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

Running title: Vitamins E, C and/or K prevent granulomatosis in meagre.

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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 dietary content of vitamin K and antioxidant vitamins E and C (23, 450 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.

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. (2012) 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.

2. Materials and methods

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 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 1.

Table 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 (%)						
14:0	1.1	1.1	1.5	1.1	0.9	1.0
16:0	2.8	2.9	3.1	2.8	2.7	2.7
18:0	0.4	0.4	0.4	0.4	0.4	0.3
Total saturated[6]	4.4	4.5	5.4	4.4	3.7	4.3
18:1n-9	2.5	2.1	2.5	2.1	2.0	2.0
Total monosaturated[7]	5.3	5.0	5.7	4.9	4.3	4.8
18:2n-6	1.2	1.1	1.2	1.1	0.9	1.1
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
18:3n-3	0.3	0.3	0.3	0.3	0.2	0.3
20:5n-3	1.3	1.4	1.4	1.3	2.2	1.4
22:5n-3	0.1	0.1	0.1	0.1	0.1	0.1
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 15:0, 17:0 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, 20:1n-9, 20:1n-7 and 20:1n-5; [8]Includes 18:3n-6, 20:2n-6, 20:3n-6 and 22:4n-6; [9]Includes 16:3n-3, 16:4n-3, 18:4n-3, 20:3n-3 and 20:4n-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.

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.

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.

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.

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).

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.

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 1.

Supplementary Table 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

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.

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.

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 2).

Supplementary Table 2. Sequences for real-time quantitative-PCR forward and reverse primers (5′–3′). The data include sequences. amplicon sizes and annealing temperatures (Ta).

Target	Primer 5´-3´	Fragment size (bp)	Ta (°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.

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 2).

2.10. 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. Results

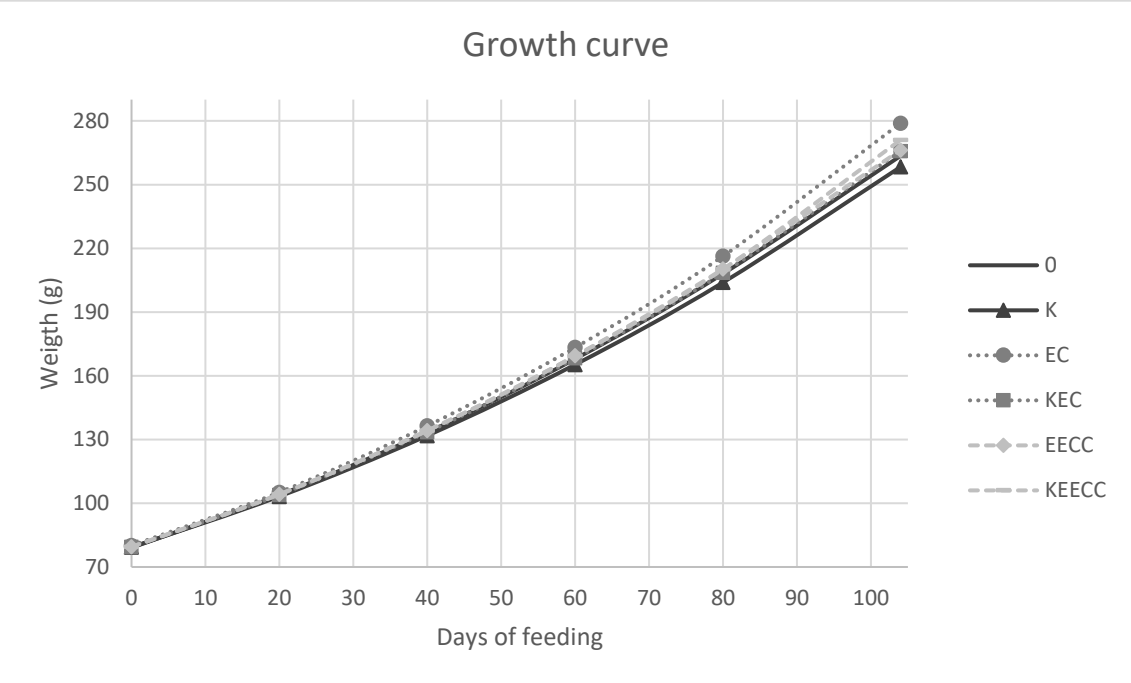
3.1. Growth performance

Inclusion of different levels of dietary vitamins E, and C with or without vitamin K affected meagre final weight and length (Table 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 1). 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 2). The feed conversion ratio (FCR) was good in all dietary treatments (0.83-0.87) but without significant differences.

Table 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.
* $p < 0.05$.
** $p < 0.01$.



Supplementary Figure 1. Growth curve of Meagre fed diets with different levels of vitamin E, C and K during 104 days.

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, 4 and 5). Similarly, no differences were observed in protein, ash or dry weight among fish fed the experimental dietary treatments (Supplementary Table 3). The fatty acid profile of the tissues of fish reflected the dietary fatty acid content (Table 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, 4 and 5, respectively). All the other fatty acids were similarly distributed in the three tissues.

Table 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 monounsaturated[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 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 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.

Supplementary Table 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.

	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.

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. 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 1). At initial stages, granulomas were observed as isolated and irregular aggregates of macrophages and some lymphocytes (Figure 1a) that later formed concentric layers (Figure 1b). These aggregates progressively led to a necrotic centre with an external layer of fibrocytes (Figure 1c). In the final stages the granuloma was completely composed of laminar material, especially observed in heart (Figure 1d).

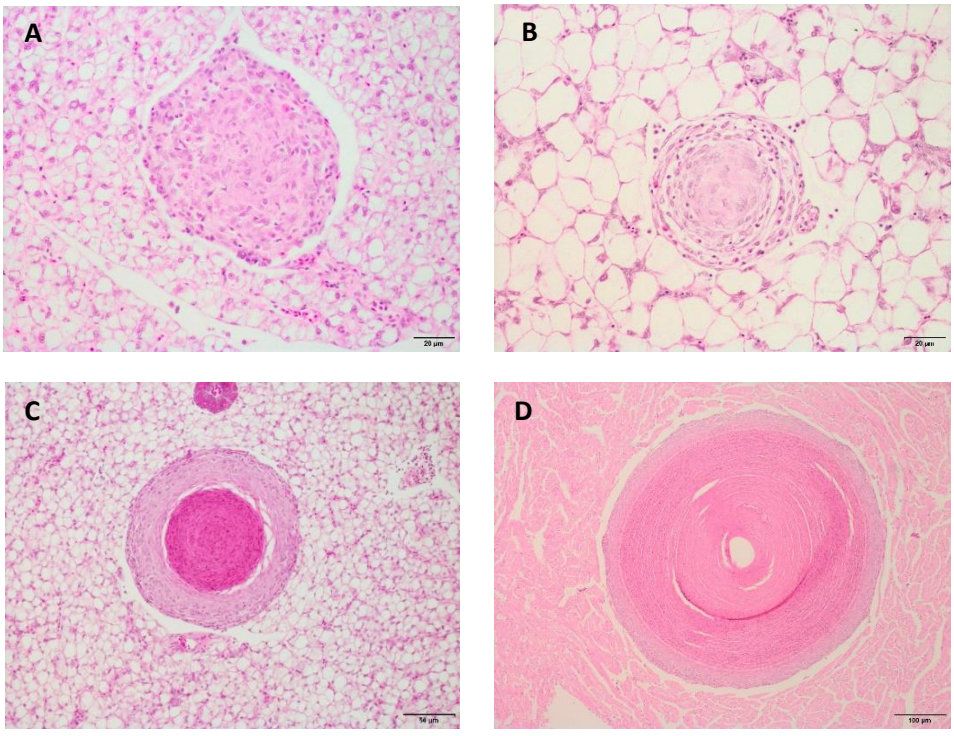


Figure 1. Different stages of granuloma formation. A) Irregular aggregates of macrophages and inflammatory cells. B) Concentric layers of macrophages and inflammatory cells. C) Necrotic centre with an external layer of fibrocytes. D) Granuloma composed completely of laminar material in heart.

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 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.

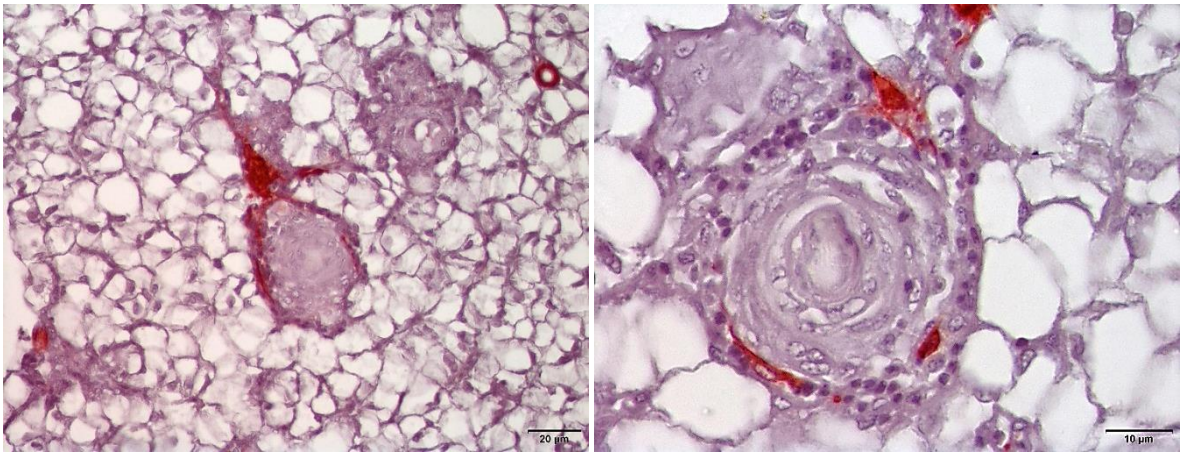


Figure 2. Positive immunoreactivity towards anti-actin antibody in hepatic granulomas.

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) (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 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 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 4c).

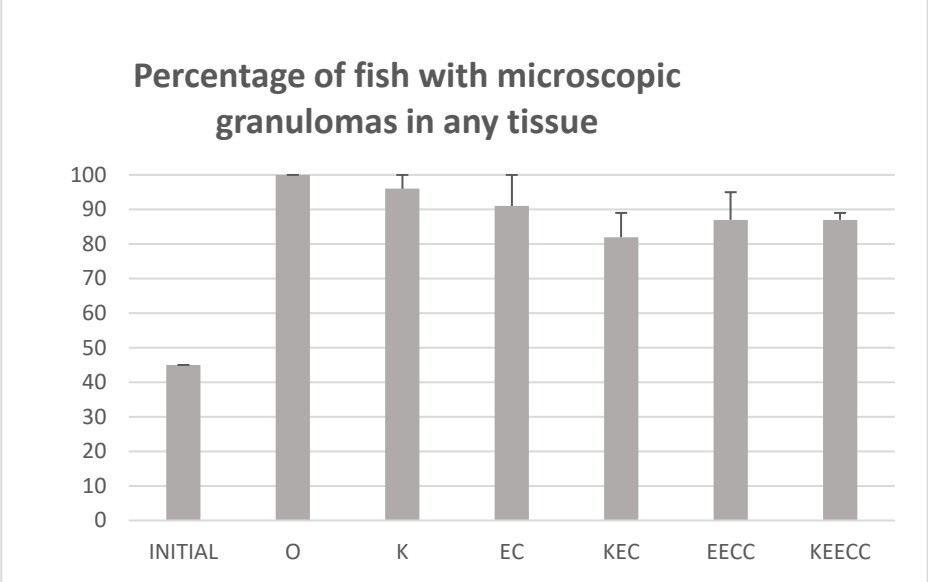
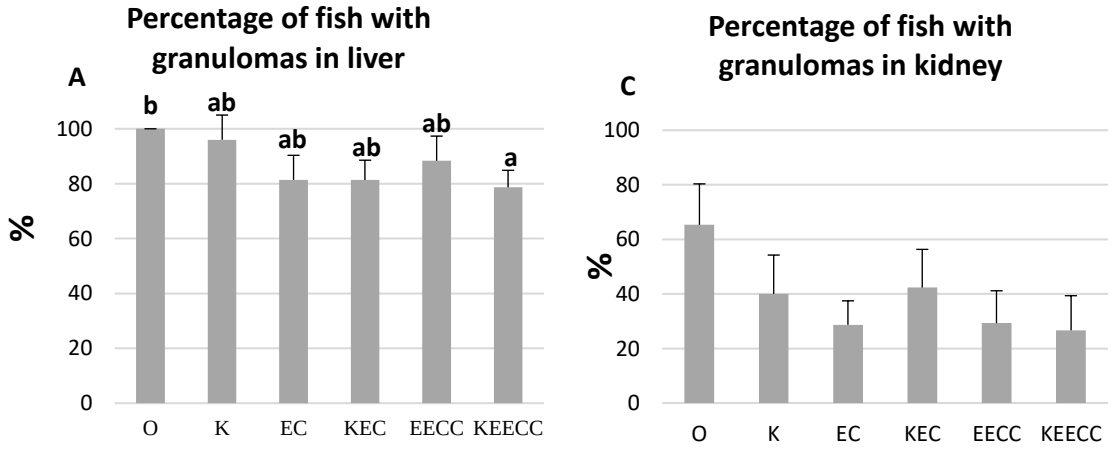
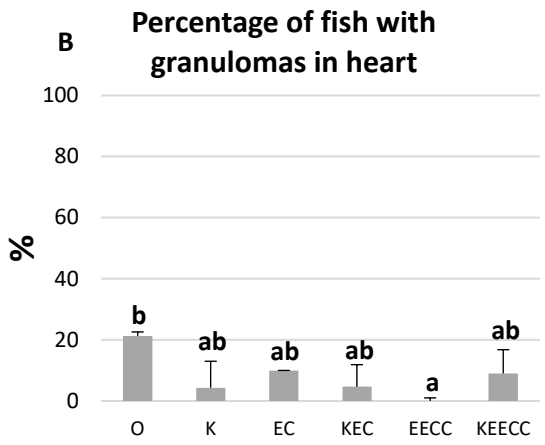


Figure 3. Percentage of fish affected with granulomas in any tissue after the microscopic evaluation of tissues of meagre fed the different experimental feeds containing graded levels of vitamins C, E and K ($p < 0.05$). Significant differences were not observed by the two-way ANOVA.





	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 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 6).

Table 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.

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 5).

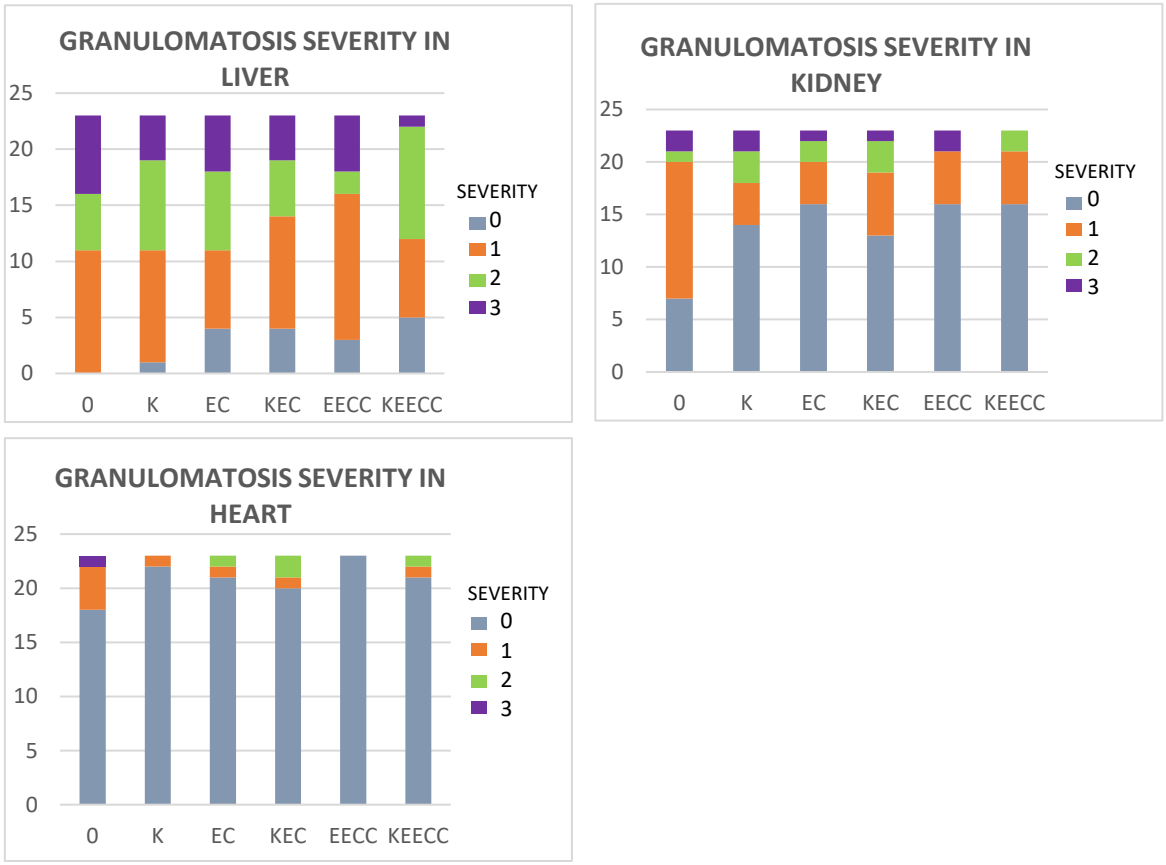


Figure 5. Distribution of fish in each severity stage in liver, kidney and heart after 104 days of feeding the experimental diets ($p < 0.05$). Significant differences were not observed by the two-way ANOVA.

3.4. 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 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 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 7).

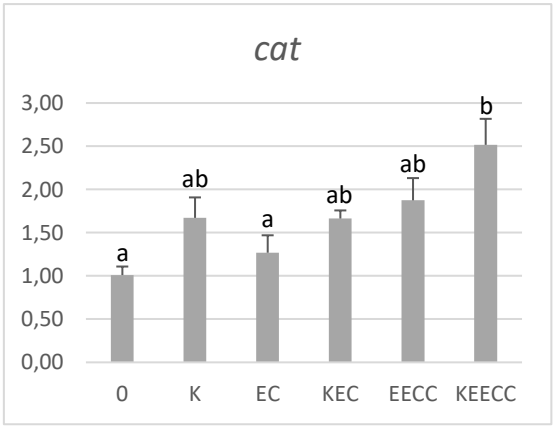
Table 7. α-tocopherol content (vitamin E) in liver, kidney and heart of meagre fed experimental diets during 104 days.

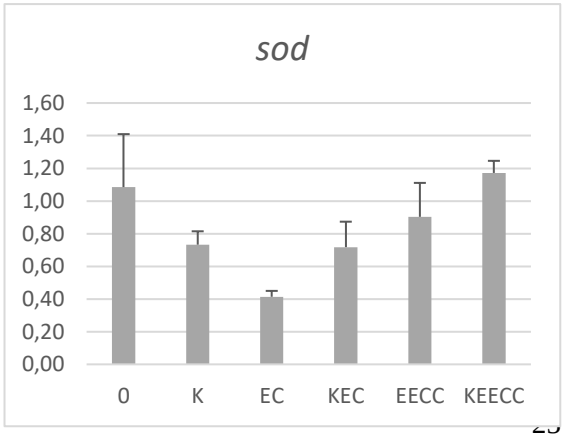
	Vitamin E content (mg kg^{-1})		
	Liver	**Kidney**	**Heart**
0	27.7 ± 2.6^{a}	27.5 ± 2.2^{a}	14.6 ± 2.5^{a}
K	26.7 ± 2.8^{a}	26.4 ± 4.7^{ab}	13.8 ± 2.0^{a}
EC	64.2 ± 1.9^{bc}	30.3 ± 3.0^{ab}	21.5 ± 2.3^{ab}
KEC	48.2 ± 8.8^{b}	32.2 ± 8.0^{abc}	23.9 ± 4.0^{b}
EECC	77.8 ± 6.8^{cd}	39.0 ± 8.3^{bc}	27.1 ± 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.

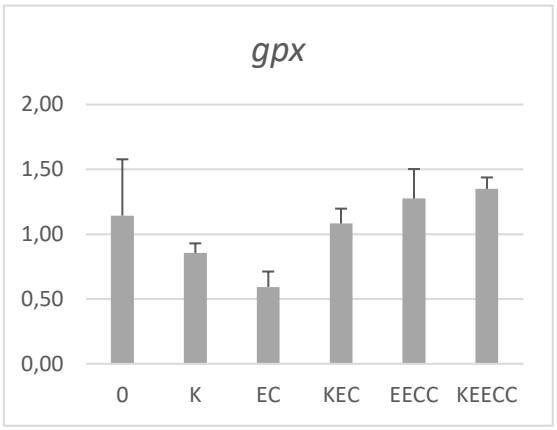
Data are means ± SD (n=3), where the means in each column with a different superscript are significantly different according to one-way ANOVA (P<0.05). The last three columns indicate the effect of vitamins E and C (Vit. E+C), vitamin K (Vit. K) or their interaction (Vit. E+C x Vit. K) according to two-way ANOVA.
* $p < 0.05$.
** $p < 0.01$.

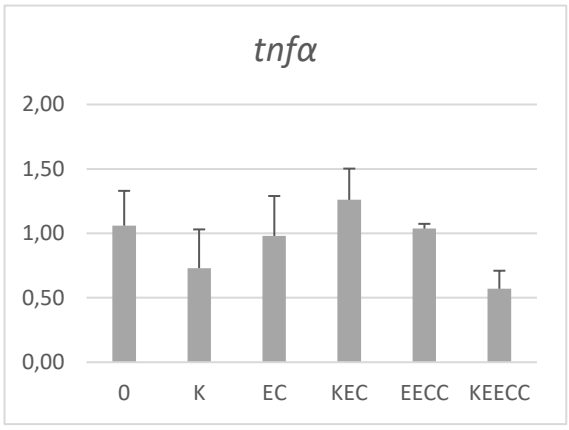
3.5. Gene expression analysis

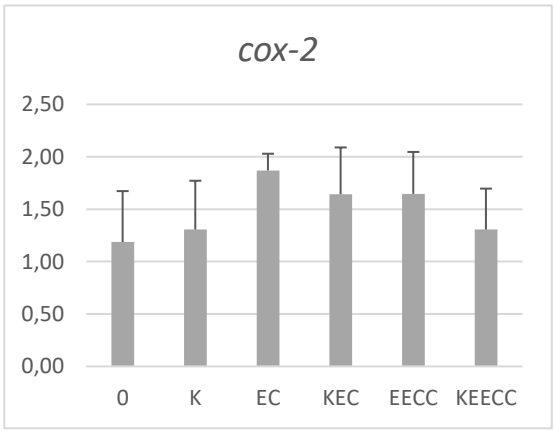
The hepatic expression of *cat* was significantly higher in the liver of fish fed the highest level of vitamin E, C and K than in fish fed diet 0, as denoted by the two-way ANOVA. Significant differences were not found in the gene expression of *sod* ($p = 0.09$) or *gpx* ($p = 0.201$) in liver but fish 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 6). No differences were observed in the expression levels of *tnfα* or *cox-2*.







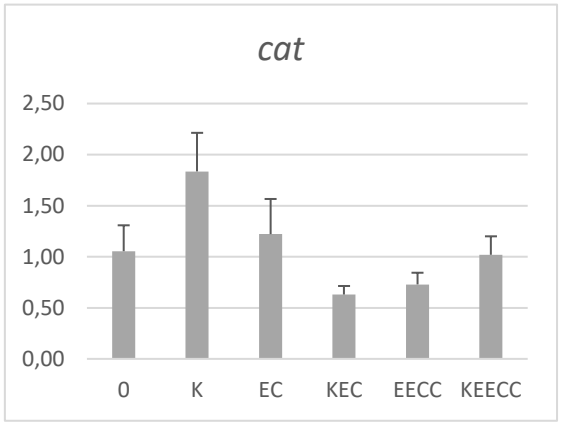


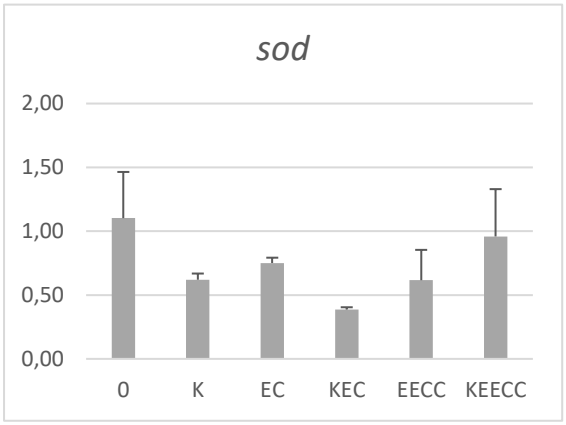


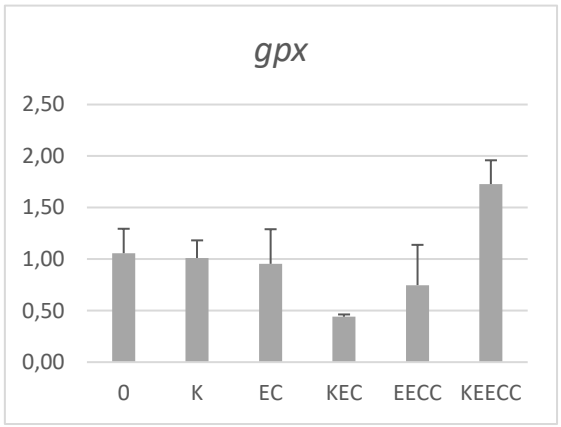
	p Value two-way ANOVA		
	Vit. E+C	Vit. K	Vit. E+C x Vit. K
cat	**	**	n.s.
sod	n.s.	n.s.	n.s.
gpx	n.s.	n.s.	n.s.
tnfα	n.s.	n.s.	n.s.
cox-2	n.s.	n.s.	n.s.

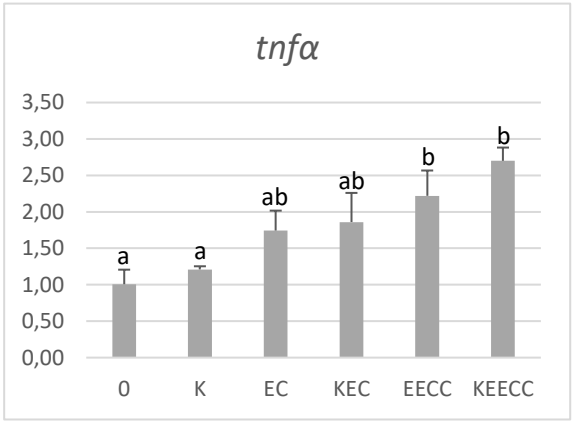
Figure 6. Expression levels of the antioxidant enzymes *cat*, *sod* and *gpx* measured by real-time PCR in liver of meagre ($p < 0.05$). Vit. E+C, vitamins E and C; Vit. K, vitamin K. * $p < 0.05$; ** $p < 0.01$.

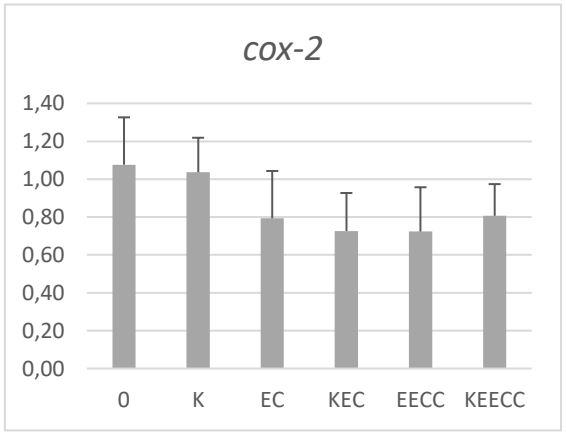
No significant differences in the gene expression of *cat*, *sod* and *gpx* were observed in kidney (Figure 7). There was an increase in the mRNAlevels of *gpx* in fish fed high levels of vitamins E, C and K (KEECC diet) albeit not significant ($p = 0.073$). The two-way ANOVA showed that the expression 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*.







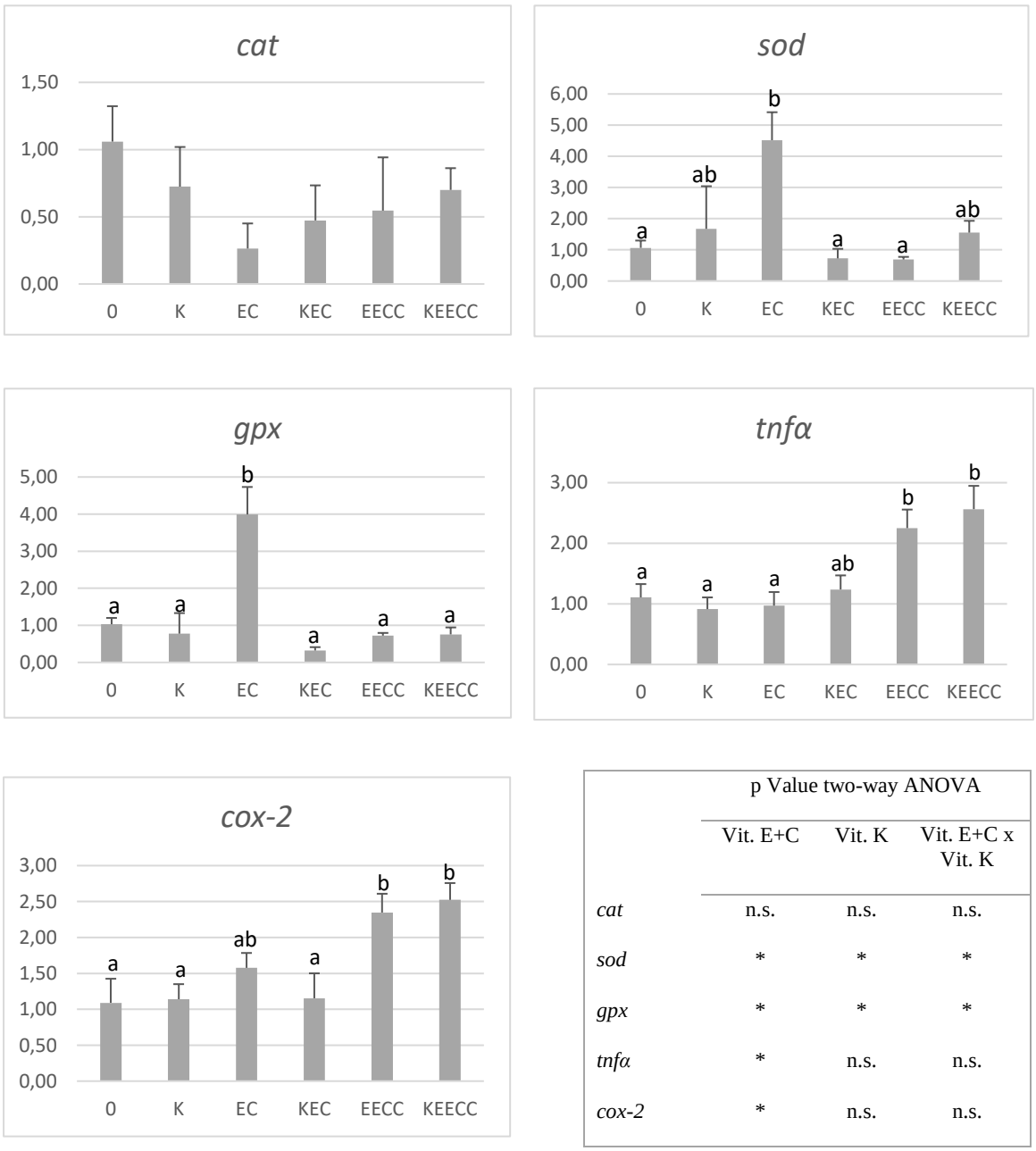




	p Value two-way ANOVA		
	Vit. E+C	Vit. K	Vit. E+C x Vit. K
cat	n.s.	n.s.	n.s.
sod	n.s.	n.s.	n.s.
gpx	n.s.	n.s.	n.s.
tnfα	*	n.s.	n.s.
cox-2	n.s.	n.s.	n.s.

Figure 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. * $p < 0.05$; ** $p < 0.01$

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 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.	n.s.	n.s.
sod	*	*	*
gpx	*	*	*
tnfα	*	n.s.	n.s.
cox-2	*	n.s.	n.s.

Figure 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. * $p < 0.05$; ** $p < 0.01$.

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., 2012). 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.

5. Acknowledgements

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