50 fish from the stock tank) and at the end of the experimental trial (90 days of feeding) (n = 30 fish per treatment, 10 fish per tank) fish were sacrificed with an overdose of anaesthetic (clove oil; Guinama, Valencia, Spain) and samples of liver, kidney, heart and spleen were collected and fixed in 4 % buffered formalin for histological analysis. Additionally, 5 fish per tank (n = 15 per treatment) were sacrificed and liver, heart and kidney removed and frozen at -20° C for biochemical analysis. 4 fish per tank (n = 12 per treatment) were also sacrificed and the same tissues collected, pooled, stabilized in RNA later (Sigma, Poole, UK) and stored at -80° C until RNA extraction.

6.2.3. Growth performance

At the beginning and end of the trial, fish were anesthetized with clove oil and individual whole body weight and standard length recorded. Fish were unfed for 24 hours before all samplings. Means and standard deviations of each triplicate were calculated for each treatment.

The data were calculated according the following equations: Survival (%) = 100*(final number fish – initial number fish)/ initial number fish; Growth (%) = ((final mean weight – initial mean weight)/initial mean weight)*100; Weight gain = (final mean weight- initial mean weight); SGR (specific growth rate) = 100 x (ln final mean weight – ln initial mean weight)/ number of days; FCR (feed conversion ratio) = feed intake (g)/ weight gain (g); K (condition factor (%)) = 100*(fish weight/ (fish length)3); FI (tank fedd intake (g)/number of fish)/ number of days.

6.2.4. Biochemical analysis

Feed and fish biochemical composition analysis were conducted following standard procedures. Lipids in liver, heart, kidney and feeds were extracted with a choloroform-methanol (2:1 v/v) mixture as described by Folch et al. (1957). Protein content (Kjeldahl method), dry matter and ash were determined according to AOAC (2010).

Fatty acids from total lipids were prepared by transmethylation as described by Christie (1982). Fatty acid methyl esters (FAMES) were separated and quantified by gas–liquid chromatography following the conditions described by Izquierdo et al. (1992).

The concentration of vitamin E was analysed in the diet. The α-tocopherol was injected (50µl) in a high performance liquid chromatograph (HPLC) with UV detection. Samples of diets were weighed, homogenized in ethanolic pyrogallol and saponified as described by McMurray et al., 1980. HPLC analysis was performed using 150 x 4.60 mm, 5 µm reverse-phase Luna and C18 column (Phenomenox, CA, USA). The mobile phase was methanol:ultrapure water (98:2 v/v) with a flow rate of 1.0 ml min^{-1} in ambient temperature. It was used a wavelength of 293 nm to determine the vitamin E concentrations and was achieved by comparison with (+)-α-tocopherol (Sigma-Aldrich) as the external standard.

The concentration of vitamin C was determined in diets. Samples were weighted, homogenised and dissolved in 0.4 M phosphate buffer (adjusted to pH 3.0 with phosphoric acid) as described by Betancor et al., (2012). The samples were centrifuged at 3.000 rpm for 5 min at room temperature. The supernatants were removed and filter through a disposable 0.45 µm filter and stored at 4 °C until the measurement. 50 µl of vitamin C were injected in the HPLC with UV detection. The mobile phase was composed by a phosphate buffer at a flow rate of 0.8 ml min^{-1}. It was used a wavelength of 254 nm and a Gemini C18 column, 5 µm particular size and 150 x 4.6 mm fitted with a Gemini pre-column of the same material to determinate the vitamin C concentration and was achieved by comparison with tris (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich) as the external standard.

The mineral analysis of zinc, selenium and manganese of the experimental diets was performed at NIFES (Bergen, Norway). Approximately 0.2 g of diet was digested in 2 mL of HNO_3 (69 % w/w) and 0.5 mL of H_2O_2 (20 % w/w) in a microwave system (Julshamn et al., 2007). The digested sample was diluted to a final volume of 25 mL with Milli-Q water. The analysis was done by inductively coupled plasma mass spectrometry (ICP-MS; iCAP-Q and FAST SC-4Q DX auto sampler, both Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) after acidic digestion of the feeds.

TBARs were measured in triplicate from extracted total fatty acids (10 mg ml^{-1}) of liver, kidney and heart according to Burk et al., (1980). Briefly, 50 µl of 0.2% (w/v) BHT in ethanol were added to 200 µl of lipid. Next, freshly prepared 0.5 ml of 1% (w/v) TBA and 0.5 ml of 10 % (w/v) trichloroacetic acid were added to the sample. All reagents were mixed in a stoppered test tube and heated in darkness at 100 °C for 20 min. Then, samples were cooled in ice for 5 min and particulate matter removed by centrifugation at 2000 g (Sigma 4K15, Osterode am Harz, Germany) for 5 min. The supernatant was read in a spectrophotometer (Evolution 300, Thermo Scientific, Cheshire, UK) at 532 nm and recorded

against a blank sample. The concentration of TBA-malondialdehyde (MDA) was expressed as μmol MDA per g of tissue and was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

6.2.5. Histopathology

Samples, previously fixed in 4 % buffered formalin, were dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 μm, fixed to the microscope slide, heated and finally stained with haematoxylin and eosin (H&E), Ziel-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco method (Fite et al., 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological evaluation. All the tissues were analysed for the presence of macroscopic granulomas.

6.2.6. Histopathology scoring

The severity of granulomatosis was individually scored in each organ as described in a previous study (Chapter 3). The severity of the granulomas was classified in each organ depending on the number of granulomas observed during the microscopy evaluation. The average severity was classified in liver, kidney and heart according to the criteria shown in Supplementary Table 6.2, Appendix 6.5.

6.2.7. Gene expression

Kidney, liver and heart were aseptically collected from 4 fish per tank at the final sampling and stored at -80 °C until further analysis. Total RNA was extracted from, approximately, 100 mg of sample using TRI Reagent® (Sigma, St Quentin Fallavier, France). Purity was assessed by spectrophotometry (A260/A280), followed by a visual quality assessment via agarose gel electrophoresis on 2% agarose gel stained with GelRed ™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

The cDNA was synthetized from 1 μg of total RNA using the iScript cDNA Synthesis Kit (BIORAD) in 20 μl reactions, which included 4 μl 5× iScript Reaction Mix, 1 μl iScript Reverse Transcriptase (Bio-Rad Laboratories, Hercules, CA), 13 μl Milli-Q sterile water and 2 μl RNA (1 μg) of the sample. The reverse transcription was done in a thermal cycler

(iCycler, Bio-Rad Laboratories, Hercules, CA) at 25 °C for 5 min, 60 min at 42 °C and finally heating samples for 5 min at 85 °C. PCR primers sequences used for the PCR amplification of the cDNAs of the target genes were *cat*, *sod* and *gpx* (Table 6.2).

Table 6.2. Sequences for real-time quantitative-PCR of the forward and reverse primers (5′–3′). The data include sequences, amplicon sizes and annealing temperatures (Tm).

Target	Primer 5´-3´	Fragment size (bp)	Tm (°C)
sod	F: GGCCCTCACTTCAATCCCTA	207	59
	R: TCCTTTTCCCAGATCGTCGG		
gpx	F: AAGCAGTTTGCCGAGTCCTA	103	57
	R: GCTGGTCTTTCAGCCACTTC		
cat	F: GCTTCCACCAACCCAGATTA	205	59
	R: GGTTCCTGTTCAGCACCATT		
bact	F: CCATCGAGCACGGTATTGT	455	60
	R: CAGCTTCTCCTTGATGTCACG		
tub	F: GGAGTACCCCGATCGTATCA	161	59
	R: AGATGTCATACAGGGCCTCG		
ef1a	F: GGTGCTGGACAAACTGAAGG	196	59
	R: GAACTCACCAACACCAGCAG		

sod, superoxide dismutase; *gpx*, glutathione peroxidase; *cat*, catalase; *bact*, β-actin; *tub*, tubulin; *ef1a*, elongation factor 1α.

The relative transcript abundance of glutathione peroxidase, superoxide dismutase and catalase was determined by quantitative real time PCR (qPCR). Primers efficiency for each gene was previously evaluated to ensure that it was close to 100 %. The relative expression values were normalized against the geometric mean of the three housekeeping genes using the method described by Pfaffl (2001). All PCRs were performed using a Biometra TOptical Thermocycler (Analytik Jena, Goettingen, Germany) in 96-well plates in duplicate using 10 µl Thermo Scientific Luminaris Color Higreen qPCR Master Mix (Bio-Rad Hercules, California), 1 µl of forward and reverse primers (100 pmol μl^{-1}), 6 µl water nuclease-free and 5 µl of a 1:10 dilution of the cDNA, with the exception of the reference genes, which were determined using 2 µL of cDNA, in a final volume of 20 µl. In addition, amplifications were carried out with a systematic negative control (NTC-non template control) containing no cDNA.

The PCR conditions were an uracil-DNA glycosylase pre-treatment at 50 °C for 2 min, a denaturation at 95 °C for 10 min, followed by 35 cycles: 15 s at 95 °C, 30 s at the annealing Tm and 30 s at 72 °C. Expression level of each gene was normalized by the corresponding expression of β-actin (*bac*t), elongation factor 1α (*ef1α*) and tubulin (*tub*).

6.2.8. Statistical analysis

All statistical analyses were performed on Statgraphics Centurion XVI (Version 16.1.11, StatPoint Technologies, Inc., Herndon, VA). Data were tested for normality with the Kolmogorov Smirnov test and homogeneity of variance was performed with the Levene test. With the variables that satisfied the normality and homogeneity was carried out a parametric one-way (ANOVA) and Tukey test post-hoc test. In order to study the effect of the stock density and the supplementation of vitamin C and minerals (Mn, Zn and Se) in the variables a two-way analysis of variance (ANOVA) with Tukey post-hoc test was carried out. A significance level of 0.05 was used.

6.3. Results

6.3.1. Growth performance

6.3.1.1. Effect of vitamins and mineral supplementation

Different dietary levels of vitamin C and Mn, Se and Zn did not affect meagre final weight, length or any other evaluated growth parameter (Table 6.3). Juvenile meagre grew from ~ 15.7 g to ~ 95.3 g in 90 days and a good food conversion ratio (FCR) was obtained among fish fed all the dietary treatments (0.75~0.80). Survival was high in all dietary treatments (97.8 - 98.3 %).

Table 6.3. Meagre (*Argyrosomus regius*) growth performance after 90 days of feeding diets with different levels of vitamin C, Mn, Zn and Se.

	Diets				
	KEC	KEC+ Mn,Zn,Se	KECC	KECCC	KECCCC
Initial weight (g)	15.6 ± 0.3	15.8 ± 0.1	15.7 ± 0.2	15.7 ± 0.4	15.9 ± 0.0
Final weight (g)	94.0 ± 1.5	94.9 ± 4.9	94.7 ± 0.7	96.8 ± 2.8	96.0 ± 2.1
Weight gain (%)	603.0 ± 9.1	601.5 ± 26.0	604.0 ± 12.3	616.6 ± 30.8	604.0 ± 12.9
Length (cm)	18.1 ± 1.6	18.1 ± 1.2	18.1 ± 1.5	18.1 ± 1.4	18.2 ± 1.5
FI (g)	0.66 ± 0.0	0.67 ± 0.0	0.66 ± 0.0	0.67 ± 0.0	0.67 ± 0.0
FCR	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0
SGR	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.1	2.0 ± 0.0
Survival (%)	98.3 ± 3.6	97.8 ± 2.0	98.0 ± 2.0	98.7 ± 3.1	97.8 ± 3.2
K factor	1.09 ± 0.0	1.07 ± 0.1	1.07 ± 0.0	1.06 ± 0.0	1.04 ± 0.0

Data are means ± SD. FCR, food conversion ratio; SGR, specific growth rate; FI, feed intake per day and fish.

6.3.1.2. Effect of stocking density and vitamin C

Growth parameters were only affected by the stock density, not existing any dietary effect or interaction between diet and density. Significantly higher final weight, weight gain and FI was obtained in fish reared under low stock density (3.20 kg m^{-3}) compared with those fish reared under high stock density (6.20 kg m^{-3}). In addition, FCR and SGR was significantly lower in fish reared under low stock density (Table 6.4). Different stocking density did not affect final survival, being high in all dietary treatments (97.4 - 98.5 %).

Table 6.4. Meagre (*Argyrosomus regius*) growth performance after 90 days of feeding diets with different levels of vitamin C and cultured under two different densities (3.20 – 6.20 kg m^{-3}).

					Two-way ANOVA		
	KECC (3.20 kg m^{-3})	KECCCC (3.20 kg m^{-3})	KECC*2 (6.20 kg m^{-3})	KECCCC*2 (6.20 kg m^{-3})	Diet	Density	Di*De
Initial weight (g)	15.7 ± 0.2	15.9 ± 0.0	15.8 ± 0.2	15.8 ± 0.1	n.s.	n.s.	n.s.
Final weight (g)	94.7 ± 0.7^{b}	96.0 ± 2.1^{b}	91.5 ± 1.8^{a}	91.7 ± 1.1^{a}	n.s.	*	n.s.
Weight gain (%)	604.0 ± 12.3^{a}	604.0 ± 12.9^{a}	580.3 ± 18.6^{b}	579.3 ± 6.1^{b}	n.s.	*	n.s.
Length (cm)	18.1 ± 1.5	18.2 ± 1.5	17.7 ± 1.8	17.8 ± 2.2	n.s.	n.s.	n.s.
FI (g)	0.66 ± 0.0^{a}	0.67 ± 0.0^{a}	0.64 ± 0.0^{b}	0.64 ± 0.0^{b}	n.s.	*	n.s.
FCR	0.76 ± 0.0^{a}	0.75 ± 0.0^{a}	0.81 ± 0.0^{b}	0.81 ± 0.0^{b}	n.s.	*	n.s.
SGR	2.0 ± 0.0^{b}	2.0 ± 0.0^{b}	1.9 ± 0.0^{a}	1.9 ± 0.0^{a}	n.s.	*	n.s.
Survival (%)	98.0 ± 2.0	97.8 ± 3.2	98.5 ± 2.2	97.4 ± 3.4	n.s.	n.s.	n.s.
K factor	1.07 ± 0.0	1.04 ± 0.0	1.06 ± 0.0	1.05 ± 0.1	n.s.	n.s.	n.s.

Data are means ± SD. where the means in each row with a different superscript are significantly different (P<0.05). FCR, food conversion ratio; SGR, specific growth rate; FI, feed intake per day and fish. Di*De, interaction diet-density. Different superscript letters denote differences among treatments identified by one-way ANOVA. n.s. not significant. * $p < 0.05$. ** $p < 0.01$

6.3.2. Histopathology

6.3.2.1. Effect of vitamins and minerals supplementation

Macroscopic granulomas were only observed in liver and kidney of two fish, not being related to any dietary treatment. The histopathological evaluation revealed the presence of granulomas at different stages of development (Supplementary Figure 6.1A, B, C; Appendix 6.5) as described previously in Chapter 3. During the microscopic

evaluation some granulomas were observed associated to the blood vessels (Supplementary Figure 6.1C; Appendix 6.5).

At the initial sampling, the percentage of fish presenting microscopic granulomas in any tissue was 62%, observing an increase in the number of fish affected with systemic granulomatosis after 90 days of feeding the experimental diets. At the end of the feeding period, significant differences were found in the percentage of fish with granulomas among the dietary treatments, being lower the percentage in fish fed diet KECCC and KECCCC (Figure 6.1). No calcification was observed at any stage or analyzed tissue.

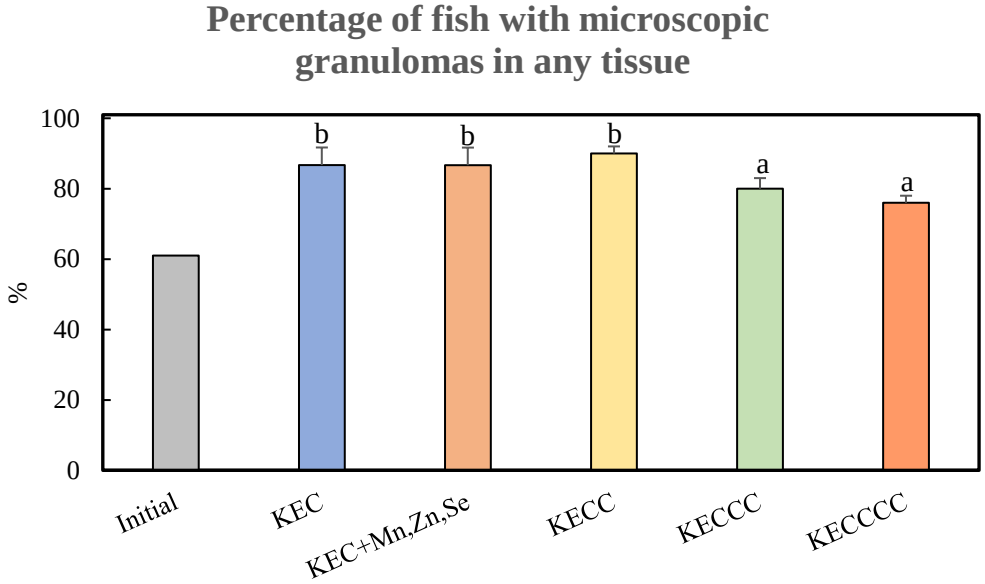


Figure 6.1. Percentage of fish affected with granulomas in any tissue during the microscopic evaluation of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se at the beginning and after 90 days of feeding.

The specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stain), were negative, discarding a possible infectious origin (Supplementary Figure 6.2; Appendix 6.5).

The most affected organ was the liver (71.4 %) followed by the kidney (62.2 %) and heart (7.6 %). No granulomas were observed in neither spleen nor muscle. The lowest number of fish with hepatic granulomas were observed when high level of vitamin C were added to the feeds (KECCC-KECCCC diet) (Table 6.5).

Table 6.5. Percentage of affected liver, kidney and heart with granulomas, of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se for 90 days.

	Liver	Kidney	Heart
Initial	38	52	3
KEC	76.7 ± 3.5^{b}	60.0 ± 6.3	16.7 ± 20.8
KEC+Mn, Zn, Se	76.7 ± 2.9^{b}	60.0 ± 0.0	6.7 ± 11.5
KECC	73.3 ± 5.8^{ab}	70.0 ± 5.0	10.0 ± 17.3
KECCC	63.3 ± 1.5^{a}	60.7 ± 4.6	3.3 ± 0.6
KECCCC	63.3 ± 1.1^{a}	56.7 ± 5.8	3.3 ± 0.6

Data are means ± SD. where the means in each row with a different superscript are significantly different ($P<0.05$). Different superscript letters denote differences among treatments identified by one-way ANOVA.

The severity score did not show significant differences among fish fed the different dietary treatments in any tissue after 90 days of feeding the experimental diets (Table 6.6). However, there was a tendency towards a decrease in the severity of granulomatosis in liver along with an increase in vitamin C content, for instance 1.37 in diet KEC vs 0.93 in diet KECCCC.

Table 6.6. Average granuloma severity scores in liver, kidney and heart of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se for 90 days.

	Liver	Kidney	Heart
Initial	0.42	0.60	0.03
KEC	1.37 ± 1.13	0.73 ± 0.78	0.17 ± 0.38
KEC+Mn, Zn, Se	1.40 ± 1.07	0.77 ± 0.97	0.07 ± 0.25
KECC	1.33 ± 1.09	1.00 ± 0.87	0.10 ± 0.31
KECCC	0.97 ± 1.03	0.91 ± 0.93	0.03 ± 0.18
KECCCC	0.93 ± 1.28	0.82 ± 0.97	0.03 ± 0.18

Data are means ± SD.

6.3.2.2. Effect of stocking density and vitamin C

The appearance of granulomas was affected by the diet, stock density and the interaction between them, being significantly lower in fish fed with the highest addition of vitamin C (KECCCC) at low density (3.20 kg m^{-3}) (Figure 6.2).

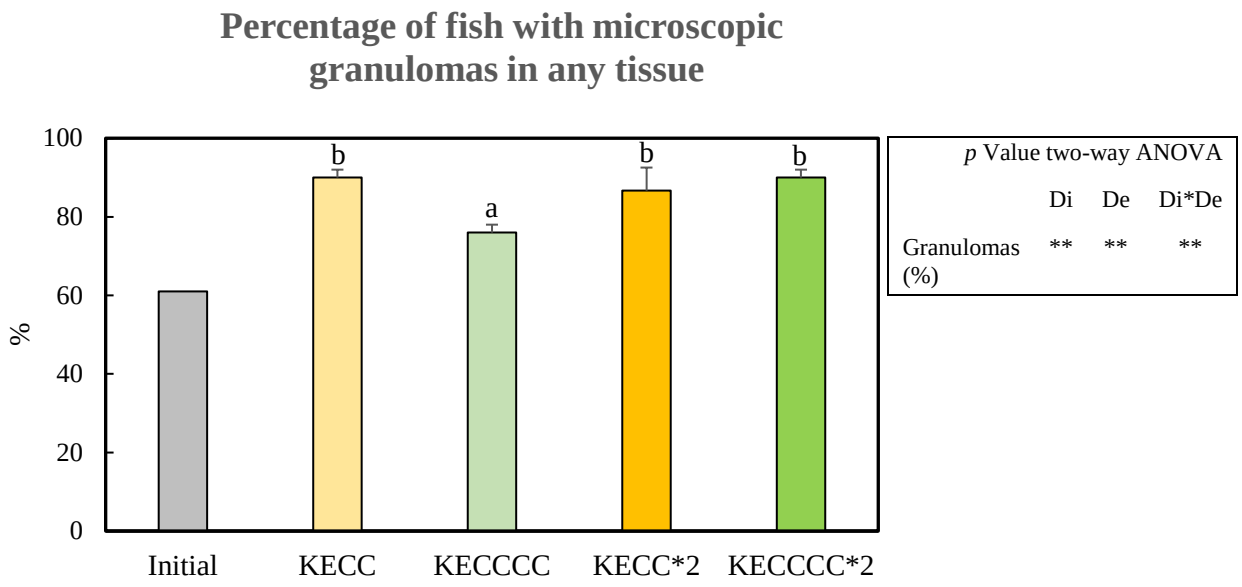


Figure 6.2. Percentage of fish affected with granulomas in any tissue during the microscopic evaluation of meagre (*Argyrosomus regius*) cultured under two different densities (3.20 – 6.20 kg m^{-3}) and fed with high or low dietary vitamin C supplementation for 90 days.

The only organ with differences in the incidence of granulomas among fish fed the different dietary treatments was liver. The incidence of granulomas was only affected by the diet, being lower in the fish fed diet whit higher addition of vitamin C, regardless of the density (63.3 % of affected fish with granulomas in liver) (Table 6.7). The scored severity in liver, kidney and heart was not affected neither the diet nor the density (Table 6.8).

Table 6.7. Percentage of liver, kidney and heart with granulomas in meagre (*Argyrosomus regius*) fed diets with different levels of C and cultured under two different densities (3.20 – 6.20 kg m^{-3}).

	Liver	Kidney	Heart
Initial	38	52	3
KECC (3.20 kg m^{-3})	83.3 ± 5.8^{c}	70.0 ± 5.0	10.0 ± 17.3
KECCCC (3.20 kg m^{-3})	63.3 ± 1.1^{a}	56.7 ± 5.8	3.3 ± 0.6
KECC*2 (6.20 kg m^{-3})	73.3 ± 2.1^{b}	65.3 ± 2.9	6.7 ± 11.5
KECCCC*2 (6.20 kg m^{-3})	63.3 ± 2.9^{a}	56.7 ± 5.8	6.7 ± 11.5
Two-way ANOVA			
Diet	*	n.s.	n.s.
Density	n.s.	n.s.	n.s.
DI*DE	n.s.	n.s.	n.s.

Data are means ± SD. where the means in each row with a different superscript are significantly different (P<0.05). DI*DE, interaction diet-density. Different superscript letters denote differences among treatments identified by two-way ANOVA. n.s. not significant. * $p < 0.05$. ** $p < 0.01$

Table 6.8. Average granuloma severity scores in liver, kidney and heart of meagre (*Argyrosomus regius*) fed diets with different levels of C and cultured under two different densities (3.20 – 6.20 kg m^{-3}).

	Liver	Kidney	Heart
Initial	0.42	0.60	0.03
KECC (3.20 kg m^{-3})	1.33 ± 1.09	1.00 ± 0.87	0.10 ± 0.31
KECCCC (3.20 kg m^{-3})	0.93 ± 1.28	0.82 ± 0.97	0.03 ± 0.18
KECC*2 (6.20 kg m^{-3})	1.23 ± 1.14	0.73 ± 0.71	0.10 ± 0.40
KECCCC*2 (6.20 kg m^{-3})	1.13 ± 1.14	0.73 ± 0.83	0.07 ± 0.25
Two-way ANOVA			
Diet	n.s.	n.s.	n.s.
Density	n.s.	n.s.	n.s.
DI*DE	n.s.	n.s.	n.s.

Data are means ± SD. where the means in each row with a different superscript are significantly different (P<0.05). n.s. not significant.

6.3.3. Tissue lipid content and fatty acid profiles

6.3.3.1. Effect of vitamins and minerals supplementation

There were not differences in the tissue proximate composition of liver, kidney and heart among the fish fed the different experimental diets (Supplementary Table 6.3; Appendix 6.5). The tissue fatty acid profile (Supplementary Table 6.4; Appendix 6.5) reflected the dietary fatty acid content (Supplementary Table 6.1; Appendix 6.5). The highest levels of total monounsaturated fatty acids were observed in liver, followed by kidney and heart, however the total omega-3 (n-3) and total polyunsaturated fatty acid (PUFA) was higher in

the heart, followed by kidney and liver. All the other fatty acids were similarly distributed in the three tissues. Eicosapentaenoic (20:5n-3; EPA), docosahexaenoic acids (22:6n-3; DHA) and arachidonic acid (20:4n-6, ARA) contents were similar between the diets, being in average higher in heart (8.9, 27.8 and 3.34 %, respectively) compare with liver (4.2, 6.9 and 0.54 %, respectively) and kidney (9.8, 17.4 and 2.12 %, respectively).

6.3.3.1.1. TBARs content

The level of lipid peroxides, as indicated by TBARS content (μmol g^{-1} tissue), was significantly lower in liver, heart and kidney of fish fed diet with high addition of vitamin C (KECCC and KECCCC) (Table 6.9). The supplementation of Mn, Zn and Se did not reduce this indicator of lipid peroxidation.

Table 6.9. TBARS content in liver, kidney and heart of juvenile meagre larvae after 90 days of feeding with the experimental diets.

	Diets				
	KEC	KEC+Mn, Zn, Se	KECC	KECCC	KECCCC
TBARS content (μmol g^{-1})					
Liver	0.07 ± 0.0^{b}	0.07 ± 0.0^{b}	0.05 ± 0.0^{a}	0.06 ± 0.0^{ab}	0.04 ± 0.0^{a}
Kidney	0.08 ± 0.0^{b}	0.09 ± 0.0^{b}	0.07 ± 0.0^{ab}	0.04 ± 0.0^{a}	0.05 ± 0.0^{a}
Heart	0.11 ± 0.0^{b}	0.11 ± 0.0^{b}	0.11 ± 0.0^{b}	0.07 ± 0.1^{a}	0.07 ± 0.0^{a}

Each value represents mean ± SD. Different superscript letters denote differences among treatments identified by one-way ANOVA ($p < 0.05$).

6.3.3.2. Effect of stocking density and vitamin C

Density did not neither affect proximate composition or fatty acid profile of liver, kidney and heart among fish fed the different dietary treatments (Supplementary Table 6.5 and 6.6; Appendix 6.5). All fatty acids were similarly distributed among all treatments.

6.3.3.2.1. TBARScontent

TBARS values were affected by the different stock density. The two-way ANOVA revealed that high stock density (6.20 kg m^{-3}) had a stronger influence in the

TBARS content than the supplementation of vitamin C, increasing the lipid peroxidation in liver, kidney and heart, being the TBARS content in heart also influenced by the interaction between diet and density (Table 6.10).

Table 6.10. TBARS content in liver, kidney and heart of juvenile meagre larvae after 90 days of feeding with the experimental diets and cultured at two different densities (3.20 – 6.20 kg m^{-3}).

	KECC (3.20 kg m^{-3})	**KECCCC** (3.20 kg m^{-3})	**KECC*2** (6.20 kg m^{-3})	**KECCCC*2** (6.20 kg m^{-3})	**Diet**	**Density**	**Di*De**
TBARS content (µmol g^{-1})							
Liver	0.04 ± 0.0^{a}	0.04 ± 0.0^{a}	0.07 ± 0.0^{b}	0.07 ± 0.0^{b}	n.s.	*	n.s.
Kidney	0.07 ± 0.0^{ab}	0.05 ± 0.1^{a}	0.08 ± 0.0^{b}	0.09 ± 0.0^{b}	n.s.	**	n.s.
Heart	0.11 ± 0.0^{b}	0.07 ± 0.1^{a}	0.10 ± 0.0^{ab}	0.12 ± 0.1^{b}	n.s.	n.s.	**

Each value represents mean ± SD. DI, diet; DE, density; DI*DE, interaction diet-density. Different superscript letters denote differences among treatments identified by two-way ANOVA. n.s. not significant. * $p < 0.05$. ** $p < 0.01$

6.3.4. Gene expression analysis

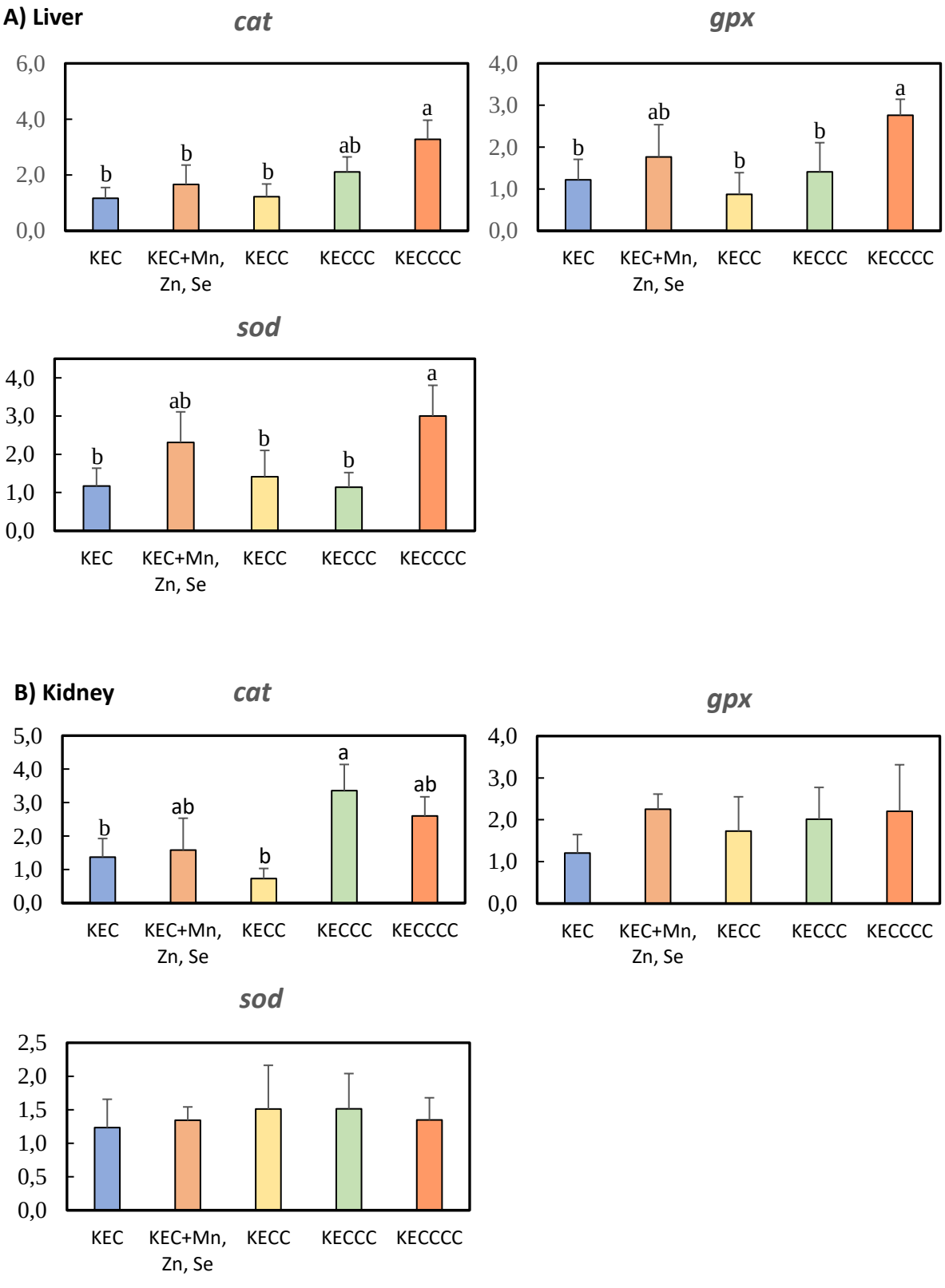
6.3.4.1. Effect of vitamins and minerals supplementation

There were significant differences in the expression levels of *cat*, *gpx* and *sod* in liver (Figure 6.3A). Expression of *cat* was significantly higher in fish fed diet KECCCC, although similar to expression levels of fish fed KECCC. The significantly highest number of mRNA copies of *gpx* and *sod* was also found in fish fed with high levels of vitamin C (KECCCC diet), although not different to that of fish fed diet KEC+Mn, Zn, Se.

Significant differences were also observed in the gene expression of *cat* in kidney in fish fed diet KECCC (Figure 6.3B) compared with the control diet (KEC) and the diet with low addition of vitamin C (KECC). No differences were observed in the expression of *sod* and *gpx* in kidney.

In heart, significant differences were obtained in the gene expression of *sod* and *gpx* (Figure 6.3C). The expression was increased in fish fed with high levels of vitamin C (KECCCC). Expression of *gpx* was significantly increased in fish fed diets with the highest dietary content of vitamin C (KECCC and KECCCC), compared with diets KEC and KEC+Mn, Zn, Se. Similarly, the expression of *sod* in heart was increased when 3,200

mg kg^{-1} of vitamin C were added to the diet (KECCCC), but without differences when only 1,200 mg $kg^{-1}$of vitamin C were added (diet KECCC). The expression of *cat* in heart was not affected by the inclusion of different levels of vitamin C or Mn, Zn and Se.



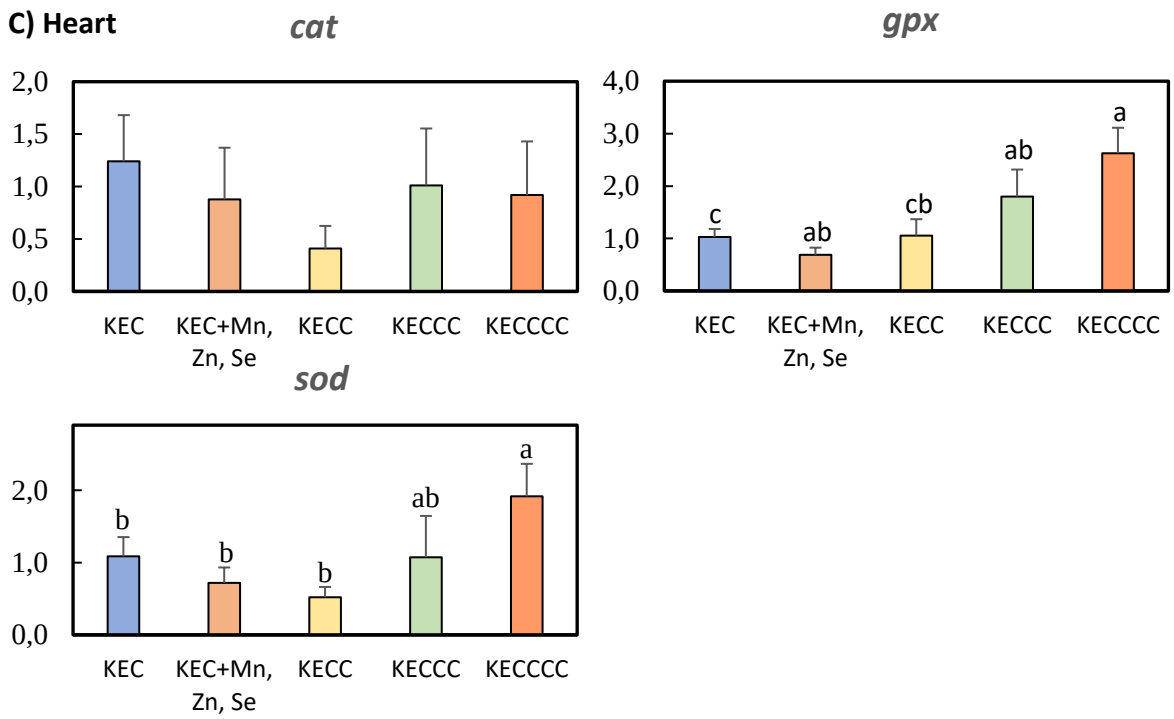
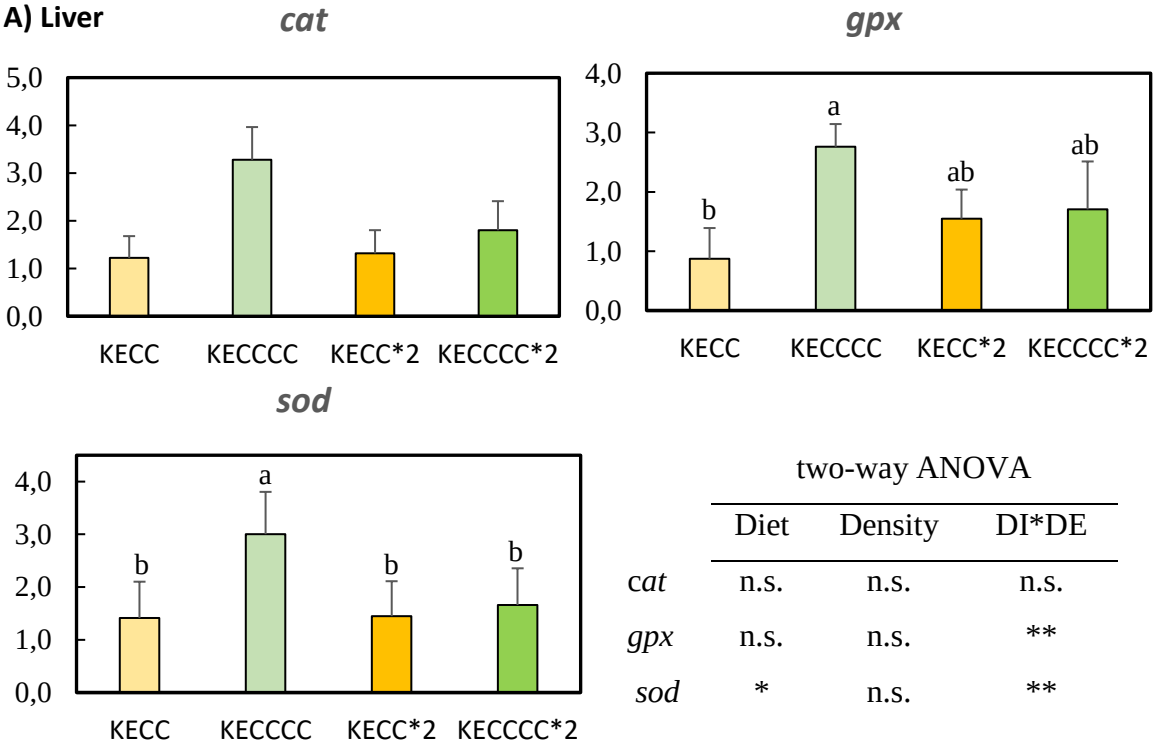


Figure 6.3. *cat*, *sod* and *gpx* expression levels measured by real-time PCR in a) liver, b) kidney and c) heart of meagre (*Argyrosomus regius*) after 90 days of feeding diets with different levels of vitamin C, Mn, Zn and Se. Values are normalized expression ratios, corresponding to an average of six individuals (n = 6) with standard errors (SEM) Different superscript letters denote differences among treatments identified by one-way ANOVA.

6.3.4.2. Effect of stocking density and vitamin C

GPX expression in liver was affected by the interaction of the diet and the stock density, the expression of this gene was only significantly higher in fish diet KECCCC at a low density (3.20 kg m^{-3}) (Figure 6.4A). SOD expression was mainly affected by the diet but also by the interaction between diet and density, being higher the expression with the addition of 3,200 mg kg^{-1} of vitamin C. The gene expression of *cat* in liver was not affected neither by the density nor the diet. In kidney, only *cat* expression was up-regulated by the effect of the diet, being significantly higher in fish fed diet KECCCC (Figure 6.4B). Significant differences were not found in *gpx* and *sod* expression. Expression of *gpx* in heart of meagre, was affected by the stock density but also by the interaction between stock density and diet (Figure 6.4C), only fish fed diet KECCCC having significantly higher expression of this gene. SOD expression was only affected by the diet, being higher in fish fed diet KECCCC and only significantly different of fish fed KECC at a low density. The expression of cat was not affected in heart.

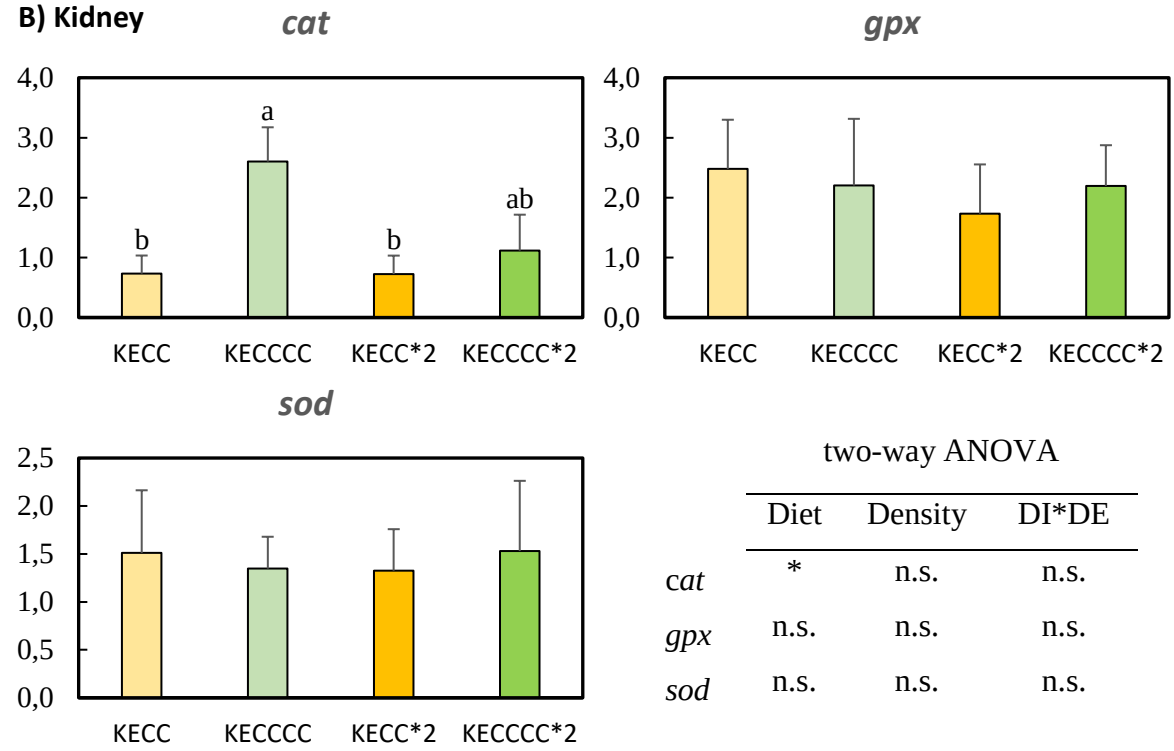
A) Liver



two-way ANOVA

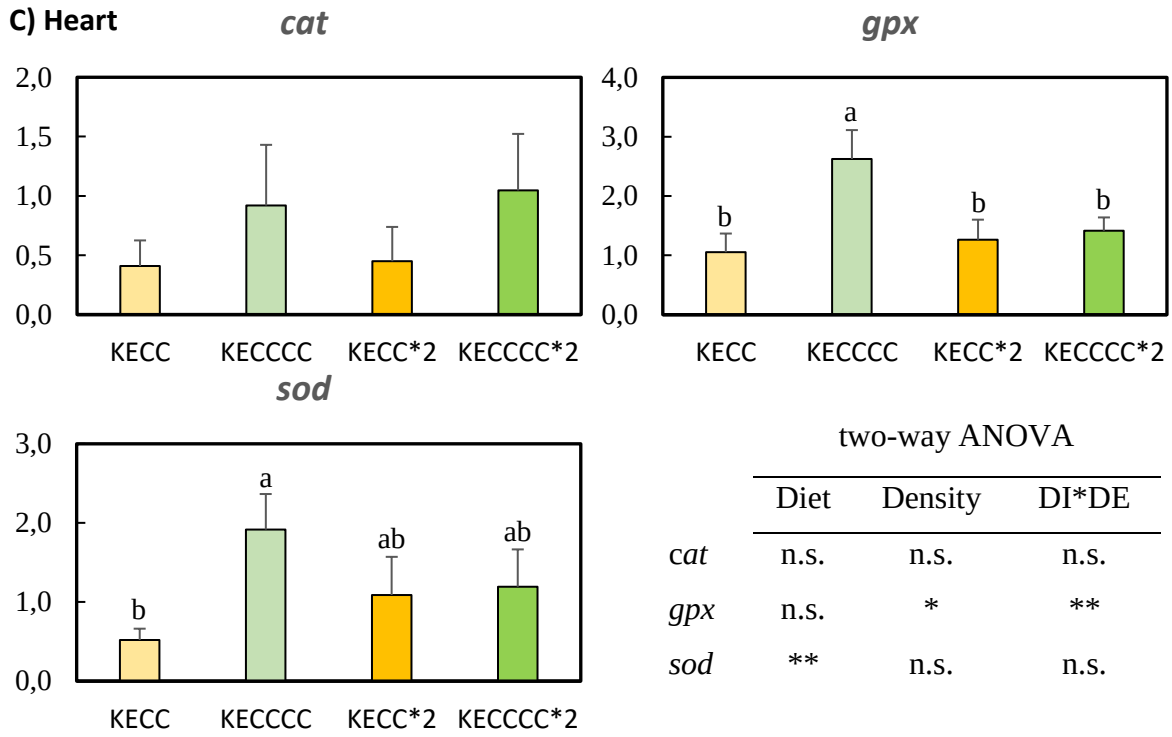
	Diet	Density	DI*DE
cat	n.s.	n.s.	n.s.
gpx	n.s.	n.s.	**
sod	*	n.s.	**

B) Kidney



two-way ANOVA

	Diet	Density	DI*DE
cat	*	n.s.	n.s.
gpx	n.s.	n.s.	n.s.
sod	n.s.	n.s.	n.s.



two-way ANOVA

	Diet	Density	DI*DE
cat	n.s.	n.s.	n.s.
gpx	n.s.	*	**
sod	**	n.s.	n.s.

Figure 6.4. *cat*, *sod* and *gpx* expression levels measured by real-time PCR in liver, kidney and heart of *Argyrosomus regius* after 90 days fed different levels of vitamin C and cultured under two different densities (3.2 – 6.2 kg m^{-3}). Di, diet; De, density; DI*DE, interaction diet-density. Different superscript letters denote differences among treatments identified by one-way ANOVA. * $p < 0.05$. ** $p < 0.01$.

6.4. Discussion

In the present study, inclusion of vitamin E, Mn, Zn, Se and different levels of vitamin C did not affect growth parameters. The results of FCR (0.75-0.76) and SGR (18.07-18.04) in low density were slightly better than those obtained by Rodriguez et al., 2017 in meagre (FCR 0.74-0.95, SGR 1.41-1.49) probably due to the larger initial size of the fish (62.9 g) in the former study. In previous studies it was observed that addition of vitamin E and C in the diet (300 and 100 mg kg^{-1}, respectively) could affect final growth in on-growing meagre (Chapter 3). However, in the present study the concentration of vitamin E was lower, which suggests that to promote growth in meagre higher levels of this vitamin are required. Significant differences in growth, SGR, FCR, and K were observed between the two stocking densities, being higher in the treatments with low density (100 fish $tank^{-1}$). The negative effect of stocking density has also been observed in other fish species, such as juvenile rainbow trout (Procarione et al., 1999), Dover sole (*Solea solea*) (Schram et al., 2006), Atlantic cod (*Gadus morhua* L.) (Lambert and Dutil,

2001) and Amur sturgeon (*Acipenser schrenckii*) (Li et al., 2012). The reduction in growth in high density stocking, could be due to heterogeneity size of fish as a consequence of the dominance of some fish in the tank. Larger sized fish exercise their dominance in the areas where the food is supplied preventing an adequate food access to those of smaller size (Grand and Grant, 1994; Alanärä and Brännäs, 1996; McCarthy et al., 1999). Moreover, it has been shown that the high stock density produce stress and, in consequence, elevation of cortisol levels (Wuertz et al., 2006). When the levels of cortisol are elevated in plasma, a variety of secondary physiological responses take place, including an increase in the metabolic rate (Lankford et al., 2005), which in turn produces a decrease in growth (Heath, 1995).

In this experiment, fish exposed to high stocking densities may have been experiencing chronic stress, as could be observed by the increase in the MDA content in all the analyzed organs. Indeed, higher levels of TBARS were found in liver and heart of fish stocked at high density (6.2 kg m^{-3}). A similar effect has been observed by Liu et al. (2016a) in Atlantic salmon (*Salmo salar*) and by Jia et al. (2016) in turbot, where the fish cultured under a high stock density showed higher levels of TBARS compared with those cultured in lower densities. It has been shown that high stock density in fish can disturb the balance between the production and removal of ROS, inducing lipid peroxidation (Sahin et al., 2014; Andrade et al., 2015). Therefore, the higher stock density might induce a higher stress resulting in an increase of lipid peroxidation and cell degradation.

Additionally, the level of lipid peroxides was reduced in all the analysed tissues with the inclusion of 1,200 or 3,200 mg kg^{-1} of vitamin C in fish farmed at low density (KECCC and KECCCC). TBARS have been the most frequently used indicator for determination of protective actions of antioxidant vitamins against lipid peroxidation (Harats et al. 1990; de Zwart et al. 1999). Similar results were obtained by Betancor et al. (2012), where the vitamin C dietary supplementation, markedly improved the protection against peroxidation, decreasing MDA contents to less than one third in sea bass larvae. In previous studies in meagre larvae, a strong correlation between the TBARS content and the appearance of granulomas was observed (Chapter 4, 5), suggesting a possible relationship between lipid peroxidation and the appearance of granulomas. In the present study, a modest correlation between the TBARS levels and the appearance of granulomas in liver ($R^2 = 0.651$, $y = 487.63x + 43.914$) and heart ($R^2 = 0.648$, $y = 206.75x + 11.087$) was observed. Probably this correlation is more attenuated than the observed in previous

studies in meagre larvae of 30 dph (R^2=0.948, y=0.084x-4.3924) (Chapter 4) and 44 dph (R^2=0.892, y=0.0446x+0.0756) (Chapter 5), due to the higher initial incidence of granulomas in juvenile meagre in the present trial (61 %).

In relation to the presence of granulomas, the increase in dietary level of vitamin C from 100 mg kg^{-1} to 1,200-3,200 mg kg^{-1}, significantly reduced the percentage of fish with granulomas in any tissue at low density, from 87 % in diet KEC to 80 and 76 % in diet KECCC and KECCCC, respectively. Percentage of fish with granulomas in liver, which was the main affected organ (71.5 %) followed by kidney (62.2 %) and heart (7.6 %), was also significantly reduced in fish fed diet KECCC and KECCCC at low density, and in fish fed diet KECCCC*2. Indeed, the percentage of granulomas in any tissue was affected by the diet, but also by the stock density. The high stock density can disturb the balance between the production of ROS and its removal (Sahin et al., 2014; Andrade et al., 2015), this imbalance potentially leading to a status of oxidative stress which may cause different diseases and lesions (Kawatsu, 1969; Cowey et al., 1984; Sakai et al., 1989; Watanabe et al., 1989; Lewis-McCrea and Lall, 2007; Betancor et al., 2012). In the present study, TBARS levels were affected by the stock density, being higher in fish farmed at high density. Therefore, the present results suggest that the high stock density is a stressful factor that can lead to oxidative stress, lipid peroxidation and in turn promote the appearance of granulomas.

On the other hand, the supplementation of Se, Mn and Zn in this study did not seem to prevent lipid peroxidation, as indicated by TBARS levels. This is probably due to the fact that this species needs a higher supplementation of these minerals in order to see an effect on the antioxidant system. For instance, Mansour et al. (2007) observed that the supplementation of 3.98 mg kg^{-1} of selenium improved the antioxidant balance and inmate immune status of juvenile meagre, while in the present experiment it was supplemented with 1.6 mg kg^{-1}. Little is known about the requirements and its effects on the antioxidant system of these three minerals.

The decrease in the percentage of granulomas and the reduction of TBARS values in fish fed with high addition of vitamin C (3,200 mg kg^{-3}) at low densities was also accompanied by the increase in the expression of some antioxidant enzymes capable to neutralize ROS, which are *sod*, *gpx* and *cat*. In the present study, the expression of theses enzymes was affected by the addition of different levels of dietary vitamin C. Expression of *sod* and *gpx* was increased in liver and heart of fish fed the diet supplemented with

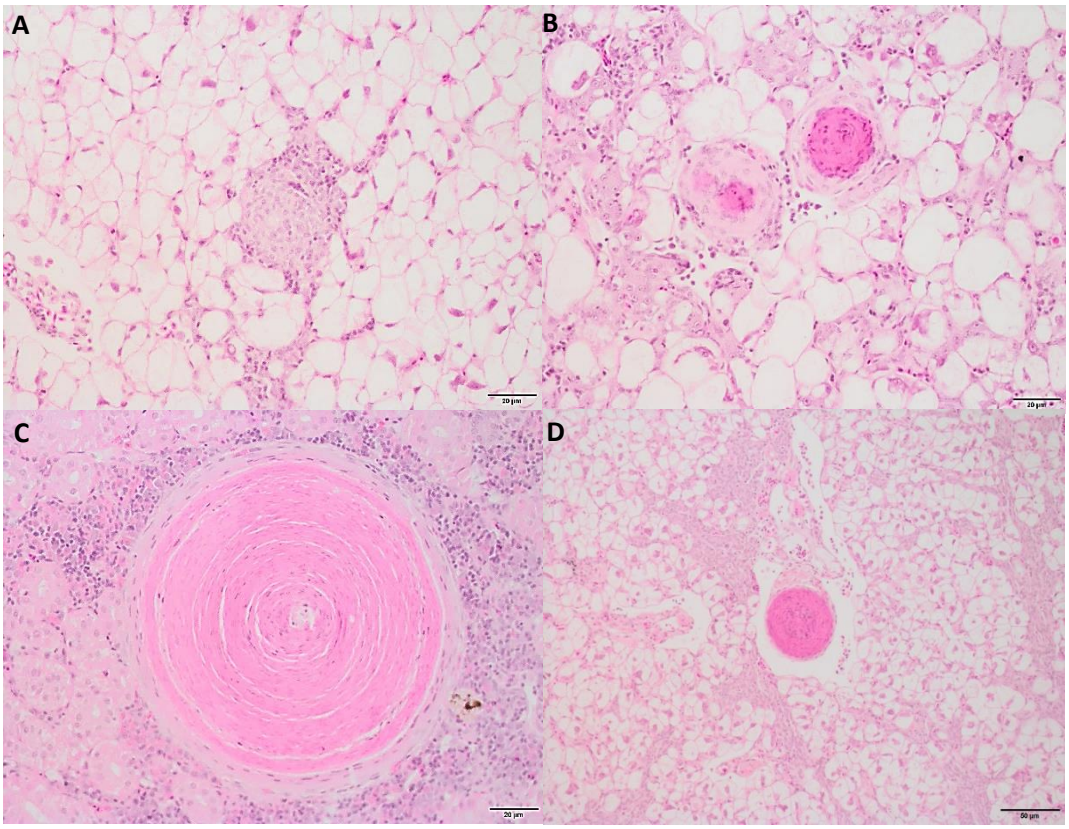
3,200 mg kg^{-1} vitamin C. Expression of *cat* was up-regulated in liver and kidney of fish fed diet supplemented 3.200 mg kg^{-1} vitamin C. SOD can catalyse the reaction of super anion transforming it to H_2O_2 and O_2, *gpx* and *cat* are ROS scavenger enzymes, which can decompose H_2O_2 into O_2 and H_2O. Therefore, the antioxidant status in fish can accurately reflect the activity of *sod*, *gpx*, and *cat* (Cheng et al., 2017). A positive correlation has been observed between the levels of vitamin C in the diet and the activity of *sod*, *gpx* and *cat* in other fish species, such as juvenile Wuchang bream (*Megalobrama amblycephala*) (Wan et al., 2013; Liu et al., 2016b), juvenile Pufferfish (*Takifugu obscurus*) (Cheng et al., 2017), Nile tilapia (*Oreochromis niloticus)* (El-Sayed et al., 2016), red seabream (*Pagrus major*) (Dawood et al., 2016) and sea bass (Betancor et al., 2012). The present results seem to indicate that dietary vitamin C has antioxidant potential by enhancing the expression of *sod* and *gpx* in liver and heart, and *cat* in liver and kidney, being influenced by the organ where they are acting. Moreover, the reduction in the TBARS levels and in the percentage of granulomas in fish fed high addition of vitamin C, suggest that this vitamin has a protective effect against oxidative stress therefore preventing the appearance of granulomas.

Nevertheless, not only the supplementation of vitamin C had an effect on the expression of antioxidant genes, but also the factor density. In liver *sod* expression was affected by the stock density, with fish fed diet KECCCC and reared at low density (3.20 kg m^{-3}) displaying a higher expression of *sod* than fish fed with the same diet but reared at a high density (6.20 kg m^{-3}). The expression of *gpx* was also up-regulated in heart of fish fed diet supplemented with 3,200 mg kg^{-1} of vitamin C at low density but was significantly lower in the same diet but at high density. Similarly to these results, Jia et al (2016) observed that in juvenile turbot reared under three different stock densities (5.1, 7.7 and 10.8 kg m^{-2}), the expression levels of *sod*, *gpx* and *cat* decreased after 80 days in fish reared under the higher stock density. The high stock density has been stablished as a source of stress that can increase the production of ROS (Vijayan et al., 1990; Braun et al., 2010). These ROS could damage cellular components and autoxidation function (Sayeed et al., 2003). It has been observed that the increase of ROS can induces a variety of secondary physiological responses, such as a decrease in the antioxidant capacity and immune function (Aksakal et al. 2011; Sadhu et al. 2014). In our experiment fish fed under high stock density, presented higher content of MDA and down-regulation of

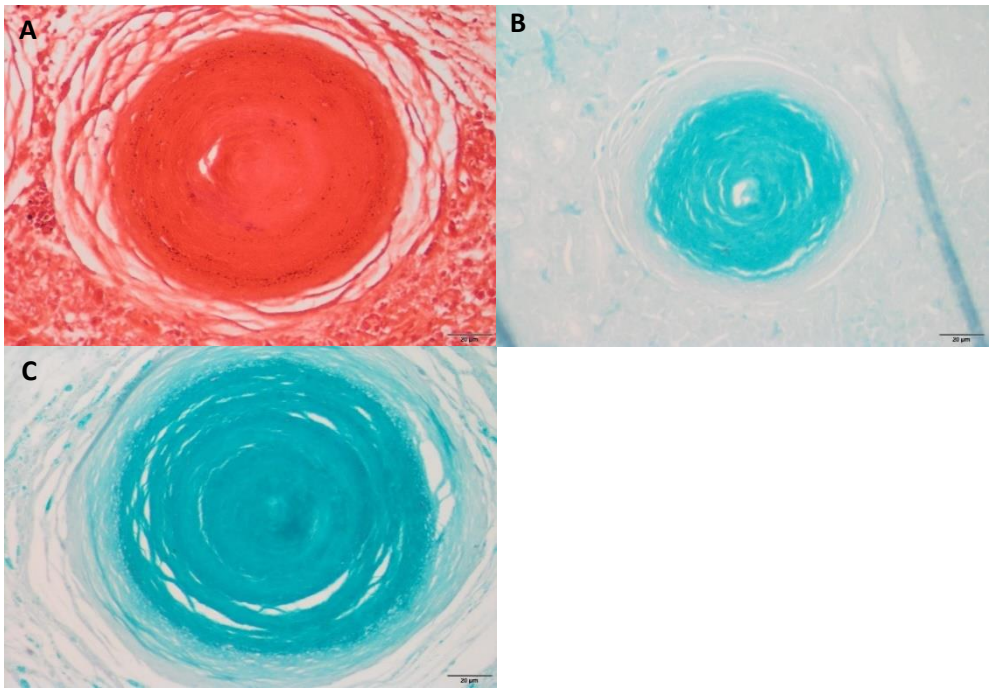
antioxidant genes, which indicates a higher oxidative stress in those fish reared under high stock density.

High stock density (6.20 kg m^{-3}) reduced growth, SGR and FCR of meagre fed the experimental diets for 90 days, regardless of the dietary content. The addition of different levels of vitamin C and supplementation of Mn, Se and Zn did not affect growth parameters. High addition of vitamin C (1,200-3,200 mg kg^{-1}) reduced TBARS values in kidney, liver and heart at low culture density. It also reduced the percentage of granulomas in any tissues and in liver, in which organ the TBARS values were highly correlated with the percentage of granulomas in liver (R^2=0.9439, y=0.003x-0.1242). As consequence of the higher initial incidence of granulomas in juvenile meagre in the present trial (61 %) a modest correlation was observed in the percentage of fish with granulomas in any tissue (R^2 = 0.651, y = 487.63x + 43.914) and heart (R^2 = 0.648, y = 206.75x + 11.087). In addition, fish fed KECCCC showed the highest expression of *cat* in liver and kidney as well as the highest expression of *sod* and *gpx* in liver and heart at low stock density. The present results show that high levels of vitamin C (1,200-3,200 mg kg^{-1}C) and low stock density (3.20 kg m^{-3}) favours the growth of juvenile meagre, reducing the lipid peroxidation indicators and decreasing the incidence of granulomas, which confirms that this pathology is mostly triggered by deficiency of antioxidant nutrients. However, a high prevalence of granulomas was observed at the beginning of the experimental trial what prompts to evaluate the combination of vitamins at earlier life stages.

6.5. Appendix



Supplementary Figure 6.1. Different stages of granuloma development. **A)** Irregular aggregated of macrophages and inflammatory cells. **B)** Necrotic centre with an external layer of fibrocytes. **C)** Granuloma composed completely of laminar material in kidney. **D)** Granuloma associated to a blood vessel in the liver of juvenile meagre larvae at the end of the feeding trial.



Supplementary Figure 6.2. A) Gram satin of granuloma in liver, B) Ziel-Neelsen stain of granuloma in kidney and C) Fite-Faraco stain in liver.

Supplementary Table 6.1. Fatty acid composition (percentage of total fatty acids) of diets used to feed meagre (*Argyrosomus regius*). Diet codes are according to vitamins and minerals supplemented to the basal diet (Diet KEC).

	KEC	KEC+Mn,Zn,Se	KECC	KECCC	KECCCC
Fatty acids (%)					
14:0	8.0	7.5	7.6	7.5	8.1
16:0	22.5	21.5	20.9	20.3	22.2
18:0	4.3	4.1	4.1	4.0	4.3
20:0	0.3	0.4	0.3	0.3	0.3
Σ Saturated[†]	36.6	35.0	34.5	33.7	34.7
16:1n-7	7.8	7.4	7.6	7.6	8.1
18:1n-9	12.1	12.9	11.2	10.9	11.6
18:1n-7	3.1	3.1	3.0	3.0	3.2
20:1n-7	3.2	3.1	2.9	2.7	3.0
22:1n-11	4.6	4.4	4.0	3.8	4.1
Σ Monosaturated[‡]	32.9	33.0	30.7	30.1	30.5
18:2n-6	6.1	6.7	5.6	5.1	5.3
18:3n-6	0.3	0.3	0.3	0.3	0.3
20:2n-6	0.2	0.2	0.2	0.2	0.2
20:3n-6	0.1	0.1	0.1	0.1	0.1
20:4n-6	0.7	0.7	0.8	0.8	0.9
Σ n-6PUFA[§]	7.6	8.2	7.2	6.8	6.9
18:3n-3	1.1	1.3	1.1	1.1	1.1
18:4n-3	1.8	1.8	2.1	2.2	2.2
20:3n-3	0.1	0.1	0.1	0.1	0.1
20:4n-3	0.5	0.5	0.5	0.6	0.6
20:5n-3	7.9	8.1	9.6	10.3	10.2
22:5n-3	0.9	0.9	1.1	1.2	1.2
22:6n-3	7.8	8.3	10.1	10.7	10.7
Σ n-3PUFA[¶]	20.1	21.1	24.6	26.1	24.8
(n-3+n-6) PUFA	27.7	29.3	31.8	32.9	31.7
Total n-3 LC-PUFA[††]	17.1	17.9	21.3	22.7	22.74

Data expressed as means of three technical replicates per batch of diet. [†]Includes 15:0 and 17:0. [‡]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9 and 20:1n-5. [§]Includes. 22:5n-6 and 22:4n-6. [¶]Includes 16:3n-3 and 16:4n-3. [††] LC- PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3).

Supplementary Table 6.2. Severity score of granulomas in liver, kidney and heart.

Score	Liver	Kidney	Heart
0	No granulomas	No granulomas	No granulomas
1	1 ≤ 10 granulomas	1 ≤ 3 granulomas	1 ≤ 1 granulomas
2	10 ≤ 30 granulomas	3 ≤ 6 granulomas	2 ≤ 2 granulomas
3	> 30 granulomas	> 6 granulomas	> 3 granulomas

Supplementary Table 6.3. Proximate composition in liver, kidney and heart of meagre (*Argyrosomus regius*) after 90 days of feeding diets with different levels of vitamin C, Mn, Zn and Se.

	Diets				
	KEC	KEC+Mn, Zn, Se	KECC	KECCC	KECCCC
Prox. composition (%)					
Liver					
Lipids	17.3 ± 1.0	16. ± 1.	15. ± 1.	17. ± 1.	14. ± 3.
Proteins	8,0 ± 0.3	8.3 ± 0.	8.3 ± 0.	8.2 ± 0.	8.3 ± 0.
Moisture	63.0 ± 0.8	64. ± 1.	63. ± 1.	62. ± 2.	64. ± 2.
Ash	0.7 ± 0.1	0.7 ± 0.	0.6 ± 0.	0.5 ± 0.	0.7 ± 0.
Kidney					
Lipids	4.7 ± 0.2	4.2 ± 0.	4.1 ± 0.	4.5 ± 0.	4.5 ± 0.
Proteins	14.4 ± 0.2	15. ± 0.	15. ± 0.	15. ± 0.	14. ± 0.
Moisture	80.0 ± 0.3	80. ± 0.	80. ± 0.	80. ± 0.	79. ± 0.
Ash	0.4 ± 0.0	0.5 ± 0.	0.4 ± 0.	0.4 ± 0.	0.5 ± 0.
Heart					
Lipids	4.5 ± 0.9	3.9 ± 0.	4.4 ± 0.	4.8 ± 0.	4.0 ± 0.
Proteins	15.8 ± 1.5	15. ± 0.	15. ± 0.	15. ± 0.	15. ± 0.
Moisture	82.7 ± 0.2	82. ± 0.	82. ± 0.	81. ± 0.	82. ± 0.
Ash	0.3 ± 0.0	0.4 ± 0.	0.3 ± 0.	0.3 ± 0.	0.4 ± 0.

Data are means ± SD.

Supplementary Table 6.4. Fatty acid composition (percentage of fatty acids) in liver, kidney and heart of meagre (*Argyrosomus regius*) after 90 days of feeding diets with different levels of vitamin C, Mn, Zn and Se.

	KEC	KEC+Mn, Zn, Se	KECC	KECCC	KECCCC
Fatty acids (%)					
Liver					
20:4n-6 (ARA)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.1	0.6 ± 0.0
20:5n-3 (EPA)	4.3 ± 0.7	3.8 ± 1.4	3.6 ± 0.9	3.9 ± 1.1	4.4 ± 0.3
22:6n-3 (DHA)	6.7 ± 1.4	6.0 ± 1.2	6.4 ± 0.9	7.5 ± 0.6	6.8 ± 0.6
Σ Saturated[1]	35.1 ± 2.8	35.9 ± 3.4	36.5 ± 1.6	35.6 ± 0.9	34.1 ± 0.6
Σ Monosaturated[2]	41.7 ± 1.1	41.5 ± 1.2	41.8 ± 0.8	41.2 ± 1.3	41.4 ± 0.9
Σ n-6PUFA[3]	6.8 ± 0.8	7.4 ± 0.9	6.6 ± 0.3	6.9 ± 0.6	7.5 ± 0.3
Σ n-3PUFA[4]	14.5 ± 2.9	13.2 ± 3.5	13.2 ± 1.9	14.7 ± 1.5	14.9 ± 0.9
(n-3+n-6) PUFA	21.2 ± 3.7	20.6 ± 4.4	19.8 ± 2.3	21.6 ± 2.1	22.4 ± 0.7
Total n-3 LC-PUFA[5]	13.0 ± 0.4	11.6 ± 1.1	11.8 ± 1.0	13.3 ± 0.7	13.2 ± 0.5
Kidney					
20:4n-6 (ARA)	1.9 ± 0.3	1.8 ± 0.6	2.6 ± 0.8	1.9 ± 0.4	2.0 ± 0.2
20:5n-3 (EPA)	9.6 ± 0.2	9.2 ± 0.8	10.3 ± 1.0	9.9 ± 0.1	9.8 ± 0.3
22:6n-3 (DHA)	16.7 ± 1.9	16.9 ± 1.7	17.1 ± 0.4	16.9 ± 1.6	17.3 ± 1.1
Σ Saturated[1]	31.1 ± 0.2	30.4 ± 1.3	32.1 ± 1.0	30.9 ± 1.0	31.3 ± 0.6
Σ Monosaturated[2]	26.3 ± 2.0	27.0 ± 2.8	23.7 ± 2.6	26.0 ± 1.9	25.4 ± 1.3
Σ n-6PUFA[3]	9.3 ± 0.2	9.5 ± 0.3	9.8 ± 0.7	9.3 ± 0.2	9.3 ± 0.1
Σ n-3PUFA[4]	30.6 ± 1.9	30.4 ± 2.2	31.3 ± 1.0	31.1 ± 1.2	31.3 ± 0.6
(n-3+n-6) PUFA	39.9 ± 2.0	39.9 ± 2.5	41.1 ± 1.7	40.4 ± 1.3	40.6 ± 0.7
Total n-3 LC-PUFA[5]	28.4 ± 2.1	28.2 ± 1.2	29.6 ± 1.0	28.9 ± 2.3	29.3 ± 0.9
Heart					
20:4n-6 (ARA)	3.3 ± 0.2	3.5 ± 0.2	3.3 ± 0.2	3.4 ± 0.1	3.1 ± 0.2
20:5n-3 (EPA)	8.9 ± 0.0	8.6 ± 0.3	9.1 ± 0.1	8.9 ± 0.3	8.7 ± 0.5
22:6n-3 (DHA)	27.3 ± 0.6	27.8 ± 0.4	27.7 ± 1.3	28.7 ± 0.6	27.4 ± 1.9
Σ Saturated[1]	30.7 ± 0.4	31.6 ± 1.0	30.4 ± 0.5	30.4 ± 0.8	29.9 ± 0.4
Σ Monosaturated[2]	16.7 ± 0.5	15.6 ± 0.9	16.7 ± 1.3	15.8 ± 0.4	17.7 ± 1.3
Σ n-6PUFA[3]	9.9 ± 0.3	10.3 ± 0.3	9.9 ± 0.1	9.9 ± 0.2	9.8 ± 0.1
Σ n-3PUFA[4]	39.5 ± 0.6	39.4 ± 0.3	39.9 ± 1.0	40.7 ± 0.6	39.4 ± 1.9
(n-3+n-6) PUFA	49.4 ± 0.4	49.7 ± 0.3	49.8 ± 1.	50.7 ± 0.4	49.3 ± 1.9
Total n-3 LC-PUFA[5]	38.6 ± 2.1	38.7 ± 2.0	39.1 ± 1.5	39.9 ± 0.9	38.5 ± 1.3

Data are means ± SD. Data expressed as means of three technical replicates per batch of tissue.[1]Includes 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0.[2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 16:1n-7, 18:1n-5, 18:1n-9, 18:1n-7, 20:1n-7, 20:1n-9, 20:1n-5 and 22:1n-11.[3]Includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3. 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6m-3. [5]LC-PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

Supplementary Table 6.5. Proximate composition in liver, kidney and heart of meagre (*Argyrosomus regius*) after 90 days of feeding diets with different levels of vitamin C and cultured at two different densities (3.20 – 6.20 kg m^{-3}).

	KECC (3.20 kg m^{-3})	KECCCC (3.20 kg m^{-3})	KECC*2 (6.20 kg m^{-3})	KECCCC*2 (6.20 kg m^{-3})	Diet	Density	Di*De
Prox. composition (%)							
Liver							
Lipids	15.9 ± 1.0	14.1 ± 3.3	16.2 ± 0.8	15.3 ± 0.0	n.s.	n.s.	n.s.
Proteins	8.3 ± 0.4	8.3 ± 0.3	8.1 ± 0.2	8.0 ± 0.1	n.s.	n.s.	n.s.
Moisture	63.6 ± 1.2	64.0 ± 2.6	63.1 ± 1.1	63.5 ± 0.9	n.s.	n.s.	n.s.
Ash	0.6 ± 0.1	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.2	n.s.	n.s.	n.s.
Kidney							
Lipids	4.1 ± 0.3	4.5 ± 0.4	4.3 ± 0.6	4.6 ± 0.3	n.s.	n.s.	n.s.
Proteins	15.1 ± 0.5	14.6 ± 0.5	15.0 ± 0.7	14.7 ± 0.3	n.s.	n.s.	n.s.
Moisture	80.5 ± 0.2	79.8 ± 0.2	79.5 ± 1.2	78.3 ± 0.5	n.s.	n.s.	n.s.
Ash	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	n.s.	n.s.	n.s.
Heart							
Lipids	4.4 ± 0.7	4.0 ± 0.3	4.9 ± 0.6	4.8 ± 0.2	n.s.	n.s.	n.s.
Proteins	15.1 ± 0.5	15.5 ± 0.2	15.3 ± 0.4	15.4 ± 0.3	n.s.	n.s.	n.s.
Moisture	82.5 ± 0.1	82.2 ± 0.4	82.1 ± 0.3	82.0 ± 0.3	n.s.	n.s.	n.s.
Ash	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	n.s.	n.s.	n.s.

Data are means ± SD. DI, diet; DE, density; DI*DE, interaction diet-density. n.s. not significant.

Supplementary Table 6.6. Fatty acid composition (percentage of fatty acids) in liver, kidney and heart of meagre (*Argyrosomus regius*) after 90 days of feeding diets with different levels of vitamin C and cultured at two different densities (3.20 – 6.20 kg m^{-3}).

	KECC (3.20 kg m^{-3})	KECCCC (3.20 kg m^{-3})	KECC*2 (6.20 kg m^{-3})	KECCCC*2 (6.20 kg m^{-3})	Diet	Density	Di*De
Fatty acids (%)							
Liver							
20:4n-6 (ARA)	0.5 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	n.s.	n.s.	n.s.
20:5n-3 (EPA)	3.6 ± 0.9	4.4 ± 0.3	4.5 ± 0.1	4.5 ± 0.3	n.s.	n.s.	n.s.
22:6n-3 (DHA)	6.4 ± 0.9	6.8 ± 0.6	7.6 ± 0.2	7.7 ± 1.0	n.s.	n.s.	n.s.
Σ Saturated[1]	36.5 ± 1.6	34.1 ± 0.6	34.6 ± 1.0	34.6 ± 0.1	n.s.	n.s.	n.s.
Σ Monosaturated[2]	41.8 ± 0.8	41.4 ± 0.9	40.0 ± 0.3	40.1 ± 0.9	n.s.	n.s.	n.s.
Σ n-6PUFA[3]	6.6 ± 0.3	7.5 ± 0.3	7.3 ± 0.3	8.0 ± 0.4	n.s.	n.s.	n.s.
Σ n-3PUFA[4]	13.2 ± 1.9	14.9 ± 0.9	14.1 ± 0.5	14.2 ± 1.4	n.s.	n.s.	n.s.
(n-3+n-6) PUFA	19.8 ± 2.3	22.4 ± 0.7	21.4 ± 0.7	22.3 ± 1.1	n.s.	n.s.	n.s.
Total n-3 LC-PUFA[5]	11.8 ± 1.0	13.2 ± 0.5	13.4 ± 1.1	12.5 ± 0.7	n.s.	n.s.	n.s.
Kidney							
20:4n-6 (ARA)	2.6 ± 0.8	2.0 ± 0.2	2.4 ± 0.1	2.4 ± 0.2	n.s.	n.s.	n.s.
20:5n-3 (EPA)	10.3 ± 1.0	9.8 ± 0.3	9.6 ± 0.2	10.2 ± 0.3	n.s.	n.s.	n.s.
22:6n-3 (DHA)	17.1 ± 0.4	17.3 ± 1.1	17.9 ± 0.5	18.1 ± 0.4	n.s.	n.s.	n.s.
Σ Saturated[1]	32.1 ± 1.0	31.3 ± 0.6	31.8 ± 0.4	31.3 ± 0.5	n.s.	n.s.	n.s.
Σ Monosaturated[2]	23.7 ± 2.6	25.4 ± 1.3	23.7 ± 0.4	23.8 ± 0.7	n.s.	n.s.	n.s.
Σ n-6PUFA[3]	9.8 ± 0.7	9.3 ± 0.1	9.6 ± 0.2	9.5 ± 0.1	n.s.	n.s.	n.s.
Σ n-3PUFA[4]	31.3 ± 1.0	31.3 ± 0.6	31.9 ± 0.8	32.9 ± 0.8	n.s.	n.s.	n.s.
(n-3+n-6) PUFA	41.1 ± 1.7	40.6 ± 0.7	41.5 ± 0.6	42.4 ± 0.8	n.s.	n.s.	n.s.
Total n-3 LC-PUFA[5]	29.6 ± 1.0	29.3 ± 0.9	30.2 ± 1.4	29.5 ± 1.2	n.s.	n.s.	n.s.
Heart							
20:4n-6 (ARA)	3.3 ± 0.2	3.1 ± 0.2	3.2 ± 0.3	3.5 ± 0.1	n.s.	n.s.	n.s.
20:5n-3 (EPA)	9.1 ± 0.1	8.7 ± 0.5	9.1 ± 0.1	9.1 ± 0.1	n.s.	n.s.	n.s.
22:6n-3 (DHA)	27.7 ± 1.3	27.4 ± 1.9	27.8 ± 1.0	28.3 ± 1.3	n.s.	n.s.	n.s.
Σ Saturated[1]	30.4 ± 0.5	29.9 ± 0.4	30.4 ± 0.3	30.1 ± 0.1	n.s.	n.s.	n.s.
Σ Monosaturated[2]	16.7 ± 1.3	17.7 ± 1.3	16.6 ± 1.3	16.1 ± 0.9	n.s.	n.s.	n.s.
Σ n-6PUFA[3]	9.9 ± 0.1	9.8 ± 0.1	9.8 ± 0.3	10.2 ± 0.1	n.s.	n.s.	n.s.
Σ n-3PUFA[4]	39.9 ± 1.0	39.4 ± 1.9	40.1 ± 1.0	40.5 ± 1.1	n.s.	n.s.	n.s.
(n-3+n-6) PUFA	49.8 ± 1.	49.3 ± 1.9	49.9 ± 1.1	50.7 ± 1.1	n.s.	n.s.	n.s.
Total n-3 LC-PUFA[5]	39.1 ± 1.5	38.5 ± 1.3	39.2 ± 1.8	39.7 ± 2.0	n.s.	n.s.	n.s.

Data are means ± SD. Data expressed as means of three technical replicates per batch of tissue.[1]Includes 14:0, 15:0, 16:0, 17:0, 18:0 and 20:0.[2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 16:1n-7, 18:1n-5, 18:1n-9, 18:1n-7, 20:1n-7, 20:1n-9, 20:1n-5 and 22:1n-11.[3]Includes 18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3. 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6m-3. [5]LC-PUFA, long-chain polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3). DI, diet; DE, density; DI*DE, interaction diet-density. n.s. not significant.

Chapter 7

General discussion

It has been the aim of the present Thesis to investigate the origin and development of systemic granulomatosis in farmed meagre. Results have highlighted the role of oxidative stress as a trigger in the appearance of the disease, without implication of pathogens (Figure 7.1). Besides, the implication of nutrients in the disease has also been evaluated. In the following paragraph, results obtained in the several sections are discussed overall.

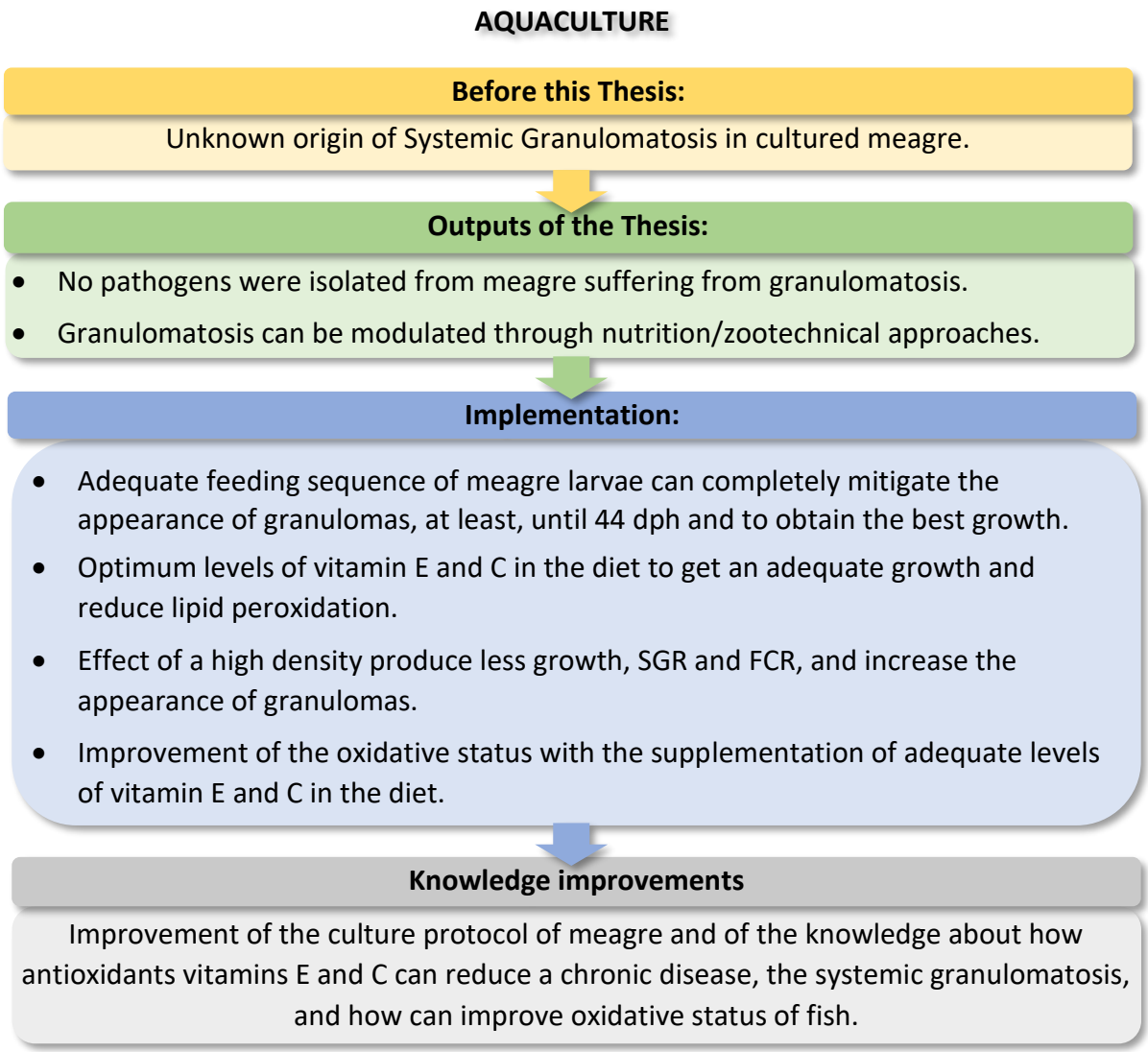


Figure 7.1. Scheme showing how this Thesis has granted new knowledge to improve health and production of meagre (*Argyrosomus regius*).

Is the *Artemia* necessary in the feeding sequence of meagre larvae?

There is still no artificial formulated feed that can completely substitute *Artemia* in the hatcheries of teleost larvae. Therefore, *Artemia* remains essential during the first stages of life in most of marine fish species (Kolkovski et al., 2004) due to its high nutritional quality and ease of use (Sorgeloos et al., 1986). In the present study the lack of enriched *Artemia* in the feeding sequence of meagre larvae reduced weight, length, SGR and survival, compared with those larvae that were fed with enriched rotifers and *Artemia* (Chapter 4). Other authors have obtained similar results. For instance, Fernández-Palacios et al. (2009b) observed that meagre larvae were significantly larger, and the survival was higher when co-fed with *Artemia* instead of rotifers only. When larvae grow, not only the range of accessible particle size increases, but also the ingested prey needs to supply adequate energy. The lack of *Artemia* in the feeding sequence lead to poor survival and growth probably because of a high energy cost/benefit related to the small size of the prey, indicating that it is necessary an intermediate feed between rotifers and microdiet. Moreover, the lack of *Artemia* in the feeding sequence had an impact in the appearance of systemic granulomatosis. Larvae fed rotifers only, had significantly higher percentage of granulomas (15.7 – 10.7 %) compared with those larvae fed with rotifers and *Artemia* (0.7 – 0.0 %) in the feeding sequence. The inclusion of *Artemia* in the feeding sequence and adequate enrichment can prevent the appearance of granulomas in meagre larvae of 30 dph, being the period before feeding with microdiet, a critical point to the development of systemic granulomatosis in this species.

Therefore, the results of Chapter 4 suggest that meagre can be weaned from rotifer to an inert food from 20 dph (12.8-14.8 % survival), but a co-feeding period with *Artemia* implies higher survival and growth of the larvae, as well as reduction in the presence of granulomas.

Can a high culture density trigger the development of systemic granulomatosis in on-growing meagre? Can it influence the oxidative status and growth parameters of the fish?

In the present Thesis, juvenile meagre were stocked at two different densities (6.20-3.20 kg m^{-3}) and were fed with different levels of ASA (100 to 3,200 mg kg^{-1}). The dietary supplementation with different levels of antioxidant vitamins can modulate the appearance of granulomas (Chapter 6). In this sense, the presence of granulomas was reduced with the addition of 3,200 mg kg^{-1} of ASA in the feeds. Nevertheless, when juvenile meagre were reared under a higher density (6.20 kg m^{-3}) the ASA supplementation cannot reduce the lipid peroxidation probably due to a higher stress, resulting in a higher presence of granulomas than fish fed the same diet but farmed at low density (3.20 kg m^{-3}). The stock density can disrupt the balance between the production and the removal of ROS, increasing the MDA content. Indeed, in the present Thesis the MDA content has been correlated with the incidence of granulomas (Chapter 4 and 5).

A higher lipid peroxidation, as indicated by TBARS values, was observed in those fish reared at a higher stock density (6.20 kg m^{-3}), , similarly as reported in other species such as Atlantic salmon (Liu et al., 2016a) or turbot (Jia et al., 2016). Under oxidative stress, lipid peroxidation has been reported to be a major contributor to the loss of cell function (Storey, 1996). It has been reported that the balance between the production of ROS and the antioxidant system can be disturbed by the stock density (Sahin et al., 2014; Andrade et al., 2015). TBARS have been suggested as a biomarker of oxidative damage (Parvez and Raisuddin, 2005), the higher stocking density induced a higher stress, resulting in an increase of lipid peroxidation and cell degradation.

The stocking density can also affect growth parameters. In this study juvenile meagre reared at a high stock density (6.20 kg m^{-3}) had significantly lower growth, SGR and FCR than those fish fed the same diets but farmed at 3.20 kg m^{-3}. In agreement with these results, the negative effect of high stocking densities has also been observed in other fish species, such as rainbow trout (Procarione et al., 1999), Dover sole (*Solea solea*; Schram et al., 2006), Atlantic cod (*Gadus morhua* L.; Lambert and Dutil, 2001) and Amur sturgeon (*Acipenser schrenckii*; Li et al., 2012). Moreover, it is known that the high stocking densities lead to a stressful condition which translates into an elevation of cortisol levels (Wuertz et al., 2006). For instance, red porgy reared at 20 kg m^{-3} displayed

higher plasma cortisol levels than fish stocked at 7 kg m^{-3} (Rotllant et al., 1997). When the levels of cortisol are elevated in plasma, a variety of secondary physiological responses take place, including an increase in the metabolic rate (Lankford et al., 2005). Indeed, stress reduces energy intake and increase energy utilisation, which indirectly reduces growth through a negative effect on energy balance (Leatherland and Cho, 1985; Ellis et al., 2002; Huntingford et al., 2006). Moreover, during a stressful period the secretion of growth hormones could be suppressed (Farbridge and Leatherland, 1992), which in turn can reduce growth. The results of the present study suggest that the high stock density is a stressful factor that can reduce growth, increase the oxidative stress, lipid peroxidation and can affect to the appearance of granulomas.

Do vitamins E and C affect the growth of meagre?

The inclusion of different dietary levels of α-TOH (40, 200, 400 and 800 mg kg^{-1}) and ASA (100, 500, 1,000 and 2,000 mg kg^{-1}) did not affect growth of meagre larvae (from 30 to 44 dph; Chapter 5). Limited information is available about the requirements of α-TOH and ASA in meagre larvae. According to the NRC (2011) requirements for α-TOH and ASA for juvenile fish are 30 and 50 mg kg^{-1}, respectively. The requirements for normal growth in several larvae fish species are 12-45 mg kg^{-1} of ASA (Boonyaratpalin et al., 1994; Merchie et al., 1997; Guillou-Coustans et al., 1998) and 30-100 mg kg^{-1} of α-TOH (Watanabe et al., 1981; Wilson et al., 1984; Hamre and Lie, 1995; Peng and Gatlin, 2009). According to these studies, low dietary levels of ASA and α-TOH are necessary for normal growth. In agreement, the results of the present Thesis suggest that low supplementation of α-TOH and ASA (40 and 100 mg kg^{-1}, respectively) probably fulfilled the requirement for normal growth.

In juvenile meagre fed the experimental diets for 13 weeks (Initial mean weight 15.75 ± 0.56 g; Chapter 6), the addition of α-TOH and ASA (300 mg kg^{-1} and 100-3,200 mg kg^{-1}, respectively) did not affect growth parameters. Only a slight increase in final weight (albeit not significant) was observed when ASA was added in higher concentration (1,200-3,200 mg kg^{-1}, average final weight of 96 g) compared with the other diets with less ASA (100-600 mg kg^{-1}, average final weight of 94 g), denoting that a longer experimental period may be necessary in order to see an effect of this vitamin on growth.

In the case of on-growing meagre (Initial mean weight 79.3 ± 0.5 g; Chapter 3) increasing the dietary levels of α-TOH (300 mg kg^{-1}) and ASA (70 mg kg^{-1}) seemed to improve final weight in meagre fed the experimental diets for 15 weeks, similarly to what has been observed in other fish species (Gao et al., 2012; Gao.et al., 2013; Gao et al., 2014; Chen et al., 2015; Rodriguez-Lozano et al., 2017). Conversely, the supplementation of α-TOH and ASA in the diet did not affect growth performance in other fish species (Tocher et al., 2002; Ai et al., 2006).

In the present Thesis, the supplementation of high levels of α-TOH and ASA does not seem to not have a strong influence in growth performance, being enough a supplementation of 40 mg kg^{-1} and 100 mg kg^{-1} in larvae, 300 and 100 mg kg^{-1} in juveniles and 300 and 70 mg kg^{-1} in on-growing meagre, respectively, to ensure adequate fish growth performance

Are vitamin E and C effective antioxidants? What about Zn, Mn and Se?

The antioxidant effect to the minerals Zn, Mn and Se has been studied in several fish species (De Rosa et al., 1980; Felton et al., 1996; Ho and Ames, 2002; Biller-Takahashi et al., 2015; Silva-Brito et al., 2016). The supplementation of 40 mg kg^{-1} of Mn, 200 mg kg^{-1} of Zn and 1.5 mg kg^{-1} of Se, did not affect the lipid peroxidation or gene expression of antioxidants enzymes (Chapter 6). The gene expression of *sod* and *gpx* was slightly increased in fish fed supplemented with these minerals. These results suggest that the supplementation of Zn, Mn and Se did not have an antioxidant effect in juvenile meagre and might indicate that a higher supplementation or of other antioxidants vitamins is needed, as observed in other fish species, where a synergism between vitamin and minerals could be observed (Poston et al., 1976; Bell and Cowey, 1985).

The protective effect of α-TOH and ASA was observed both in meagre larvae and on-growing juveniles in this Thesis. Meagre larvae fed a microdiet supplemented with α-TOH (800 mg kg^{-1}) and ASA (2,000 mg kg^{-1}) had lower TBARS content, what indicates less lipid peroxidation compared with those larvae fed with lower dietary levels of these vitamins. In general terms, larvae microdiets had high levels of n-3 LC-PUFA, and when antioxidant vitamins are not included in adequate concentration an imbalance between

prooxidants and antioxidants occurs, leading to oxidative stress. Similarly results, were observed in sea bass, where increasing levels of ASA and α-TOH, up to 1,500 and 3,600 mg kg^{-1} proved to be successful in compensating the effect of lipid oxidation and reducing TBARS (Betancor et al., 2012).

In on-growing meagre the supplementation of α-TOH and ASA (300 and 1,200 – 3,200 mg kg^{-1}, respectively) also reduced TBARS content in all analysed organs (liver, kidney and heart). ASA promotes a sparing effect on α-TOH (Tappel, 1972; Shiau and Hsu, 2002) and oxidized α-TOH could be regenerated to its reduced form by ascorbate. This sparing action has been suggested is some fish species (Ortuño et al., 2001; Mourente et al., 2007; Yildirim-Aksoy et al., 2008; Hamre, 2011; Betancor et al., 2012) having an influence in growth, tissue composition or immune response. Probably low addition of α-TOH in the diet increases the necessity of ASA for its recycling, impeding the use of these vitamins for other functions such as growth or collagen formation (Ai et al., 2006; Belin et al., 2010; Zhou et al., 2012; Darias et al., 2011; Gao et al., 2014). This suggests that it is necessary to balance the supplementation of both vitamins to prevent oxidative stress.

The dietary α-TOH and ASA regulated the expression of the antioxidant enzymes *sod*, *gpx* and *cat*, although the number of transcripts were influenced by the organ analysed. The supplementation of 300 mg kg^{-1} α-TOH and 3,200 mg kg^{-1} ASA on juvenile meagre (from 15.8 g to 94 g) increased gene expression levels of *sod* and *gpx* in liver and heart whereas the expression of *cat* was also up-regulated in liver and kidney (Chapter 6). The expression of *cat* was also affected in on-growing meagre (from 79.3 to 265 g), being significantly higher in fish fed the diet supplemented with the highest level of α-TOH, ASA and vitamin K (450, 230 and 23 mg kg^{-1}, respectively) compared with the control (150 mg kg^{-1} E, 20 mg kg^{-1} C and no vitamin K) (Chapter 3). The expression levels of *sod* and *gpx* in liver only showed a tendency to increase with high levels of vitamins in the diet, albeit not significantly.

What causes systemic granulomatosis in cultured meagre in the Canary Islands?

The systemic granulomatosis was not associated with any infectious agent in this Thesis. Several specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stains), isolation in culture media (blood agar, tryptone soya agar and Lowenstein-Jensen) and a PCR for Nocardia were performed, being all of them negative, what discarded a possible infectious origin and reinforced the hypothesis of a nutritional origin of the disease. Curiously, granulomas were observed in very early life stages of meagre larvae (10-30 dph) while in adults this disease can affect almost the total of the population. Different stages of development of the granulomas could be observed during the life cycle of meagre. At initial stages, granulomas were observed as isolated and irregular aggregated of macrophages and some lymphocytes that later were forming concentric layers. Then, the aggregates progressively lead to a necrotic centre with an external layer of fibrocytes. These stages of development were observed both in meagre juveniles and larvae. Final stages of development were only observed in juvenile fish only, in which the granulomas were completely composed of laminar material.

As observed in the present Thesis, granulomas can first appear in very early stages of life of meagre larvae (20 dph), but the appearance of them can be avoided in this stage using an adequate feeding sequence protocol (Chapter 4). In this study differences in the appearance of granulomas were found in meagre larvae (2-30 dph) fed under different feeding sequences. The lack of enriched *Artemia* in the feeding sequence increased the percentage of granulomas, being affected after 30 dph a 10.7-15.3 %, depending on the enrichment, compared with larvae fed with enriched *Artemia* and rotifer, where the incidence was 0-0.7 %. The analysed content of α-TOH (368-179.9 mg kg^{-1}) and ASA (279.8-287.5 mg kg^{-1}) in rotifers was lower than in *Artemia* (410.7- 310.1 and 1037.4-1033 mg kg^{-1}, respectively). Moreover, α-TOH was also influenced by the enrichment media, being higher the concentration in the diets enriched with Easy DHA Selco compared with Ori-Green enrichment. The composition of rotifer and *Artemia* has been previously studied by Meeren et al. (2008) being the level of α-TOH slightly lower in rotifer but those of ASA clearly lower (220.1 µg g^{-1}) compared with *Artemia* (530.6 µg g^{-1}), similarly to what was observed in the present study. Therefore, the results of this study showed that a combination of enriched *Artemia* and rotifers greatly decreased the

incidence of granulomas, not finding any at 30 dph and a 0.7-0.0 % at dph, depending on the enrichment media.

Following the best feeding sequence (RAS, Chapter 4), another experiment with meagre larvae (30-44 dph) was carried out using microdiets with different levels of α-TOH and ASA (40/100, 400/1000, 200/500, 800/2000 mg kg^{-1}, α-TOH and ASA respectively). A high correlation was observed between the the incidence of granulomas and the dietary levels of α-TOH ($y = -0.0079x + 6.3987$, $R^2 = 0.9056$) and ASA ($y = -0.0032x + 6.3987$, $R^2 = 0.9056$) . Granulomas were only observed when low levels of ASA and α-TOH were added to the diet (from 40/100 to 400/1000 mg kg^{-1}), and no granulomas were observed after 44 dph only in larvae fed the diet with higher amount of these vitamins (800/2,000 mg kg^{-1}). Thus, the decrease in the incidence of systemic granulomatosis observed in the present study, when high levels of ASA and α-TOH were employed, could be due to the protective effect of these two antioxidant nutrients and the decreased of lipid oxidation as indicates TBARS values, which were also correlated with the presence of granulomas (R^2=0.892, y=0.0446x+0.0756).

In on-growing meagre (from 15.8 g to 94 g) the supplementation with 300 mg kg^{-1} α-TOH and 1,200-3,200 mg kg^{-1} ASA significantly reduced the percentage of fish with granulomas in any tissue and in liver, which was the main affected organ. Also, the supplementation with 450 mg kg^{-1} and 230 mg kg^{-1} of α-TOH and ASA respectively, reduced the percentage of granulomas in liver and heart of juvenile fish (from 79.3 to 265 g) and also showed a tendency to decrease the severity in kidney and liver compared with the control group. This, in juvenile meagre the appearance of systemic granulomatosis can be reduced with the supplementation of high levels of α-TOH and ASA in the diet.

It has been observed that granulomas can begin to appear in meagre larvae of 20 dph, but these could be avoided with the correct feeding sequence and supplementation, almost until 44 dph. The incidence of granulomatosis in juvenile meagre is high and the supplementation of α-TOH and ASA in the diet can delay and/or avoid the appearance of new granulomas but the existing granulomas cannot be eliminated, being necessary to act at earlier stages. The results show that the combination of high dietary content of antioxidant vitamins α-TOH and ASA can have an influence on the incidence of the granulomatosis in meagre, which suggests that this pathology could be mediated by nutritional factors. It has been showed that the systemic granulomatosis can first appear at 20 dph, being the first stages of life a critical point where it is necessary to act. It is

pivotal to continue studying the nutritional deficiencies in these life stages, in order to prevent the appearance of the disease, given that in juvenile and in adults the prevalence is very high, and the granulomas cannot be eliminated.

How do the antioxidant vitamins E and C affect the development of systemic granulomatosis in meagre?

Limited information is available on the effect of antioxidant vitamins in the formation of granulomas. Previously, this disease has been associated with a nutritional imbalance in other species. Paperna et al. (1980) observed granulomas with inflammatory response and necrotic lesions, caused by a deficiency of ASA in the diet in sea bream. Tixerant et al. (1984) were the first to link a so called Granulomatous Syndrome observed on farmed turbot to a disorder in tyrosine metabolism and later this was confirmed by Baudin-Laurencin et al. (1989) and Coustans et al. (1990). They reported that deficiency in ASA in turbot can be the cause of hypertyrosinaemia and of tissue tyrosine deposits, given that fact that ASA is involved in the catabolism of tyrosine and a deficiency of this nutrient could cause a precipitation of tyrosine. These crystal deposits could be able to induce local an inflammatory granulomatous response (Goldsmith, 1978). Furthermore, a deficiency of antioxidants in the diet can cause primary lesions in the tissues and these lesions could potentially lead to the development of granulomas. The antioxidant effect of ASA and α-TOH depends on the concentration of both in the diet, due to ascorbic acid is involved in α-tocopherol metabolism, reducing α-tocopheroxyl radicals and regenerating them to α-tocopherol. It has been observed by Betancor et al. (2012) that the increase of ASA from 1,800 to 3,600 mg kg^{-1} in European sea bass larvae, reduced the occurrence of muscular dystrophy, tissue TBARS and increases tissue contents in α-tocopherol, denoting its sparing effect over dietary α-TOH. The deficiency of α-TOH and ASA has been associated to structural deformities, internal haemorrhages, appearance of granulomas, muscle injuries, oxidative damage and reduction of growth and survival (Tixerant et al., 1984; Sau, et al., 2004; NRC, 2011; Zhou et al., 2012; Betancor et al., 2011; Xu et al., 2016; Pan et al., 2017).

Irregular aggregates of cells and granulomas were observed around the blood vessels during the macroscopic evaluation. Immunostaining for acting showed a layer of

action around granulomas, suggesting a possible origin of the granulomas in blood vessels. α-TOH and ASA are involved in the normal function of blood vessels, for instance ASA participates in the synthesis of collagen, an important protein used to generate blood vessels (Lim and Lovell, 1978; Nusgen et al., 2001). ASA and α-TOH are involved in the prevention of the endothelial dysfunction in humans, the dysfunction increasing the tendency for arterial blockage due to a blood clot, or thrombosis (Riitta et al., 2003; Marguerite et al., 2003). The appearance of granulomas inside and around of blood vessels suggests that their origin could be in the blood vessels and the antioxidant vitamins, apart from protecting from oxidative stress, exert a role on the formation of blood vessels and other components.

Is there a relation between oxidative status and immunity/inflammation genes expression?

In fish, *tnfα* has been described as a regulator and effector in innate and adaptive immune responses (Locksley et al., 2001), while *cox*-2 is a prostaglandin which plays a key role in inflammation (Ishikawa et al., 2007a,b) and innate immune response (Xu et al., 2008; Legler et al., 2010). The transcript copy number of *tnfα* and *cox*-2 was increased when fish diet was supplemented with 450 mg kg^{-1} α-TOH and 230 mg kg^{-1} ASA (Chapter 3). Indeed, in teleost the expression of *cox*-2 has been associated with an increase in the pro-inflammatory cytokine *tnfα* (Wang et al., 2016). In mammals, α-TOH and ASA supplementation inhibit nuclear factor-kB thus reducing *tnfα* and *cox*-2 mRNA levels (Cárcamo et al., 2002; Han et al., 2004; Huey et al., 2008; Lee et al., 2008; Nakamura and Omaye, 2009), which is opposite to the regulation observed in the present study. Little is known about the effect of ASA and α-TOH in the expression of *tnfα* and *cox*-2 in fish as most of the studies are focused in mammalians. These divergent results could suggest a different regulatory mechanism of *tnfα* and *cox*-2 in fish and in mammals.

In conclusion, as observed in the results the supplementation of α-TOH and ASA can reduce lipid peroxidation in on-growing juveniles and meagre larvae and improve the gene expression of antioxidant genes (*sod*, *cat* and *gpx*) and immune genes (*tnfα* and *cox*-2). The requirements of these vitamin in order to have an effective antioxidant effect depend on the meagre life stage, being higher in larvae and fingerling meagre, due to its higher growth and metabolic rate than in juvenile and adult fish.

Chapter 8

Conclusions

1.- The incidence of systemic granulomatosis is very high in juvenile and adult meagre, being the larval stage, a critical point to intervene.

2.- In on-growing meagre the incidence of granulomas was modulated when high addition of α-TOH, ASA and vitamin K was present in the diet (450, 230 and 23 or 100, 1,200 and 23 mg kg^{-1}, respectively).

3.- The supplementation in the diet of Mn, Zn and Se (40 mg kg^{-1}, 200 mg kg^{-1} and 1.5 mg kg^{-1}, respectively) did not have any effect on the incidence of granulomas in on-growing meagre.

4.- Dietary supplementation of ASA, α-TOH and vitamin K and minerals Zn, Mn and Se did not affect growth performance in on-growing meagre, what suggests that low addition of these vitamins (70 C and 300 E mg kg^{-1} or 100 C and 100 E mg kg^{-1}) and minerals (40 Mn, 200 Zn and 1.5 Se mg kg^{-1}) are necessary for normal growth.

5.- The first appearance of granulomas occurs at 20 dph when *Artemia* is not included in the feeding sequence.

6.- The appearance of granulomas could be avoided up to 44 dph when Easy DHA Selco was used as enrichment for both rotifers and *Artemia* and larvae where later fed a microdiet supplemented with 800 mg kg^{-1} of α-TOH and 2,000 mg kg^{-1} of ASA.

7.- The stock density can trigger the appearance of granulomas in meagre. Juvenile fish fed high levels of ASA (1,200 - 3,200 mg kg^{-1}) had lower percentage of granulomas at low density (3.2 kg m^{-3}), although at high density (6.2 kg m^{-3}) the appearance of granulomas could not be prevented through the diet.

8.- The culture stock density of meagre can affect growth parameters, as the increase of the initial density from 3.20 to 6.20 kg m^{-3} reduced final growth, SGR and FCR.

9.- A correlation between TBARS, the expression levels of antioxidant genes (catalase, superoxide dismutase and glutathione peroxidase) and dietary/tissue vitamin levels was observed which suggests that lipid peroxidation is involved in the appearance of systemic granulomatosis.

10.- Given that the initial stage granulomas have been localized surrounding blood vessels, this seems to indicate that the origin of the oxidative tissue damage is vascular.

Capítulo 9

Resumen en Español

9.1. Producción global de acuicultura

La acuicultura comprende una serie de actividades enfocadas a la cría y cultivo de especies acuáticas (peces, moluscos, crustáceos y plantas), cubriendo su ciclo biológico y con el objetivo de concentrar especies para alimentarlas, protegerlas de depredadores, controlar enfermedades, realizar selección genética y mejorar la producción. La acuicultura juega un papel crucial en los esfuerzos por erradicar el hambre y la malnutrición, proporcionando comidas ricas en proteínas, aceites esenciales, vitaminas y minerales a un gran sector de la población. Las capturas de pesca no han incrementado significativamente desde 1990´s (90-95 millones de toneladas), la sobreexplotación de las áreas de pesca tradicionales y las limitaciones debido a los cambios en las políticas y acuerdos de pesca en áreas internacionales han causada una restricción en la suplementación de productos marinos. La acuicultura ha sido responsable, debido a su gran crecimiento, de proporcionar desde un 7 % del pescado consumido en 1974, a un 26 % en 1994, un 39 % en 2004 y un 46% en 2016 (FAO, 2018).

En cuanto a la producción global de acuicultura, China permanece líder con 63.721.768 millones de toneladas de producción en 2016, un 4,5 % más alto que en 2015, seguida de Indonesia (16.616.002 millones de toneladas), India (5.703.002 millones de toneladas) y Vietnam (3.634.531 millones de toneladas). La producción de acuicultura de la Unión Europea en 2016 fue de 1,3 millones de toneladas, siendo España la principal productora con 283.831 toneladas (22 %), seguida de Reino Unido (194.492 toneladas, 15 %) y Francia (166.640 toneladas, 12.9%) (APROMAR, 2018). La producción de acuicultura en España ha estado principalmente enfocada a la dorada (*Sparus aurata*) y lubina (*Dicentrarchus labrax*). Sin embargo, la producción de estas especies ha saturado el mercado, con un decrecimiento del precio final del producto. El sector de la acuicultura de la lubina y dorada ha alcanzado su madurez y ha forzado a buscar nuevas especies alternativas que puedan cubrir otros nichos del mercado. En este sentido, los consumidores exhiben otros gustos y preferencias, por lo que el mercado necesita ser más diverso y abarcar una mayor variedad de productos procesados procedentes de estos animales acuáticos

9.2. Producción de corvina

La corvina (*Argyrosomus regius*) posee un gran potencial para la diversificación de la acuicultura en el Mediterráneo. La producción Mediterránea de corvina en 2017 fue de 7.934 toneladas, siendo un 17,2 % más alto que en 2016. El principal productor dentro de la Unión Europea fue Turquía (3.500 toneladas), seguido de España (2.298 toneladas) y Grecia (2.200 toneladas) (APROMAR, 2018). En cuanto a las diferentes especies, las principales producidas en España fueron el mejillón (*Mytilus sp.*; 215.855 toneladas), seguido de la lubina (23.445 toneladas) y la dorada (13.740 toneladas), con la corvina ocupando la octava plaza (2.298 toneladas).

La corvina es una especie con alto valor económico en algunas regiones de Europa, donde ha sido tradicionalmente consumida. Sin embargo, debido al reducido volumen de pesca y al reciente inicio de su producción en acuicultura, es poco conocida en muchos mercados. A pesar de ello, la corvina tiene un gran potencial para la diversificación de la acuicultura debido a la alta calidad del filete, buen sabor (Poli et al., 2003), rápido crecimiento y buenos ratios de conversión (0.9-1.2) (Jiménez et al., 2005; Duncan et al., 2013), así como la capacidad de crecer bien bajo un amplio rango de salinidades (5-45 g L^{-1}) (Márquez, 2010). Además, posee un filete con bajo contenido en grasa, incluso bajo condiciones de cultivo intensivo (Piccolo et al., 2008) y tiene buena capacidad para adaptarse a la cría en cautividad (El-Shebly et al., 2007).

9.3. Descripción general de la corvina

La corvina, *Argyrosomus regius*, es una especie Teleóstea perteneciente a la familia Sciaenidae dentro del orden Percomorphi (Perciformes), suborden Percoidei. Posee un cuerpo alargado y ligeramente comprimido de color gris-plateado, con una cabeza relativamente grande y una boca terminal ancha pero ligeramente oblicua (Cárdenas, 2010; Figura 9.4), siendo parecida a la lubina, pero de mayor tamaño. La cavidad de la boca es de color dorada y tiene dientes pequeños dispuestos en varias filas. Se trata de una especie corpulenta y muy ágil (Piccolo et al., 2008, Poli et al., 2001). Tiene escamas grandes y ctenoides en todo el cuerpo, excepto en algunas partes de la cabeza donde son pequeñas y cicloides. La línea lateral es bastante visible, debido a su intenso brillo. La aleta dorsal está dividida en dos partes por una peque hendidura, laparte delantera tiene 9-10 radios duros mientras que la trasera posee 1 duro y 26 radios blandos. Según a la FAO, 2005 la corvina

puede llegar a alcanzar los 2 metros de longitud y pesar más de 50 kg, aunque el individuo más grande que se ha capturado medía 2,3 metros de longitud y pesaba 103 kg (Quero y Vayne, 1987). Esta especie tiene características biológicas adecuadas para la comercialización en acuicultura (Jiménez et al., 2005; Piccolo et al., 2008). La corvina se distribuye a lo largo del Mediterráneo, el Mar Negro y las costas Atlánticas de Europa y el oeste de África (Chao, 1986; Haffray et al., 2012).

9.4. Retos del cultivo de la corvina

En los últimos años, la corvina y otras especies como la seriola (*Seriola dumerili*) o el halibut Atlántico (*Hippoglossus hippoglossus*) han sido identificadas como nuevas especies para la diversificación de la acuicultura mediterránea. Estas especies se seleccionaron en base a su potencial biológico y económico. Sin embargo, en el caso de la corvina se han identificado varios cuellos de botella que pueden afectar a su producción en la cría, crecimiento y reproducción de esta especie.

9.4.1. Cría de corvina

Inicialmente, los protocolos de cría de corvina se adaptaron de otras especies de peces. Sin embargo, en la actualidad, ya existen protocolos específicos para esta especie con tasas de supervivencia de larvas de entre el 15 y el 40% a los 30 días después de la eclosión (dph) y del 15% a los 60 dph (PLANACOR 2009). El canibalismo es una de las causas más importantes de mortalidad en las etapas iniciales de la vida de larvas de corvina, el cual se ve aumentado drásticamente después de 21 dph con la inclusión del alimento artificial.

9.4.2. Engorde

Actualmente no existe una dieta comercial específica ni para larvas ni para juveniles y adultos de corvina. Además, no se conocen todos los requisitos nutricionales de esta especie, lo que restringe su potencial de rápido crecimiento. De hecho, el uso de dietas no específicas para la corvina se relaciona con un crecimiento pobre y pérdidas económicas.

Los requerimientos mínimos de proteína para esta especie son alrededor del 50 % (Martínez-Llorens et al., 2011, Chatzifotis et al., 2012) siendo más altos que los de otros Sciaenidae (McGoogan y Gatlin, 1999; Lee et al., 2002, Turano et al., 2002, Pirozzi et al., 2010), mientras que el nivel de lípidos de las dietas es más bajo que el de otras especies carnívoras (aproximadamente el 17%). La información es limitada en cuanto a los requerimientos de vitaminas en corvina. En el estudio de El Kertaoui et al. (2017) se observó que las dietas con niveles altos α-tocoferol (α-TOH) y ácido ascórbico (ASA; 1,500 y 1,800 mg kg^{-1}, respectivamente) mejoraban el crecimiento y la protección contra el estrés oxidativo en larvas de corvina, lo que sugiere que los requisitos óptimos de estas vitaminas podrían ser más altos que el de otras especies con menor tasa de crecimiento. La deficiencia de vitaminas y micronutrientes en la dieta puede conducir a trastornos metabólicos y enfermedades debido a su influencia negativa en el sistema fisiológico en los peces (Percival, 1995; Watanabe et al., 1997; Lin y Shiau, 2005). Además, se ha estimado que los requerimientos de ácidos grasos poliinsaturados de cadena larga (LC-PUFA) omega 3 (n-3) en corvina, al menos, son del 2 % de materia seca de la dieta (Carvalho et al., 2019). Se ha sugerido que es necesario para juveniles de corvina una suplementación en la dieta de 451 mg kg^{-1} de α-TOH, lo que mejora el crecimiento, la calidad del pescado y la estabilidad de almacenamiento de esta vitamina (Rodriguez-Lozano et al., 2017). Además, una deficiencia o exceso de algunos de estos nutrientes puede deteriorar la salud de los peces.

9.4.3. Reproducción

En cautividad la corvina presenta disfunción reproductiva, es decir, es necesario el uso de hormonas gonadotrofinas (GnRH) para desencadenar el desove. Durante el periodo del 2005 al 2008 se desarrolló el Plan Nacional de Cría de la Corvina (PLANACOR) del proyecto JACUMAR, realizado por seis centros de I + D (IFAPA, LIMIA, ICCM, IRTA, IMIDA y UPV), y cuyo objetivo principal fue la producción experimental de larvas de corvina. Con este proyecto se consiguió obtener los primeros desoves de corvina en cautividad en 2006 (Grau et al., 2007, Duncan et al., 2007a, b). Mediante la inducción del desove con GnRH se obtuvieron fecundaciones de 498.141 huevos kg^{-1} de hembras, 59 millones de huevos fertilizados, 36 millones de larvas y supervivencias de entre el 10 al 63% después de 30 dph (Cárdenas et al., 2007). Dentro

del proyecto DIVERSIFY (2013-2018), se han llevado a cabo algunos estudios con el objetivo desarrollar protocolos de fertilización *in vitro* y estudiar la variabilidad genética de la cría comercial, así como las bases genéticas del crecimiento de la corvina (Mylonas et al., 2013b, 2016, 2017). En el estudio de Mylonas et al. (2013a) se observó que un método de inducción hormonal del desove con GnRHa (agonista de la hormona liberadora de gonadotropina), resultaba en una supervivencia larvaria del 73% del número total de huevos desovados, demostrando que se trata de un método eficaz y eficiente que conduce a una alta fecundidad y calidad de los huevos.

9.4.4. Salud en el cultivo de la corvina

La salud es uno de los aspectos mas importantes en la cría de peces y esto no es diferente en el cultivo intensivo de la corvina. Se han descrito en corvina enfermedades infecciósas causadas por trematodos (*Calceostoma* spp. y *Sciaenocotyle* spp.) (Duncan et al., 2008; Ternengo et al., 2010), nematodos (*Philometra* sp.) (Moravec et al., 2007), dinoflagelados (*Amyloodinium ocellatum*) (Soares et al., 2012), monogeneos (*Sciaenocotyle pancerii*, *Microcotyle pancerii*, *Benedenia sciaenae* y *Diplectanum sciaenae*) (Toksen et al., 2007; Merella et al., 2009; Quilichini et al., 2009; Ternengo et al., 2010; Andree et al., 2015) y bacterias (*Vibrio tapetis, Photobacterium damselae* subsp. *piscicida, Nocardia* spp.) (Bottari et al., 2009; Labella et al., 2010; Cardenas, 2011; Elkesh et al., 2013). Además, la mayoría de la población cultivada se encuentra afectada por la granulomatosis sistémica, tratándose de una patología con un gran impacto en el cultivo de la corvina (Ghittino et al., 2004). De hecho, esta enfermedad cursa de forma crónica, produciendo baja mortalidad pero con una gran prevalencia e intensidad, lo que hace que sea una de las causas que impiden la expansión de la producción. Pese a que la granulomatosis sistemica es una enferdad de etiología desconocida, se ha pensado que pueden ser dos las causas que la producen: deficienda/desquilibrio nutricional o un agentes infecciosos.

Por un lado, esta enfermead fue descrita por primera vez en dorada (Paperna et al., 1980) y posteriormente en rodaballo (Psetta maximaes) (Coustans et al., 1990) y la causa de su aparación se asoció con un desiquilibrio nutricional (vitamia C y B) (Ghittino et al., 2004). La granulomatosis sistemica esta caracterizada por la presencia de multiples granulomas en organos internos, que progresivamente van formando un centro necrótico

rodeado de una capa de celular epiteliales y macrofagos. Esta enfermedad afecta principalmente al hígado y riñón, donde es posible observar en estadios muy avanzados del desarrollo de la enfermedad nódulos macroscópicos de diferente tamaño. En los últimos estadios del desarrollo también se pueden observar estos nódulos en otros tejidos como en bazo, corazón, piel y ojos pudiendo llevar a producir exoftalmia y cataratas (Ghittino et al., 2004).

Por otro lado, hay determinados patógenos que pueden producir granulomas, como son *Mycobacterium* spp. y *Nocardia* sp. (Bowser, 2009; Labrie, 2008). La micobacteriosis es un tipo de granulomatosis sistemica que cursa de forma crónica y que afecta a diferentes especies marinas (Decostere et al., 2004). En peces esta enfermead esta causada principalmente por tres especies de este patogeno, las cuales son *Mycobacterium marinum*, *Mycobacterium fortuitum* y *Mycobacterium chelonae* (Frerichs, 1993). Se trata de bacterias imobiles, Grampositivas, aeróbicas y acido-resistentes (Kato-Meda et al., 2011). Los signos clínicos externos no son específicos e incluyen perdidas de escamas y ulceración de la piel, cambio de pigmentación, comportamiento anormal, inanición y ascitis (Gauthier y Rhodes, 2009). Los signos internos de la infección incluyen endurecimiento del bazo, riñón e hígado, y la aparición de nódulos blanco-grisaceos en órganos internos (Frerichs, 1993). Se pueden observar normalmente granulomas visceralas compuestos de una capsula dura de células epiteliales que rodean un centro necrótico, donde un gran número de bacilos acido-resistentes se encuentran concentrados (Toranzo et al., 2005).

La nocardiosis es una enfermedad que afecta tanto a peces de agua salada como dulce y es producida por actinomicetos del género Nocardia (Woo y Bruno, 2011). El primer caso de nocardiosis descrito en peces fue causado por *Nocardia asteroides* en tetra neón (*Hyphessobrycon innesi*) (Valdez y Conroy, 1963). En 2013, en el estudio de Elkesh et al. se describió por primera vez la presencia de Nocardia en una población mediterránea de corvina en Creta (Grecia). La nocardiosis ha sido considerada económicamente importante, debido a que es una enfermedad crónica que causa bajas mortalidades, pero que puede producir pérdidas importantes (Labrie et al., 2008). La nocardiosis puede ser malinterpretada y confundida por la enfermedad causada por micobacterias, ya que ambas poseen signos clínicos similares. La nocardiosis es una enfermedad producida por bacterias Grampositivas, aeróbicas y filamentosas (Brown-Elliott et al., 2006). Se trata de

una enfermedad sistémica que produce lesiones en la piel y órganos internos, formando estructuras típicas de granulomas (Wang et al., 2005).

Dado que diversos agentes infecciosos son capaces de producir granulomas en peces, es necesario llevar a cabo una serie de test para poder descartar un origen infeccioso de la enfermedad, como son los test microbiológicos (Rhodes et al., 2004; Pourahmad et al., 2009), histológicos, imunohistoquímicos (Chinabut, 1999; Noga, 2000) y moleculares (Puttinaowarat et al., 2002; Roth et al., 2003) para *Mycobacterium* sp. o en el caso de *Nocardia* sp. tambien serían necesarios test microbiológicos (Chen et al., 2000; Elkesh et al., 2011; Elkesh et al., 2013), histológicos, imunohistoquímicos (Labrie et al., 2008) y moleculares (Kono et al., 2001) (Itano et al., 2006).

9.4.5. Descubrir las posibles causas de la aparición de la granulomatosis sistemica en corvina

Los estudios sobre las posibles causas de la aparición de la granulomatosis sistemica en corvina son muy escasos, sin embargo, la ausencia de un agente infeccioso y la relación observada entre la aparición de esta enfermedad y la secuencia de alimentación en larvas de corvina refuerzan la hipótesis de un origen nutricional de la enfermedad (Cotou et al., 2016). Se ha observado la presencia de granulomatosis visceral en rodaballos alimentados con dietas deficientes en ASA (Messager et al., 1986). En el estudio de Coustans et al. (1990) se confirmó la realación entre la deficiencia de ASA y la aparición de la granulomatosis visceral en rodaballo, y además, se observó que la condición patológica se agravaba con una hipovitaminosis del complejo B. En dorada, la deficiencia de ASA también ha sido responsable de la aparición de granulomas en el riñón (Alexis et al., 1997). En estos estudios ASA parece tener un papel importante en la aparición de la enfermedad. Esta vitamina también participa en el metabolismo de α-TOH, reduciendo los radicales α-tocopheroxyl y regenerándolos de nuevo a α-TOH (Niki et al., 1985). En consecuencia, los niveles óptimos de α-TOH en la dieta están influenciados por los niveles de ASA (Hamre et al., 1997; Chen et al., 2004; Atalah et al., 2010). El desequilibrio entre prooxidantes y antioxidantes puede llevar a un estado de estrés oxidativo (Rando, 2002) y conducir a diferentes enfermedades (Cowey et al., 1984; Lewis-McCrea y Lall, 2007; Betancor et al., 2012), como podría ser la granulomatosis sistémica.

El estrés oxidativo se produce debido a la acumulación de especies reactivas al oxígeno "Reactive oxygen species" (ROS), las cuales se forman continuamente como producto de diferentes rutas metabólicas en todos los organismos. Los organismos aeróbicos multicelulares requieren de oxígeno (O_2), siendo este susceptible de la formación de radicales debido a su estructura electrónica. A bajas concentraciones, ROS pueden ser beneficiosas o incluso indispensables para determinados procesos, como es la defensa contra microorganismos, ya que contribuyen a la actividad fagocitaria bactericida. Sin embargo, cuando hay un desequilibrio entre la eliminación de ROS y su formación, se puede producir un estado de estrés oxidativo (Nita, et al., 2016). El estrés oxidativo puede producir la oxidación de diferentes constituyentes celulares como son los lípidos, proteínas o ADN, causando alteraciones que pueden ocasionar diferentes daños celulares, llegando incluso a su muerte (Halliwell y Gutteridge, 1995).

La acción de las ROS puede ser inhibida o retrasada mediante los antioxidantes, los cuales evitan la iniciación o la propagación de las reacciones en cadena de oxidación (Figura 9.8) (Pokorny y Korczak, 2001). Los antioxidantes pueden reaccionar con los radicales libre y neutralizarlos mediante la donación de uno de sus electrones (Halliwell y Gutteridge, 1990). Para prevenir el daño de células y tejidos, hay una variedad de componentes antioxidantes que pueden actuar contra los radicales libres y neutralizarlos (Yu, 1994), estos se pueden clasificar en dos categorías, de origen endógeno y exógeno (Jacob, 1995). Los antioxidantes de origen endógeno o enzimáticos que se encuentran principalmente en peces son el superóxido dismutasa (SOD), glutatión peroxidasa (GPX), glutatión reductasa (GR) y catalasa (CAT) (Rudneva, 1997). Los antioxidantes de origen exógeno o no enzimáticos, son aquellos que no pueden ser sintetizado por el cuerpo y tienen que ser aportados necesariamente por la dieta, como son el caso de ASA y α-TOH.

Los antioxidantes exógenos o no enzimáticos no solo protegen los componentes de las células de posibles daños, sino que también juegan un papel importante en el crecimiento de los peces, control de la peroxidación de ácidos grasos insaturados, en la respuesta inmune y malformaciones (Putnam y Comben, 1987; Kumari y Sahoo, 2005; Zhou et al., 2012). Sin embargo, hay otros nutrientes que juegan un papel importante en el crecimiento y salud de los peces como son los ácidos grasos (Rodríguez et al., 1994; Izquierdo, 1996; Salhi et al., 1997; Izquierdo y Koven, 2011; Carvalho et al., 2018), la vitamina K (Phillips et al., 1963; Olson, 1999; Sheehan et al., 2001; Udagawa, 2006; Roy y Lall, 2007; Udagawa, 2008; Choi et al., 2016) y los minerales Manganeso (Hurley y

Keen, 1987; Cossarini-Dunier et al., 1988; Wang y Zhao, 1994; Maage et al., 2000; Zhang et al., 2016), Selenio (Felton et al., 1996; Arteel y Sies, 2001; Lall, 2002; Kohlmeier, 2003; Biller-Takahashi et al., 2015; Silva-Brito et al., 2016; Mansour et al., 2017;) y Zinc (Bray y Bettger, 1990; Eisler, 1993; Watanabe et al., 1997; Yamaguchi, 1998; Powell, 2000; Ovesen et al., 2001; Ho y Ames, 2002; Yamaguchi y Fukagawa, 2005).

La prevalencia de la granulomatosis sistémica es muy alta, llegando a ser del 100 % en adultos de corvina cultivados, sin embargo no ha podido ser asociada a ningún agente infeccioso, siendo un paso clave el estudio de la implicación de diferentes nutrientes sobre le desarrollo de esta enfermedad.

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9.5. Objetivos

Se ha propuesto a la corvina como un buen candidato para la diversificación de la acuicultura debido a sus buenas características biológicas. Sin embargo, el cultivo intensivo de esta especie se ve afectado por una enfermedad conocida como granulomatosis sistémica. Eventualmente, esta enfermedad se ha asociado a agentes infecciosos en corvinas cultivadas. Sin embargo, se ha hipotetizado que la granulomatosis sistémica también se puede producir por un desequilibrio nutricional, ya que en la gran mayoría de casos en los que se ha observado la enfermedad, no se ha detectado ningún agente infeccioso. Por lo tanto, parece que mediante el uso de dietas balanceadas, podría modularse la aparición de la granulomatosis sistémica en corvina, sin embargo, poco se sabe acerca de los requerimientos nutricionales de esta especie. Dado que la corvina es una especie de rápido crecimiento, se espera que los requerimientos nutricionales sean más altos que los de otras especies de peces teleósteos cultivadas. Por esta razón, los objetivos de esta Tesis fueron:

1.- Determinar que nutrientes en la dieta pueden modular la aparición de la granulomatosis sistémica. Se evaluó el papel de diferentes nutrientes sobre la aparición de la enfermedad.

1.1- *Vitaminas antioxidantes E (α-TOH) y C (ASA)*: α-TOH y ASA son potentes antioxidantes capaces de neutralizar especies reactivas al oxígeno. La deficiencia de estas vitaminas puede producir alteraciones, incluyendo la aparición de los granulomas. Se probó la adición de diferentes niveles de α-TOH y ASA y su efecto antioxidante tanto en larvas como en juveniles de corvina. Se evaluó la incidencia y severidad de la enfermedad, el efecto sobre la expresión de diferentes genes y el efecto sobre los parámetros de crecimiento.

1.2- *Otros nutrientes*: Con el fin de alcanzar este objetivo, se evaluó el efecto de la alimentación con dietas suplementadas con diferentes niveles de vitamina K, y de los minerales Se, Zn y Mn sobre la aparición de la granulomatosis sistémica en juveniles de corvina

2.- Determinar la primera aparición de la granulomatosis sistémica en larvas de corvina. Nunca antes se había estudiado cuando comenzaban a aparecer los granulomas

en larvas de corvina. Mediante la determinación de su primera aparición, es posible modular su incidencia en estadios de desarrollo más tardíos. Esto se ha estudiado mediante:

2.1- *Protocolo de alimentación*: Con el fin de prevenir la aparición de granulomas fue necesario establecer un protocolo de alimentación adecuado que evitará la aparición de la enfermedad. Para alcanzar este objetivo, se estudió el efecto de la ausencia de la *Artemia* en la secuencia de alimentación, alimentando las larvas de corvina directamente desde rotíferos a microdieta.

2.2- *Nutrientes en la dieta*: Se evaluó el efecto de diferentes medios de enriquecimiento comerciales, con diferentes perfiles de vitaminas, sobre la aparición de la granulomatosis sistémica.

3.- Evaluar el efecto de las condiciones de cultivo sobre la aparición de la granulomatosis sistémica en corvina. Las corvinas cultivadas se encuentran expuestas a factores de estrés que potencialmente podrían conducir a la aparición de granulomas. En esta Tesis se evaluó el efecto de la densidad sobre la aparición de esta enfermedad en juveniles de corvina.

9.6. Resúmenes de los experimentos

9.6.1. Capítulo 3: La combinación en la dieta de vitamina E, C y K afecta al crecimiento, actividad antioxidante y la incidencia de la granulomatosis sistémica en corvina (*Argyrosomus regius*).

La granulomatosis sistémica es una enfermedad con una alta morbilidad que afecta a la mayoría de las corvinas cultivadas. La imposibilidad de aislar un agente infeccioso ha hecho sugerir que esta enfermedad puede ser de origen nutricional. Con el objetivo de investigar el origen nutricional de esta enfermedad, se alimentaron juveniles de corvina durante 15 semanas con 6 dietas diferentes que contenían diferentes niveles de vitamina E y C y con adición y sin ella de vitamina K: Dieta 0 (premix basal, sin K, 150 mg kg^{-1} E, 20 mg kg^{-1}C), K (23 mg kg^{-1} K, 150 mg kg^{-1} E, 20 mg kg^{-1}C), EC (300 mg kg^{-1} E,70 mg kg^{-1}C), KEC (23 mg kg^{-1} K, 300 mg kg^{-1} E, 70 mg kg^{-1}C), EECC (450 mg kg^{-1} E, 230 mg kg^{-1}C) y KEECC (23 mg kg^{-1} K, 450 mg kg^{-1} E, 230 mg kg^{-1}C). Se observó que la dieta EC incrementó significativamente el crecimiento en términos de peso final y longitud. Los peces alimentados con los niveles más altos de vitamina E y C presentaron un menor porcentaje de granulomas en hígado y corazón, en comparación con aquellos peces alimentados con la dieta 0. Se observó una tendencia a una reducción de la severidad de los granulomas en hígado y riñón (principales órganos afectados) con el aumento dieta de vitamina E, C y la adición de vitamina K (de 1.83 dieta 0 a 1.3 dieta KEECC y de 0.91 dieta 0 a 0.39 dieta KEECC). En el hígado, la dieta KEECC aumento significativamente la expresión de la catalasa en comparación con la dieta 0. En el riñón, se incrementó significativamente la expresión de *tnfα* en corvinas alimentadas con las dietas EECC y KEECC. En el corazón, niveles bajos de vitamina E y C (300 y 70 mg kg^{-1}, respectivamente) aumentaron significativamente la expresión de superóxido dismutasa y glutatión peroxidasa, mientras que una alta adición de estas vitaminas aumentaba la expresión de *tnfα* y *cox-2* (0 o 23 mg kg^{-1} K, 450 mg kg^{-1} E y 230 mg kg^{-1} C, dieta EECC y KEECC). Los resultados muestran que la combinación de un alto contenido en la dieta de vitamina K y vitaminas antioxidantes E y C (23, 450 y 230 mg kg^{-1}, respectivamente) influyeron en la incidencia de la granulomatosis, sugiriendo que esta patología podría estar mediada por factores nutricionales.

9.6.2. Capítulo 4: La incidencia de la granulomatosis sistémica es modulada por la secuencia de alimentación y el tipo de enriquecimiento en larvas de corvina (*Argyrosomus regius*).

La granulomatosis sistémica es la enfermedad más frecuente en juveniles y adultos de corvina, pero no existen estudios sobre la primera aparición de estos granulomas en larvas. Con el fin de evaluar esto, se alimentaron larvas de corvina con cuatro secuencias de alimentación diferentes de la siguiente manera: RS y RO (rotífero enriquecido con Easy DHA Selco u Ori-Green de 3-30 dph, respectivamente), RAS y RAO (rotífero enriquecido con Easy DHA Selco u Ori-Green de 3-21 dph y *Artemia* enriquecida con Easy DHA Selco u Ori-Green de 12-30 dph, respectivamente). Las larvas de todos los tratamientos también se alimentaron con microdietas comerciales de 20-30 dph. A 30 dph el peso, la longitud, la tasa de crecimiento específica y la supervivencia fueron significativamente mayores en las larvas alimentadas con *Artemia*, independientemente del enriquecedor utilizado. La primera aparición microscópica de granulomas se observó en larvas de 20 dph alimentadas con RS y RO. A 30 dph, los valores de granulomas y sustancias reactivas al ácido tiobarbitúrico (TBARS) fueron significativamente más altos en larvas alimentadas con RS y RO que en las larvas alimentadas con RAS y RAO. Los resultados mostraron que la primera aparición de granulomas en larvas de corvina ocurre en 20 dph cuando estas fueron alimentadas únicamente con rotíferos y microdieta en la secuencia de alimentación. Sin embargo, la inclusión de *Artemia* en la secuencia de alimentación redujo la presencia de granulomas y la peroxidación lipídica, lo que refuerza la hipótesis de un origen nutricional de la granulomatosis sistémica.

9.6.3. Capítulo 5: La aparición de la granulomatosis sistémica es modulada por la suplementación de vitamina E y C en microdietas inertes de larvas de corvina (*Argyrosomus regius*).

En el anterior estudio se observó que las granulomatosis sistémica se puede prevenir en larvas de corvina con la secuencia de alimentación y enriquecedores adecuados. Por lo tanto, el control de esta enfermedad podría prevenirse a través de los componentes nutricionales de la dieta, siendo los antioxidantes la clave del éxito. Por este motivo, en el presente estudio, se alimentaron larvas de corvina de 30 dph con cinco microdietas experimentales isonitrogenadas e isolipídicas con diferentes niveles de vitamina E y C: C- (40 mg kg^{-1} E, 100 mg kg^{-1} C), C + (400 mg kg^{-1} E, 1,000 mg kg^{-1} C), Krill (400 mg kg^{-1} E, 1,000 mg kg^{-1} C y sustitución de aceite de pescado por aceite de krill), EC (200 mg kg^{-1} E, 500 mg kg^{-1} C) y EECC (800 mg kg^{-1} E, 2,000 mg kg^{-1} C). Antes de ser alimentadas con estas microdietas, las larvas se alimentaron conjuntamente con rotíferos y *Artemia* siguiendo el protocolo descrito en el anterior estudio en el que se evitó la aparición de granulomas hasta 30 dph. La sustitución del aceite de pescado por aceite de krill incrementó significativamente los niveles de ácido eicosapentaenoico (EPA, 16,6%) y ácido docosahexaenoico (DHA, 17,6%) en las larvas de corvina, incrementando el índice de peroxidación, lo que a su vez se tradujo en una mayor incidencia de granulomas. Aunque incluso con niveles bajos de vitamina E y C (40 mg kg^{-1} E, 100 mg kg^{-1} C; C-) se observó un crecimiento adecuado de las larvas, estos no fueron suficientes para prevenir la aparición de granulomas, requiriéndose niveles superiores de ambas vitaminas antioxidantes (800 mg kg^{-1} E y 2,000 mg kg^{-1} C) para poder mitigar la granulomatosis sistémica. Esta reducción fue simultánea con la disminución del contenido de TBARS en larvas, observándose una alta correlacionadas entre el contenido de TBARS y la presencia de granulomas ($R2 = 0.892$, $y = 0.0446x + 0.0756$). Se observó una fuerte correlación negativa entre los niveles de vitamina E ($y = -0.0098x + 11.174$, $R2 = 0.8766$, valor de $p = 0.019$, $r = -0.93$) y vitamina C ($y = -0.0022x + 6.4777$, $R2 = 0.9278$, valor de $p = 0.003$, $r = -0.96$) en la dieta y el porcentaje de larvas con granulomas. Los resultados mostraron que la aparición de la granulomatosis sistémica parece estar asociada al estado de peroxidación de las larvas, por lo que niveles altos de vitamina E y C (800 y 2,000 mg kg^{-1}, respectivamente; Dieta EECC), redujeron la peroxidación de lípidos e impidieron completamente la aparición de granulomas en larvas de corvina de 44 dph.

9.6.4. Capítulo 6: **Efecto de la densidad y la suplementación de la vitamina C y micronutrientes (Mn, Zn y Se) en el desarrollo de la granulomatosis sistémica en juveniles de corvina (*Argyrosomus regius*).**

La granulomatosis sistemática es una enfermedad crónica que afecta a la mayoría de las corvinas cultivadas, aunque el origen de la patología no está claro. Recientemente, el estrés oxidativo se ha asociado a la aparición de granulomas, pero poco se sabe acerca de los requisitos nutricionales de algunas vitaminas y minerales en esta especie. Además, en la acuicultura intensiva, los peces se cultivan a altas densidades y el hacinamiento puede producirles estrés. Desequilibrios nutricionales y altas densidades pueden aumentar el riesgo de sufrir estrés oxidativo y, por consiguiente, afectar la incidencia de granulomatosis. Para tratar de conocer mejor el origen nutricional de los granulomas y estudiar el efecto de la densidad de la población en la incidencia de la granulomatosis, se alimentaron juveniles de corvina con cinco dietas isolipídicas (16,7%) e isoproteicas (49,6%) de harina y aceite de pescado, a las que se les añadió diferentes niveles de vitamina C y minerales (Mn, Zn, Se), además de vitamina E y K (100 y 35 mg kg^{-1}, respectivamente): Dieta KEC (100 mg kg^{-1} C), Dieta KEC + Mn / Zn / Se (100 mg kg^{-1} C, 40 mg.kg^{-1} Mn, 200 mg kg^{-1} Zn, 1,5 mg kg^{-1} Se), dieta KECC (600 mg kg^{-1} C), dieta KECCC (1.200 mg kg^{-1} C), dieta KECCCC (3.200 mg kg^{-1} C). Todas las dietas se probaron con 100 peces / tanque a baja densidad (3,20 kg m^{-3}), pero además, las dietas KECC y KECCCC también se probaron a una densidad más alta (175 tanque de $peces^{-1}$; 6,20 kg m^{-3}). Los parámetros de crecimiento (peso final, SGR, FCR y K) solo se vieron afectados por la densidad de población, siendo menores cuando la densidad fue alta (6,20 kg m^{-3}). El porcentaje de peces con granulomas en cualquier tejido fue significativamente menor en los peces alimentados con dietas suplementadas con niveles altos de vitamina C (KECCC y KECCCC) a baja densidad (80 y 76%, respectivamente), en comparación con los peces alimentados con las otras dietas (87-90). %). Se observo un menor número de peces con granulomas en el hígado en peces alimentados con las dietas KECCC y KECCCC y cultivados a baja densidad, así como en peces alimentados con la dieta KECCCC con alta densidad. El contenido de TBARS se correlacionó con el porcentaje de granulomas en el hígado ($R2 = 0.9439$, $y = 0.003x-0.1242$) lo que denota la participación de un desequilibrio del estado oxidativo en la aparición de los granulomas. Además, los peces alimentados con la dieta KECCCC mostraron mayor expresión de *cat* en hígado y riñón, así como mayor expresión de *sod* y *gpx* en hígado y corazón. Los

resultados muestran que altos niveles de vitamina C (1,200-3,200 mg kg^{-1} C) y baja densidad de cultivo (3,20 kg m^{-3}) favorecen el crecimiento de los juveniles de corvina, reduce la peroxidación lipídica y disminuyendo la incidencia de granulomas, lo que confirma que esta patología se desencadena principalmente por la deficiencia de nutrientes antioxidantes, particularmente la vitamina C.

9.7. Conclusiones

1.- La incidencia de la granulomatosis sistémica es muy alta en juveniles y adultos de corvina, siendo la fase larvaria el punto crítico en el que intervenir.

2.- En juveniles de corvina la incidencia de granulomas se moduló cuando se añadió en la dieta niveles altos de α-TOH, ASA y vitamina K (450, 230 y 23 o 100, 1.200 y 23 mg kg^{-1}, respectivamente).

3.- La suplementación en la dieta de Mn, Zn y Se (40 mg kg^{-1}, 200 mg kg^{-1} y 1,5 mg kg^{-1}, respectivamente) no afecto a la incidencia de granulomas en juveniles de corvina.

4.- La suplementación en la dieta de ASA, α-TOH, vitamina K y de los minerales Zn, Mn y Se no afectó a los parámetro de crecimiento en juveniles de corvina, lo que sugiere que una baja adición de estas vitaminas (70 C y 300 E mg kg^{-1} o 100 C y 100 E mg kg^{-1}) y minerales (40 Mn, 200 Zn y 1,5 Se mg kg^{-1}) son necesarias para un crecimiento normal.

5.- La primera aparición de granulomas ocurre a los 20 dph cuando la *Artemia* no está incluida en la secuencia de alimentación.

6.- La aparición de granulomas se puede prevenir al menos hasta 44 dph cuando los rotíferos y *Artemia* de la secuencia de alimentación son enriquecidos con Easy DHA Selco y posteriormente las larvas son alimentadas con una microdieta suplementada con 800 mg kg^{-1} de α-TOH y 2.000 mg kg^{-1} de ASA.

7.- La densidad de cultivo puede afectar a la aparición de granulomas en corvina. Los juveniles de corvina alimentados con niveles altos de ASA (1.200 – 3.200 mg kg^{-1}), presentaron un menor porcentaje de peces afectados con granulomas cuando fueron cultivados a baja densidad (3,2 kg m^{-3}), ya que a altas densidades (6,2 kg m^{-3}) no se puedo reducir la aparición de granulomas mediante la dieta.

8.- La densidad de cultivo de la corvina puede afectar a los parámetros de crecimiento, el incremento de la densidad inicial desde 3,20 hasta 6,20 kg m^{-3} redujo el crecimiento final, SGR y FCR.

9.- Se observó una correlación entre los niveles de TBARS, la expresión de genes antioxidantes (catalasa, superóxido dismutasa y glutatión peroxidasa) y los niveles en la

dieta/tejidos de vitaminas antioxidantes, lo que sugiere que la peroxidación de lípidos está relacionada con la aparición de la granulomatosis sistémica.

10.- Dado que los estadios iniciales de los granulomas se han observado alrededor de vasos sanguíneos, esto parece indicar que el origen de los granulomas podría deberse a un daño oxidativo en los tejidos vasculares.

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