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ORIGINAL ARTICLE

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*)

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Abstract

Systematic granulomatosis is a chronic disease that affects the majority of farmed meagre (*A. regius*). Nutritional imbalances and overcrowding can increase the risk to suffer oxidative stress, and consequently, impact the incidence of granulomatosis. In order to evaluate this, 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) with constant vitamin E and K (300 and 35 mg/kg respectively): Diet KEC (100 mg/kg C), Diet KEC+Mn/Zn/Se (100 mg/kg C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg C), Diet KECCC (1200 mg/kg C), Diet KECCCC (3200 mg/kg C). All diets were tested at 3.20 kg/m^3, but diets KECC and KECCCC were also tested at 6.20 kg/m^3. Growth performance was only affected by stocking density, being lower at high density. The percentage of fish with granulomas was significantly lower in fish fed with the highest dietary vitamin C contents (KECCC and KECCCC) at low density. TBARS content was correlated with the percentage of granulomas in the liver (R^2 = 0.9439, y = 0.003x − 0.1242) denoting the involvement of an imbalance oxidative status in the appearance of granulomas. The present results show that high levels of vitamin C (1200–3200 mg/kg 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 the deficiency of antioxidant nutrients, particularly vitamin C.

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

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 impacted by systemic granulomatosis. The pathology of this chronic disease demonstrates a large impact in farmed meagre, causing low mortalities, but with high prevalence and intensity (Ghittino et al., 2004). Although some infectious agents like the bacteria *Nocardia* spp. can produce granulomatosis in meagre (Elkesh et al., 2012), 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; Ruiz et al., 2018, 2019a,2019b; Carvalho et al. 2018).

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 (Ruiz et al., 2019a) and by using a microdiet with high enough levels of the antioxidant vitamins E and C (Ruiz et al., 2019b). Inadequate levels of these two vitamins could cause a nutritional imbalance between pro-oxidants and antioxidants, 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 (450 mg/kg vitamin E, 230 mg/kg vitamin C) in feeds for on-growing meagre the incidence and severity of granulomas was lower in liver together with a promotion of fish growth (Ruiz et al., 2018). The effects of dietary inclusion of vitamin D_3 have also been studied in the development of systemic granulomatosis in juvenile meagre, although the dietary inclusion of this vitamin did not seem to prevent the appearance of this disease (Cotou et al., 2016). A deficiency of antioxidants in the diet can cause primary lesions in the tissues and these lesions could potentially lead to a status of oxidative stress (Abdel-Haimed et al. 2012; Betancor et al., 2012). Oxidative stress is caused by the presence of reactive oxygen species (ROS) which are continuously produced in various metabolic pathways in all organisms. A consequence of ROS attack is an oxidative modification of membrane-bound lipids resulting in membrane bilayer damage, together with alterations in the regulation of cellular metabolism (Farooqui & Horrocks, 1998). Indeed, the oxidative stress and ROS have been related to the appearance of granulomas in humans (Facchetti et al., 1999). Nevertheless, ROS action can be inhibited or delayed by the effect of the antioxidant nutrients and enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Pokorny & Korczak, 2001). These antioxidant enzymes can prevent the cascade of oxidant reactions, intercepting and inactivation the reactive intermediates of oxygen and their expression could be related to the appearance of granulomas.

Additionally, there are other micronutrients that can act as antioxidants. Zinc (Zn) has antioxidant properties and protects tissue from oxidative damage (Ho & Ames, 2002). Selenium (Se) is an exogenous antioxidant involved in the prevention of oxidative stress (Biller-Takahashi et al., 2015; Felton et al., 1996; Silva-Brito et al., 2016) being one of the components of glutathione peroxidase (GPX) reducing hydroperoxides at the expense of reduced glutathione (Arteel & 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 enzyme systems, such as Mn superoxide dismutase (Mn-SOD) (Andreini et al., 2008; De Rosa et al., 1980).

One strategy to increase the benefits of intensive aquaculture is to optimize the space with high stock densities. However, overcrowding can be a stressful factor (Ashley, 2007; Wendelaar Bonga, 1997) and negatively affect fish growth, reproduction and immune system (Barton & Iwama, 1991; Di Marco et al., 2008; Pickering, 1998; Shubha & Reddy, 2011). Dietary supplementation of vitamin C and E has been shown to modulate the stress response (Kolkovski et al., 2001; Montero et al., 1998) including in fish reared under different stocking density (Belo et al., 2005; Montero et al., 1999), but little is known about its effects on meagre reared at 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 the resistance to a challenge (Estévez et al., 2007; Roo et al., 2007). Additionally, negative effects have been associated with 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 the 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 the antioxidant minerals 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 two different levels of vitamin C levels (600 and 3200 mg/kg respectively). A basal dietary vitamin E level of 300 mg/kg was used given that it has been previously demonstrated that potentiates the growth of meagre (Ruiz et al., 2018). To reach this objective, diets containing graded levels of vitamin C were fed to juvenile meagre and growth, survival, biochemical composition (total lipids, fatty acid profiles and thiobarbituric acid reactive substances), histopathological evaluation and gene expression of antioxidant enzymes (*catalase*, *superoxide dismutase* and *glutathione peroxidase*) were determined. Additionally, another experimental feed was formulated to contain higher levels of Mn, Zn and Se. The relationship between the presence of granulomas and the oxidative status in the different tissues affected with this condition is evaluated in the present trial.

2 | MATERIALS AND METHODS

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 of F2 broodstock at the ECOAQUA facilities. Meagre larvae were fed with a standard feeding protocol. From 3 to 21 days after hatching (dah) were fed with enriched rotifer with Ori-Green (0.15–0.25 g/million rotifers) twice a day. From 12 to 30 dah were fed with enriched Artemia with Ori-Green (0.8 g/million Artemia) twice a day (8:00 a.m. and 15:00 p.m.). During the co-feeding period, microdiets Gemma Micro 150 and 300 (Skretting, France) were used (15% biomass/day). Juveniles were fed a commercial diet until the start of the experiment. 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 the addition of vitamin E and K (300 and 35 mg/kg respectively) by Skretting ARC (Stavanger, Norway). Diet KEC (100 mg/kg C), Diet KEC+Mn/Zn/Se (100 mg/kg C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg C), Diet KECCC (1200 mg/kg C), Diet KECCCC (3200 mg/kg C). The inclusion level of vitamin E was based in a previous trial (Ruiz et al., 2019) where 250 mg/kg of vitamin E proved to maximize meagre growth without potentiating the appearance of granulomas. The formulation, proximate composition and fatty acid content of each feed are shown in Table 1 and Table S1. The juvenile meagre were fed three times per day (8:00, 11:30, 15:00), 6 days per week for 90 days with the different experimental diets. All the uneaten feed was daily collected 1 h after feeding from each tank with a strainer through the tank drain and dried 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 per tank (3.20 kg/m^3) for all the diets. Additionally, diets KECC and KECCCC were also tested at higher density (175 fish per tank, 6.20 kg/m^3, treatments 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 to 21.5°C and from 5.5 to 6.5 mg/L respectively. Fish were reared under natural light conditions throughout the feeding trial.

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).

2.2 | Sample collection

At the beginning (day 0, prior to the start of the feeding trial; $n = 50$ 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 in ice and liver, heart and kidney removed and frozen at −20°C for biochemical analysis. Four 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.

2.3 | Growth performance

At the beginning (day 0, prior to the start of the feeding trial) and end of the trial, fish were anaesthetized with clove oil (50–70 mg/L) and individual whole body weight and standard length recorded. All fish in the tanks were measured/weighed. Fish were unfed for 24 h before all samplings. Means and standard deviations of each triplicate were calculated for each treatment.

The data 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 × (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 feed intake (g)/number of fish)/ number of days.

2.4 | 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 & Martoja-Pearson, 1970), Fite–Faraco (Fite et al., 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological evaluation. At the sampling, all the tissues were analysed to see if macroscopic granulomas were present. The H&E stain was used to analyse the presence of granulomas in the target tissues, while the stainings of ZN, Fite–Faraco and Gram stain were used to detect the presence of a possible bacteria that could produce the presence of granulomas.

TABLE 1 Formulation and proximate composition of the experimental feeds fed to juvenile meagre (*Argyrosomus regius*) for 90 days

	KEC	KEC+Mn, Zn, Se	KECC	KECCC	KECCCC
Ingredients (%)					
Wheat[a]	17.1	17.0	16.9	16.7	16.1
Corn gluten[a]	5.0	5.0	5.0	5.0	5.0
Wheat gluten[a]	6.8	6.8	6.9	6.9	7.2
Soya concentrate[a]	25.1	25.1	25.0	25.0	24.7
Fish meal[a]	35.0	35.0	35.0	35.0	35.0
Fish oil[a]	10.4	10.4	10.4	10.4	10.4
Mineral Vitamin premix[b]	0.1	0.1	0.1	0.1	0.1
Vitamin E[c]	0.03	0.03	0.03	0.03	0.03
Vitamin C[d]	0.01	0.01	0.06	0.12	0.32
Vitamin K[e]	0.003	0.003	0.003	0.003	0.003
Se[f]	0.001	0.002	0.001	0.001	0.001
Mn[f]	0.003	0.004	0.003	0.003	0.003
Zn[f]	0.016	0.02	0.016	0.016	0.016
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 E (mg/kg)	228.0	242.0	243.0	241.0	255.0
Vitamin C (mg/kg)$^{-1}$	98.0	96.0	586.0	1180.0	2835.0
Vitamin K (mg/kg)	23.0	23.0	23.0	22.0	23.0
Se (mg/kg)	1.1	1.6	1.1	1.1	1.2
Mn (mg/kg)	37	49	34	34	35
Zn (mg/kg)	130	180	130	140	140

Note: Diet codes indicate the levels of vitamins and minerals supplemented to the basal diet (Diet KEC).

[a]Skretting, Stavanger, Norway;

[b]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);

[c]Lutavit E-50, Trouw Nutrition, Boxmeer, the Netherlands.

[d]Lutavit C Aquastab 35%, Trouw Nutrition, Boxmeer, the Netherlands.

[e]Menadione dimethypyrimidinol bisulphite 43.7%, Trouw Nutrition, Boxmeer, the Netherlands.

[6]$ZnSO_4$, Na_2SeO_3 and $MnSO_4 \cdot H_2O$.

2.5 | Biochemical analysis

The analysis of feed and fish biochemical composition was conducted following standard procedures. Total lipids in liver, heart, kidney and feeds were extracted with a chloroform-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 chromatography (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 × 4.60 mm, 5 μm reverse-phase Luna and C18 column (Phenomenox, CA, USA). The mobile phase was methanol: ultra-pure water (98:2 v/v) with a flow rate of 1.0 ml/min 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, homogenized 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 1610 *g* 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 into the HPLC with UV detection. The mobile phase was composed of a phosphate buffer at a flow rate of 0.8 ml/min. It was used a wavelength of 254 nm and a Gemini C18 column, 5 μm particular size and 150 × 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 autosampler, 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) of the liver, kidney and heart according to Burk et al. (1980). Thiobarbituric acid (TBA) is reacted with malondialdehyde (MDA, a product from the oxidation) which is resulting in a colour compound, which can be determined spectrophotometrically. Briefly, 50 μl of 0.2% (w/v) butylated hydroxytoluene (BHT) in ethanol was 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}.

2.6 | Histopathology scoring

The severity of granulomatosis was individually scored in each organ as described in a previous study (Ruiz et al., 2018). 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 Table S2.

2.7 | Gene expression

Kidney, liver and heart were aseptically collected from four fish per tank at the final sampling and stored at −80°C until further analysis. Total RNA was extracted from, ~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 synthesized 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* (*catalase*), *sod* (*superoxide dismutase*) and *gpx* (*glutathione peroxidase*) (Ruiz et al., 2018) (Table 2).

The relative transcript abundance of *gpx*, *sod* and *cat* was determined by quantitative real-time PCR (qPCR). Primer 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, β-actin (*bact*), elongation factor 1α (*ef1α*) and tubulin (*tub*), 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), 6 μl water nuclease-free and 5 μl of a 1:10 dilution of the cDNA, with the exception of the housekeeping 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 pretreatment at 50°C for 2 min, a denaturation at 95°C for 10 min,

TABLE 2 Sequences of the forward and reverse primers (5′–3′) used for real-time quantitative-PCR

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		
bact	F: CCATCGAGCACGGTATTGT	455	60
	R: CAGCTTCTCCTTGATGTCACG		
tub	F: GGAGTACCCCGATCGTATCA	161	59
	R: AGATGTCATACAGGGCCTCG		
ef1a	F: GGTGCTGGACAAACTGAAGG	196	59
	R: GAACTCACCAACACCAGCAG		

Note: The data include sequences, amplicon sizes and annealing temperatures (Ta).

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

followed by 35 cycles: 15 s at 95°C, 30 s at the annealing Tm and 30 s at 72°C. The expression level of each gene was normalized by the corresponding expression of *bact*, *ef1α* and *tub*.

2.8 | Statistical analysis

All statistical analyses were performed on Statgraphics Centurion XVI (Version 16.1.11, StatPoint Technologies, 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. Correlations between the TBARS levels and the appearance of granulomas were analysed with Pearson's correlation coefficient. A significance level of 0.05 was used.

3 | RESULTS

3.1 | Growth performance

3.1.1 | Effect of vitamins and mineral supplementation

The different dietary levels of vitamin C, Mn, Se and Zn did not affect meagre final weight, length or any other evaluated growth parameter (Table 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%).

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 was significantly lower and SGR was significantly higher in fish reared under low stock density (Table 4). Different stocking densities did not affect final survival, being high in all dietary treatments (97.4%–98.5%).

3.2 | Histopathology

3.2.1 | Effect of vitamins and minerals supplementation

Macroscopic granulomas were only observed in the 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 (Figure S1a–c). During the microscopic evaluation, some granulomas were observed associated with the blood vessels (Figure S1c).

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 between the dietary treatments, being lower the percentage in fish-fed diet KECCC and KECCCC (Figure 1). No calcification was observed at any stage or analysed tissue.

The specific stainings (Ziehl–Neelsen, Fite–Faraco and Gram stain), were negative, discarding a possible infectious origin (Figure S2.).

The tissue presenting the highest number of granulomas was the liver followed by kidney and heart, whereas no granulomas were observed in neither spleen nor muscle. The lowest number of fish with

TABLE 3 Meagre (*Argyrosomus regius*) growth performance after 90 days of feeding diets with different levels of vitamin C, Mn, Zn and Se

	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

Notes: Data are means ± *SD*. FCR, food conversion ratio; SGR, specific growth rate; FI, feed intake per day and fish (n = 300). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

TABLE 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)

	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)	Two-way ANOVA		
					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.

Notes: 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, diet–density interaction. Different superscript letters denote differences between treatments identified by one-way ANOVA. n.s. not significant. *$p < 0.05$. **$p < 0.01$ ($n = 300$). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

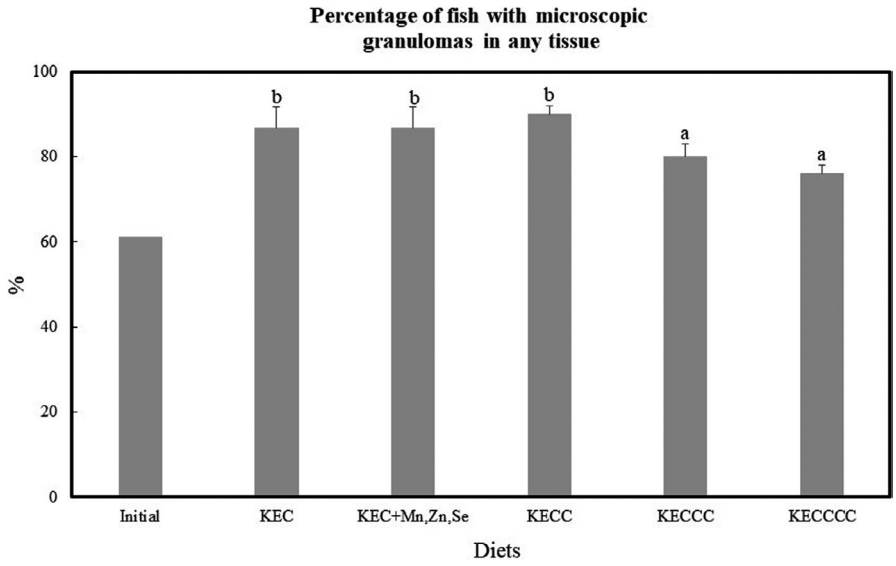

FIGURE 1 Percentage of meagre (*Argyrosomus regius*) fed diets with different levels of C and Mn, Zn and Se with microscopic granulomas in any tissue at the beginning and after 90 days of feeding the experimental diets ($p < 0.05$). Different superscript letters denote differences between treatments identified by one-way ANOVA ($n = 30$)

TABLE 5 Percentage of meagre (*Argyrosomus regius*) presenting granulomas at either liver, kidney and heart, fed diets with different levels of vitamin C and Mn, Zn and Se. Samples were collected at the beginning of the trial (stock tank) and after 90 days of feeding the experimental diets

	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

Notes: Data are mean ± *SD*. where the means in each row with a different superscript are significantly different ($p < 0.05$). Different superscript letters denote differences between treatments identified by one-way ANOVA ($n = 30$). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

hepatic granulomas was observed when the high level of vitamin C was added to the feeds (KECCC-KECCCC diet) (Table 5).

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). However, there was a tendency towards a decrease in the severity of granulomatosis in the liver along with an increase in vitamin C content, for instance 1.37 in diet KEC versus 0.93 in diet KECCCC.

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 2).

The only organ with differences in the incidence of granulomas among fish fed the different dietary treatments was the liver. The incidence of granulomas was only affected by the diet, being lower in the fish fed the diet with higher addition of vitamin C, regardless of the density (63.3% of affected fish with granulomas in liver) (Table 7). The scored severity in liver, kidney and heart was not affected neither the diet nor the density (Table 8).

3.3 | Tissue lipid content and fatty acid profiles

3.3.1 | Effect of vitamins and minerals supplementation

There were no differences in the tissue proximate composition of liver, kidney and heart among the fish fed the different experimental diets (Table S3). The tissue fatty acid profile (Table S4) reflected the dietary fatty acid content (Table S1). The highest levels of total monounsaturated fatty acids were observed in the liver, followed by the kidney and heart; however, the total omega-3 (n-3) and total polyunsaturated fatty acid (PUFA) were higher in the heart, followed by the kidney and liver. All the other fatty acids were similarly distributed in the three tissues. Eicosapentaenoic (20:5n-3; EPA), docosahexaenoic (22:6n-3; DHA) and arachidonic acid (20:4n-6, ARA) contents were similar between the diets, being in average higher in the heart (8.9%, 27.8% and 3.34% respectively) compare with the kidney (9.8%, 17.4% and 2.12% respectively) and liver (4.2%, 6.9% and 0.54% respectively).

TABLE 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 at the beginning and after 90 days of feeding the experimental diets

	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

Notes: Data are mean ± *SD* ($n = 30$). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

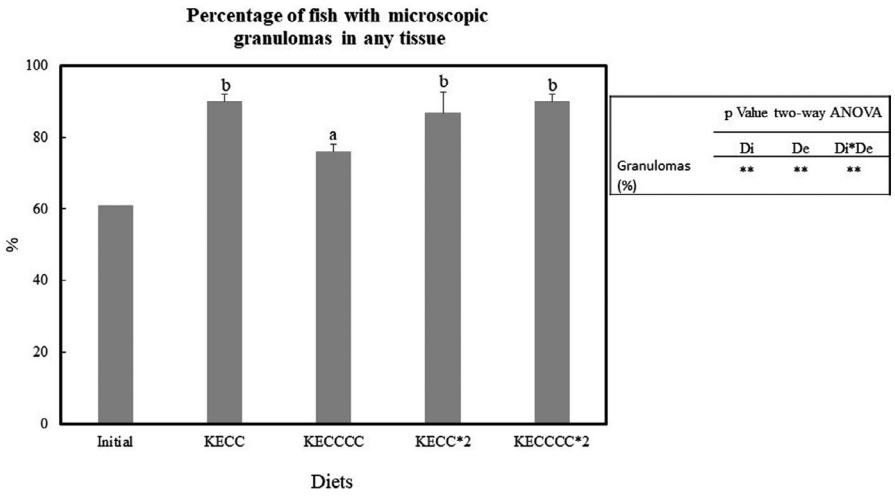

FIGURE 2 Percentage of meagre (*Argyrosomus regius*) reared under two different densities and fed with high or low dietary vitamin C supplementation for 90 days with granulomas in any tissue ($p < 0.05$). Di, diet; De, density; DI*DE, diet–density interaction. Different superscript letters denote differences between treatments identified by one-way ANOVA. *$p < 0.05$. **$p < 0.01$ ($n = 30$)

TABLE 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), at the beginning and after 90 days of feeding

	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.

Notes: Data are mean ± *SD*. where the means in each row with a different superscript are significantly different ($p < 0.05$). DI*DE, diet–density interaction. Different superscript letters denote differences between treatments identified by two-way ANOVA. n.s. not significant. $^*p < 0.05$. $^{**}p < 0.01$ ($n = 30$). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

TABLE 8 Average granuloma severity scores in liver, kidney and heart of meagre (*Argyrosomus regius*) fed diets with different levels of C at the beginning and after 90 days of feeding the experimental diets

	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.

Notes: Displayed in brackets is the density at which the fish were grown (3.20–6.20 kg/m^3). Data are mean ± *SD*. where the means in each row with a different superscript are significantly different ($p < 0.05$). n.s. not significant ($n = 30$). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

TABLE 9 Thiobarbituric reactive substances (TBARS) content in liver, kidney and heart of juvenile meagre (*Argyrosomus regius*) after 90 days of feeding the experimental diets

	KEC	KEC+Mn, Zn, Se	KECC	KECCC	KECCCC
TBARS content (μmol/g)					
Liver	167.7 ± 7.5^b	167.0 ± 6.8^b	111.9 ± 7.1^a	142.2 ± 4.5^{ab}	92.6 ± 9.7^a
Kidney	187.6 ± 9.8^b	211.3 ± 8.6^b	173.3 ± 9.3^b	99.9 ± 5.3^a	113.9 ± 12.6^a
Heart	261.2 ± 6.1^b	272.0 ± 10.5^b	268.3± 7.0^b	166.2 ± 3.1^a	178.1 ± 11.7^a

Notes: Each value represents mean ± *SD*. Different superscript letters denote differences between treatments identified by one-way ANOVA ($p < 0.05$) ($n = 15$). Diet KEC + Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

TBARs content

The level of lipid peroxides, as indicated by TBARS content (μmol/g tissue), was significantly lower in liver, heart and kidney of fish-fed diets with high addition of vitamin C (KECCC and KECCCC) (Table 9). In liver ($R^2 = 0.651$, $y = 487.63x + 43.914$) and heart ($R^2 = 0.648$, $y = 206.75x + 11.087$) a modest positive correlation between the TBARS levels and the

appearance of granulomas was observed. The supplementation of Mn, Zn and Se did not reduce this indicator of lipid peroxidation.

3.3.2 | Effect of stocking density and vitamin C

Density did not affect the proximate composition or fatty acid profile of the liver, kidney and heart between fish fed the different dietary treatments (Tables S5, S6). All fatty acids were similarly distributed among all treatments.

TBARS content

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 the liver, kidney and heart, being the TBARS content in the heart also influenced by the interaction between diet and density (Table 10).

3.4 | Gene expression analysis

3.4.1 | Effect of vitamins and minerals supplementation

There were significant differences in the expression levels of *cat*, *gpx* and *sod* in the liver (Figure 3a). The 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 expression levels of *cat* in kidney in fish-fed diet KECCC (Figure 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 the kidney.

In the heart, significant differences were obtained in the gene expression of *sod* and *gpx* (Figure 3c). The expression was increased in fish fed with high levels of vitamin C (KECCCC). The mRNA copies of *gpx* were 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 the heart was increased when 3200 mg/kg of vitamin C was added to the diet (KECCCC), but without differences when only 1200 mg/kg of vitamin C was added (diet KECCC). The expression of *cat* in the heart was not affected by the inclusion of different levels of vitamin C or Mn, Zn and Se.

3.4.2 | Effect of stocking density and vitamin C

GPX expression in the 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 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 3200 mg/kg 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 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 4c), only fish-fed diet KECCCC having a 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 the heart.

4 | DISCUSSION

In the present study, the inclusion of vitamin E, Mn, Zn, Se and different levels of vitamin C did not affect growth parameters. The mean results of FCR (0.75–0.76) and SGR (1.95–2.04) in low density were slightly better than those obtained by Rodríguez Lozano et al. (2017) in meagre (FCR 0.74–0.95, SGR 1.41–1.49) probably due to

TABLE 10 Thiobarbituric reactive substances (TBARS) content in liver, kidney and heart of juvenile meagre (*Argyrosomus regius*) 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)							
Liver	111.8 ± 5.1^a	92.6 ± 5.5^a	168.04 ± 5.4^b	165.66 ± 2.6^b	n.s.	*	n.s.
Kidney	166.2 ± 25.1^{ab}	113.9 ± 18.2^a	190.5 ± 6.4^b	204.2 ± 3.9^b	n.s.	**	n.s.
Heart	268.3 ± 15.0^b	178.0 ± 11.7^a	231.6 ± 18.1^{ab}	283.3 ± 12.3^b	n.s.	n.s.	**

Notes: Each value represents mean ± *SD*. DI, diet; DE, density; DI*DE, diet–density interaction. Different superscript letters denote differences between treatments identified by two-way ANOVA. n.s. not significant. *$p < 0.05$. **$p < 0.01$ ($n = 15$). Diet KEC+Mn/Zn/Se (100 mg/kg vitamin C, 40 mg/kg Mn, 200 mg/kg Zn, 1.5 mg/kg Se), Diet KECC (600 mg/kg vitamin C), Diet KECCC (1200 mg/kg vitamin C), Diet KECCCC (3200 mg/kg vitamin C).

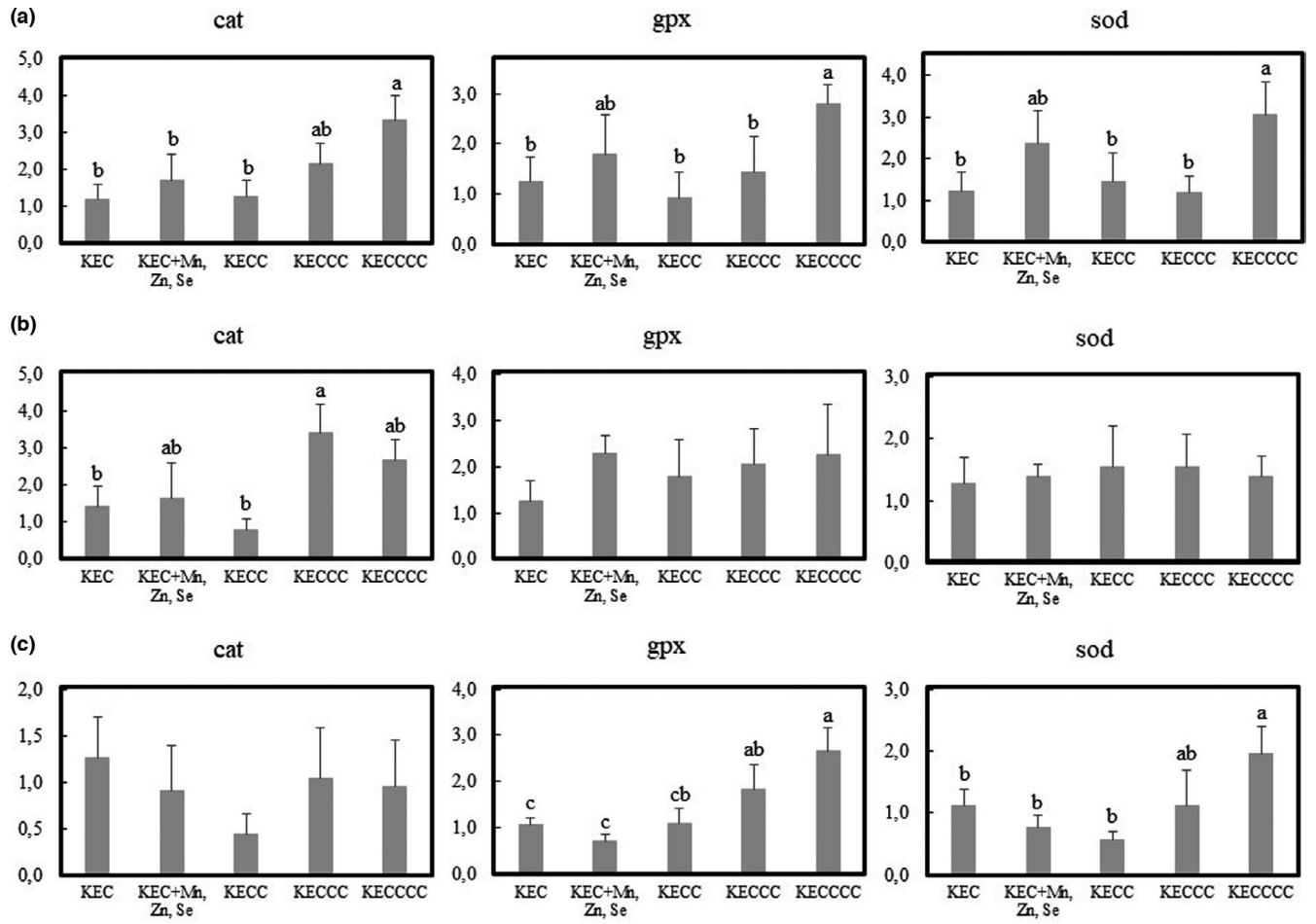

FIGURE 3 Expression levels of *cat*, *sod* and *gpx* measured by real-time PCR in (a) liver, (b) kidney and (c) heart of meagre (*Argyrosomus regius*) after 90 days of feeding diets with different levels of vitamin C, Mn, Zn and Se. Values are normalized expression ratios, corresponding to an average of six individuals ($n = 6$) with standard errors (SEM). Different superscript letters denote differences between treatments identified by one-way ANOVA ($n = 6$)

the larger initial size of the fish (62.9 g) in the former study. In previous studies, it was observed that the addition of vitamin E and C in the diet (300 and 70 mg/kg respectively) could affect final growth in on-growing meagre (Ruiz et al., 2018). 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 and FCR were observed between the two stocking densities, being better in the treatments with low density (100 fish per tank). 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 & Dutil, 2001) and Amur sturgeon (*Acipenser schrenckii*) (Li et al., 2012). The reduction in growth at high stock density, could be due to the heterogeneous size of the fish as a consequence of the dominance of some fish in the tank. In this sense, larger sized fish exercise their dominance in the areas where the food is supplied preventing an adequate food access to those of smaller size (Alanärä & Brännäs, 1996; Grand & Grant, 1994; McCarthy et al., 1999). Moreover, it has been shown that the high stock density produces stress and, in consequence, the elevation of cortisol levels in different fish species (Ellis et al., 2002; North et al., 2006; Ortuño et al., 2001; Turnbull et al., 2005; 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 leads to 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 analysed organs. A similar effect was observed by Liu et al. (2016) in Atlantic salmon (*Salmo salar*) and by Jia et al. (2016) in turbot, where the fish farmed at a high stock density showed higher levels of TBARS compared with those 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 (Andrade et al., 2015; Sahin et al., 2014). Therefore, the higher stock density might induce a higher stress resulting in an increase in lipid peroxidation and cell degradation.

Additionally, the level of lipid peroxides was lower in all the analysed tissues with the inclusion of 1200 or 3200 mg/kg of vitamin C in fish farmed at low density (KECCC and KECCCC). TBARS have

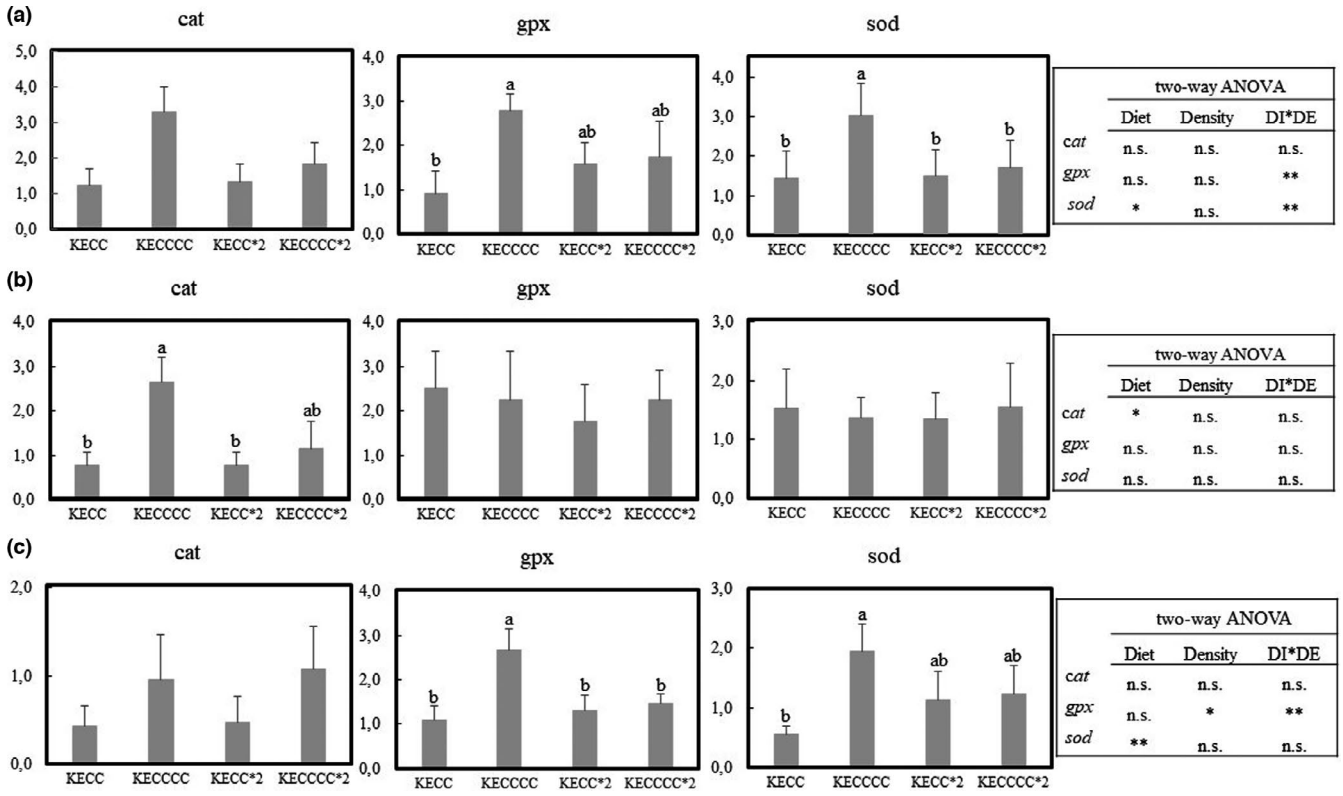

FIGURE 4 Expression levels of *cat*, *sod* and *gpx* measured by real-time PCR in liver, kidney and heart of meagre (*Argyrosomus regius*) after 90 days fed different levels of vitamin C and cultured under two different densities (Diets KECC and KECCCC at 3.20 kg/m^3 and diets KECC*2 and KECCCC*2 at 6.20 kg/m^3). Di, diet; De, density; DI*DE, diet–density interaction. Values are normalized expression ratios, corresponding to an average of six individuals ($n = 6$) with standard errors (SEM). Different superscript letters denote differences between treatments identified by one-way ANOVA. n.s., not significant. $^*p < 0.05$. $^{**}p < 0.01$ ($n = 6$)

been the most frequently used indicator for the 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 positive correlation between the TBARS content and the appearance of granulomas was observed (Ruiz et al., 2019a,2019b), 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 at 30 dph ($R^2 = 0.948$, $y = 0.084x - 4.3924$) (Ruiz et al., 2019a) and 44 dph ($R^2 = 0.892$, $y = 0.0446x + 0.0756$) (Ruiz et al., 2019b), 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 the dietary level of vitamin C from 100 mg/kg to 1200–3200 mg/kg, the percentage of fish with granulomas in any tissue at low density was significantly lower, from 87% in diet KEC to 80 and 76% in diet KECCC and KECCCC respectively. The liver was the main affected organ (71.5%) followed by kidney (62.2%) and heart (7.6%), and the incidence of granulomas in liver was significantly lower in fish-fed diet KECCC and KECCCC at low density, and in fish-fed diet KECCCC*2. Indeed, the percentage of fish with 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 (Andrade et al., 2015; Sahin et al., 2014), this imbalance potentially leading to a status of oxidative stress which may cause different diseases and lesions (Betancor et al., 2012; Cowey et al., 1984; Kawatsu, 1969; Lewis-McCrea & Lall, 2007; Sakai et al., 1989; Watanabe et al., 1989). In the present study, the 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 promote the appearance of granulomas.

On the other hand, in the present study the supplementation of Se, Mn and Zinc 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. (2017) observed that the supplementation of 3.98 mg/kg 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. Little is known about the requirements and its effects on the antioxidant system of these three minerals.

The lower percentage of granulomas and TBARS values in fish fed with a high addition of vitamin C (3200 mg/kg^3) at low densities was also accompanied by the increase in the expression of some antioxidant enzymes capable to neutralize ROS, named *sod*, *gpx* and *cat*. In the present study, the expression of these 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 3200 mg/kg vitamin C. Expression of *cat* was up-regulated in liver and kidney of fish-fed diet supplemented 3200 mg/kg vitamin C. SOD can catalyse the reaction of super anion transforming it to H_2O_2 and O_2, whereas 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/expression of *sod*, *gpx* and *cat* in other fish species, such as juvenile Wuchang bream (*Megalobrama amblycephala*) (Liu, Wan, et al., 2016; Wan et al., 2013), 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, suggests 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 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 3200 mg/kg of vitamin C at low density but was significantly lower in the same diet but at high density. Similar to these results, Jia et al. (2016) observed that juvenile turbot reared under three different stock densities (5.1, 7.7 and 10.8 kg/m^2), showed a lower expression levels of *sod*, *gpx* and *cat* after 80 days when reared at the higher stock density. The high stock density has been established as a source of stress that increases the production of ROS (Braun et al., 2010; Vijayan et al., 1990). These ROS could damage cellular components and lead to autoxidation (Sayeed et al., 2003). It has been observed that the increase in ROS can induce 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 a high content of MDA together with down-regulation of antioxidant genes, which indicates a higher oxidative stress in those fish reared at 6.20 kg/m^3.

To conclude, meagre farmed at a high stock density (6.20 kg/m^3) for 90 days showed reduced growth, SGR and a higher FCR, regardless of the dietary content. The addition of different levels of vitamin C and the supplementation of Mn, Se and Zn did not affect growth parameters. TBARS values in kidney, liver and heart at low stocking density were lower when fish were fed with high addition of vitamin C (1200–3200 mg/kg). Also, the percentage of granulomas in any tissue and particularly in liver, where the TBARS values were highly correlated with the percentage of granulomas ($R^2 = 0.9439$, $y = 0.003x - 0.1242$) was lower in fish farmed at low density. 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 low stock density (3.20 kg/m^3) favours the growth of juvenile meagre and that high levels of vitamin C (1200–3200 mg/kg C) can reduce the lipid peroxidation indicators as well as decrease the incidence of granulomas. The results seem to suggest that this pathology is mostly triggered by a deficiency of antioxidant nutrients, as high levels of vitamin C led to a lower percentage of granulomas in the liver. However, the percentage of fish with granulomas increased along the experimental trial regardless of the dietary treatment, which opens the possibility that other factors may be involved in the appearance of the disease. Given that systemic granulomatosis has been diagnosed in very early life stages, a genetic origin of the pathology could be speculated. Nevertheless, this pathology has also been described in several other locations such as Italy or Greece, it is highly unlikely that the genetic background of the fish plays a role in the appearance of the disease.

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AUTHORS' CONTRIBUTIONS

Miguel Ángel Ruiz: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft, Visualization. **Mónica Betancor:** Conceptualization, Methodology, Writing - Review & Editing. **Daniel Montero:** Conceptualization, Project administration, Supervision, review & Editing. **María José Caballero:** Conceptualization, Methodology, Writing - Review & Editing. **Carmen María Hernández:** Conceptualization, Project administration, Supervision. **Grethe Rosenlund:** Conceptualization, Resources. **Ramón Fontanillas:** Conceptualization, Resources. **María Soledad Izquierdo:** Conceptualization, Project administration, Supervision.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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REFERENCES

Abdel-Haimed, N.-A. H., Abidi, S. F., & Khan, M. A. (2012). Dietary vitamin E requeriment for maximizing the growth conversion efficiency, biochemical composition and haemotological status of fingerling *Channapunctatus*. *Aquaculture Research*, *43*, 226–238. https://doi.org/10.1111/j.1365-2109.2011.02819.x.

Aksakal, E., Ekinci, D., Erdogan, O., Beydemir, S., Alım, Z., & Ceyhun, S. B. (2011). Increasing stocking density causes inhibition of metabolic–antioxidant enzymes and elevates mRNA levels of heat shock protein 70 in rainbow trout. *Livestock Science*, *141*, 69–75. https://doi.org/10.1016/j.livsci.2011.07.006.

Alanärä, A., & Brännäs, E. (1996). Dominance in demand-feeding behaviour in Arctic charr and rainbow trout: The effect of stocking density. *Journal of Fish Biology*, *48*, 242–254. https://doi.org/10.1111/j.1095-8649.1996.tb01116.x.

Andrade, T., Afonso, A., Pérez-Jiménez, A., Oliva-Teles, A., de las Heras, V., Mancera, J. M., Serradeiro, R., & Costas, B. (2015). Evaluation of different stocking densities in a Senegalese sole (*Solea senegalensis*) farm: Implications for growth, humoral immune parameters and oxidative status. *Aquaculture*, *438*, 6–11. https://doi.org/10.1016/j.aquaculture.2014.12.034.

Andreini, C., Bertini, I., Cavallaro, G., Holliday, G. L., & Thornton, J. M. (2008). Metal ions in biological catalysis: from enzyme databases to general principles. *JBIC Journal of Biological Inorganic Chemistry*, *13*, 1205–1218. https://doi.org/10.1007/s00775-008-0404-5.

AOAC. (2010). *Officials Methods of Analysis*, 17th ed. Association of Official Analytical Chemists.

Arteel, G. E., & Sies, H. (2001). The biochemistry of selenium and the glutathione system. *Environmental Toxicology and Pharmacology*, *10*, 153–158. https://doi.org/10.1016/S1382-6689(01)00078-3.

Ashley, P. J. (2007). Fish welfare: current issues in aquaculture. *Applied Animal Behaviour Science*, *104*, 199–235. https://doi.org/10.1016/j.applanim.2006.09.001.

Barton, B. A., & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases*, *129*, 3–26. https://doi.org/10.1016/0959-8030(91)90019-G.

Belo, M. A. A., Schalch, S. H. C., Moraes, F. R., Soares, V. E., Otoboni, A. M. M. B., Moraes, J. E. R. (2005). Effect of Dietary Supplementation with Vitamin E and Stocking Density on Macrophage Recruitment and Giant Cell Formation in the Teleost Fish, Piaractus mesopotamicus. *Journal of Comparative Pathology*, *133* (2-3), 146–154. http://dx.doi.org/10.1016/j.jcpa.2005.04.004.

Betancor, M. B., Caballero, M. J., Terova, G., Corà, S., Saleh, R., Benítez-Santana, T., Bell, J. G., Hernández-Cruz, C. M., & Izquierdo, M. (2012). Vitamin C enhances vitamin E status and reduces oxidative stress indicators in sea bass larvae fed high DHA microdiets. *Lipids*, *47*, 1193–1207. https://doi.org/10.1007/s11745-012-3730-x.

Biller-Takahashi, J. D., Takahashi, L. S., Mingatto, F. E., & Urbinati, E. C. (2015). The immune system is limited by oxidative stress: Dietary selenium promotes optimal antioxidative status and greatest immune defense in pacu *Piaractus mesopotamicus*. *Fish & Shellfish Immunology*, *47*, 360–367. https://doi.org/10.1016/j.fsi.2015.09.022.

Braun, N., de Lima, R. L., Baldisserotto, B., Dafre, A. L., & de Oliveira Nuñer, A. P. (2010). Growth, biochemical and physiological responses of *Salminus brasiliensis* with different stocking densities and handling. *Aquaculture*, *301*, 22–30. https://doi.org/10.1016/j.aquaculture.2010.01.022.

Burk, R. F., Trumble, M. J., & Lawrence, R. A. (1980). Rat hepatic cytosolic GSH-dependent enzyme protection against lipid peroxidation in the NADPH microsomal lipid peroxidation system. *Biochimica Et Biophysica Acta*, *618*, 35–41. https://doi.org/10.1016/0005-2760(80)90051-X.

Carvalho, M., Castro, P., Peres, H., Fontanillas, R., Rosenlund, G., Monter, D., Acosta, F., Robaina, L., & Izquierdo, M. (2018) Effect of increasing dietary levels of n-3 long-chain polyunsaturated fatty acids on liver composition and histopathology of meagre (Argyrosomus regius, Asso 1801) fingerlings. 18th International Symposium on Fish Nutrition and Feeding, 3-7 June 2018, Las Palmas de Gran Canaria. Spain.

Cheng, C. H., Liang, H. Y., Guo, Z. X., Wang, A. L., & Ye, C. X. (2017). Effect of Dietary Vitamin C on Growth Performance, Antioxidant Status and Innate Immunity of Juvenile Pufferfish (*Takifugu obscurus*). *Israeli Journal of Aquaculture*, *69*, 13.

Christie, W. W. (1982). *Lipid analysis*. Pergamon.

Costas, B., Aragão, C., Mancera, J. M., Dinis, M. T., & Conceição, L. E. C. (2008). High stocking density induces crowding stress and affects amino acid metabolism in Senegalese sole *Solea senegalensis* (Kaup 1858) juveniles. *Aquaculture*, *39*, 1–9. https://doi.org/10.1111/j.1365-2109.2007.01845.x.

Cotou, E., Fountoulaki, E., Hager-Theodorides, A. L., Theodorou, G., Tsertou, M., Loukanari, T., Katharios, P., & Kounna, C. H. (2016). The effects of dietary inclusions of vitamin D3 associated to CYP27A1, antioxidant enzymes and non-infectious systemic granulomatosis in meagre (*Argyrosomus regius*). In *International Symposium on Fish Nutrition and Feeding, 5-10, 2016, Sun Valley, Idaho*.

Cowey, C. B., Degener, E., Tacon, A. G. J., Youngson, A., & Bell, J. G. (1984). The effects of vitamin E and oxidised fish oil on the nutrition of rainbow trout (*Salmo gairdneri*) grown at natural, varying water temperature. *British Journal of Nutrition*, *51*, 443–451. https://doi.org/10.1079/BJN19840050.

Dawood, M. A. O., Koshio, S., Ishikawa, M., & Yokoyama, S. (2016). Immune responses and stress resistance in red sea bream (*Pagrus major*) after oral administration of heat-killed Lactobacillus plantarum and vitamin C. *Fish & Shellfish Immunology*, *54*, 266–275. https://doi.org/10.1016/j.fsi.2016.04.017.

De Rosa, G., Keen, C. L., Leach, R. M., & Hurley, L. S. (1980). Regulation of superoxide dismutase activity by dietary manganese. *Journal of Nutrition*, *110*, 795–804. https://doi.org/10.1093/jn/110.4.795.

de Zwart, L. L., Meerman, J. H. N., Commandeur, J. N. M., & Vermeulen, N. P. E. (1999). Biomarkers of free radical damage: applications in experimental animals and in humans. *Free Radical Biology and Medicine*, *26*, 202–226. https://doi.org/10.1016/S0891-5849(98)00196-8.

Di Marco, P., Priori, A., Finoia, M. G., Massari, A., Mandich, A., & Marino, G. (2008). Physiological responses of European sea bass *Dicentrarchus labrax* to different stocking densities and acute stress challenge. *Aquaculture*, *275*, 319–328. https://doi.org/10.1016/j.aquaculture.2007.12.012.

Elkesh, A., Kantham, K. P. L., Shinn, A. P., Crumlish, M., & Richards, R. H. (2012). Systemic nocardiosis in a Mediterranean population of cultured meagre, *Argyrosomus regius* Asso (Perciformes: Sciaenidae). *Journal of Fish Diseases*, *36*, 141–149. https://doi.org/10.1111/jfd.12015.

Ellis, T., North, B., Scott, A. P., Bromage, N. R., Porter, M., & Gadd, D. (2002). The relationships between stocking density and welfare in farmed rainbow trout. *Journal of Fish Biology*, *61*, 493–531. https://doi.org/10.1111/j.1095-8649.2002.tb00893.x.

El-Sayed, Y. S., El-Gazzar, A. M., El-Nahas, A. F., & Ashry, K. M. (2016). Vitamin C modulates cadmium-induced hepatic antioxidants' gene transcripts and toxicopathic changes in Nile tilapia, *Oreochromis niloticus*. *Environmental Science and Pollution Research International*, *23*, 1664–1670. https://doi.org/10.1007/s11356-015-5412-8.

Estévez, A., Treviño, L., & Gisbert, E. (2007). La densidad larvaria inicial afecta al crecimiento pero no a la supervivencia de las larvas de corvina (*Argyrosomus regius*) en cultivo. In A. Cerviño, A. Guerra, & C. Pérez (Eds.), *Actas XI Congreso Nacional de Acuicultura, 24–28 Septiembre, Vigo* (pp. 747–750). Pontevedra.

Facchetti, F., Vermi, W., Fiorentini, S., Chilosi, M., Caruso, A., Duse, M., Notarangelo, L. D., & Badolato, R. (1999). Expression of inducible nitric oxide synthase in human granulomas and histiocytic reactions. *The American Journal of Pathology*, *154*(1), 145–152. https://doi.org/10.1016/S0002-9440(10)65261-3.

Farooqui, A. A., & Horrocks, L. A. (1998). Lipid peroxides in the free radical pathophysiology of brain diseases. *Cellular and Molecular Neurobiology*, *18*, 599–608. https://doi.org/10.1023/a:10206 25717298.

Felton, S. P., Landolt, M. L., Grace, R., & Palmisano, A. (1996). Effects of selenium dietary enhancement on hatcheryreared coho salmon, *Oncorhynchus kisutch* (Walbaum), when compared with wild coho: hepatic enzymes and seawater adaptation evaluated. *Aquaculture Research*, *27*, 135–142. https://doi.org/10.1111/j.1365-2109.1996.tb00977.x.

Fite, G. L., Cambre, F. J., & Turner, M. H. (1947). Procedures for demonstrating lepra bacilli in paraffin sections. *Archives of Pathology*, *43*, 624–625.

Folch, J. M., Lees, M., & Stanley Sloane, G. H. (1957). A simple method for the isolation and purification of total lipids from the animal tissues. *Journal of Biological Chemistry*, *226*, 497–509.

Ghittino, C., Manuali, E., Latini, M., Agnetti, F., Rogato, F., Agonini, R., Colussi, S., & Prearo, M. (2004). Caso di granulomatosi sistemica in ombrina boccadoro (*Argyrosomus regius*) e raffronto con le lesioni istologiche presenti nell'orata. *Ittiopatologia*, *1*, 59–67.

Grand, T. C., & Grant, J. W. A. (1994). Spatial predictability of food influences, its monopolisation and defence by juvenile convict cichlids. *Animal Behavior*, *47*, 91–100. https://doi.org/10.1006/anbe.1994.1010.

Gregersen, T. (1978). Rapid method for distinction of gram-negative from gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology*, *5*, 123–127. https://doi.org/10.1007/BF00498806.

Harats, D., Ben-Naim, M., Dabach, Y., Hollander, G., Havivi, E., Stein, O., & Stein, Y. (1990). Effect of vitamin C and E supplementation on susceptibility of plasma lipoproteins to peroxidant induced by acute smoking. *Atherosclerosis*, *85*, 47–54. https://doi.org/10.1016/0021-9150(90)90181-H.

Heath, A. G. (1995). *Water Pollution and Fish Physiology*. Lewis.

Ho, E., & Ames, B. N. (2002). Low intracellular zinc induces oxidative DNA damage, disrupts p53, NFkappa B, and AP1 DNA binding, and affects DNA repair in a rat glioma cell line. *Proceedings of the National Academy of Sciences of USA*, *99*, 16770–16775. https://doi.org/10.1073/pnas.222679399.

Izquierdo, M. S., Arakawa, T., Takeuchi, T., Haroun, R., & Watanabe, T. (1992). Effect of n-3 HUFA levels in Artemia on growth of larval Japanese flounder (*Paralichthys olivaceous*). *Aquaculture*, *105*, 73–82. https://doi.org/10.1016/0044-8486(92)90163-F.

Jia, R., Liu, B. L., Feng, W. R., Han, C., Huang, B., & Lei, J. L. (2016). Stress and immune responses in skin of turbot (*Scophthalmus maximus*) under different stocking densities. *Fish & Shellfish Immunology*, *55*, 131–139. https://doi.org/10.1016/j.fsi.2016.05.032.

Julshamn, K., Maage, A., Norli, H. S., Grobecker, K. H., Jorhem, L., Fecher, P., Hentschel, A., Martin de la Hinojosa, I., Viehweger, L., Mindak, W. R., Lindholm, K., Holm, K., Olsson, B., Engman, J., Habernegg, R., Hammer, D., Lewis, J., van der Wielen, J., Hovind, H., & Vadset, M. (2007). Determination of arsenic, cadmium, mercury, and lead by inductively coupled plasma/mass spectrometry in foods after pressure digestion: NMKL Interlaboratory Study. *Journal of AOAC International*, *90*, 844–856. https://doi.org/10.1093/jaoac/90.3.844.

Katharios, P., Kokkari, K., Papadaki, M., & Papandroulakis, N. (2011). Systemic granulomas in cultured meagre, *Argyrosomus regius*. Aquaculture Europe 11, 18-21 October 2011, Rhodos, Greece. pp. 537–538.

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

Kolkovski, S., Czesny, S., Yackey, C., Moreau, R., Cihla, F., Mahan, D., & Dabrowski, K. (2001). The effect of vitamins C and E in (n-3) highly unsaturated fatty acids-enriched Artemia nauplii on growth, survival and stress resistance of fresh water walleye *Stizostedion vitreum* larvae. *Aquaculture Nutrition*, *6*, 199–206. https://doi.org/10.1046/j.1365-2095.2000.00112.x.

Laiz-Carrión, R., Viana, I. R., Cejas, J. R., Ruiz-Jarabo, I., Jerez, S., Martos, J. A., Eduardo, A. B., & Mancera, J. M. (2012). Influence of food deprivation and high stocking density on energetic metabolism and stress response in red porgy, *Pagrus pagrus* L. *Aquaculture Research*, *20*, 585–599. https://doi.org/10.1007/s10499-011-9488-y.

Lambert, Y., & Dutil, J. (2001). Food intake and growth of adult Atlantic cod (*Gadus morhua* L.) reared under different conditions of stocking density, feeding frequency and sizegrading. *Aquaculture*, *192*, 233–247. https://doi.org/10.1016/S0044-8486(00)00448-8.

Lankford, S. E., Adams, T. E., Miller, R. A. Jr, & Cech, J. J. (2005). The cost of chronic stress: impacts of a nonhabituating stress response on metabolic variables and swimming performance in sturgeon. *Physiological and Biochemical Zoology*, *78*, 599–609. https://doi.org/10.1086/430687.

Lewis-McCrea, L. M., & Lall, S. P. (2007). Effects of moderately oxidized dietary lipid and the role of vitamin E on the development of skeletal abnormalities in juvenile atlantic halibut (*Hippoglossus hippoglossus*). *Aquaculture*, *262*, 142–155. https://doi.org/10.1016/j.aquaculture.2006.09.024.

Li, D., Liu, Z., & Xie, C. (2012). Effect of stocking density on growth and serum concentrations of thyroid hormones and cortisol in Amur sturgeon. *Acipenser schrenckii*. *Fish Physiology and Biochemistry*, *38*, 511–520. https://doi.org/10.1007/s10695-011-9531-y.

Liu, B., Liu, Y., & Sun, G. (2016). Effects of stocking density on growth performance and welfare-related physiological parameters of Atlantic salmon *Salmo salar* L. in recirculating aquaculture system. *Aquaculture Research*, *48*, 2133–2144. https://doi.org/10.1111/are.13050.

Liu, B., Wan, J., Ge, X., Xie, J., Zhou, Q., Miao, L., Ren, M., & Pan, L. (2016). Effects of dietary Vitamin C on the physiological responses and disease resistance to Ph stress and *Aeromonas Hydrophila* infection of *Megalobrama Amblycephala*. *Turkish Journal of Fisheries and Aquatic Sciences*, *16*, 421–433. https://doi.org/10.4194/1303-2712-v16_2_22.

Mansour, A. T., Goda, A. A., Omar, E. A., Khalil, H. S., & Esteban, M. A. (2017). Dietary supplementation of organic selenium improves growth, survival, antioxidant and immune status of meagre, *Argyrosomus regius*, juveniles. *Fish & Shellfish Immunology*, *68*, 516–524. https://doi.org/10.1016/j.fsi.2017.07.060.

Martoja, R., & Martoja-Pearson, M. (1970). *Técnicas de Histología Animal*, 1st ed. Toray-Masson S.A.

McCarthy, I. D., Gair, D. J., & Houlihan, D. F. (1999). Feeding rank and dominance in *Tilapia rendalli* under defensible patterns of food distribution. *Journal of Fish Biology*, *55*, 854–867. https://doi.org/10.1111/j.1095-8649.1999.tb00722.x.

McMurray, C. H., Blanchflower, W. J., & Rice, D. A. (1980). Influence of extraction techniques on determination of α-tocopherol in animal feedstuffs. *Journal of Association of Official Analytical Chemists*, *63*, 1258–1261. https://doi.org/10.1093/jaoac/63.6.1258.

Montero, D., Marrero, M., Izquierdo, M. S., Robaina, L., Vergara, J. M., & Tort, L. (1999). Effect of vitamins E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus auratus*) juveniles subjected to crowding stress. *Aquaculture*, *171*, 269–278. https://doi.org/10.1016/S0044-8486(98)00387-1.

Montero, D., Tort, L., Izquierdo, M. S., Robaina, L., & Vergara, J. M. (1998). Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. *Fish Physiology and Biochemistry*, *18*, 399–407. https://doi.org/10.1023/A:1007734720630.

North, B. P., Turnbull, J. F., Ellis, T., Porter, M. J., Migaud, H., Bron, J., & Bromage, N. R. (2006). The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *255*, 466–479. https://doi.org/10.1016/j.aquaculture.2006.01.004.

NRC (2011). *Nutrient requirements of fish and shrimp*. The National Academies Press Washington.

Ortuño, J., Esteban, M. A., & Meseguer, J. (2001). Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L.) innate immune response. *Fish & Shellfish Immunology*, *11*, 187–197. https://doi.org/10.1006/fsim.2000.0304.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, *1*, 29–45. https://doi.org/10.1093/nar/29.9.e45.

Pickering, A. D. (1998). Stress responses of farmed fish. In K. Black, & A. Pickering (Eds.), *Biology of farmed fish* (pp. 222–382). Sheffield Academic Press.

Pokorny, J., & Korczak, J. (2001). Preparation of natural antioxidant. In J. Pokorny, N. Yanishlieva, & M. Gordon (Eds.), *Antioxidants in Food: Practical Applications* (pp. 311–330). Wood head Publishing Limited.

Procarione, L. S., Barry, T. P., & Malison, J. A. (1999). Effects of high rearing densities and loading rates on the growth and stress responses of juvenile rainbow trout. *N. Am. J. Aquac.*, *61*, 91–96. https://doi.org/10.1577/1548-8454(1999)061<0091:EOHRDA>2.0.CO;2.

Rodríguez Lozano, A., Borges, P., Robaina, L., Betancor, M., Hernández-Cruz, C. M., Romero García, J., Caballero, M. J., Vergara, J. M., & Izquierdo, M. (2017). Effect of different dietary vitamin E levels on growth, fish composition, fillet quality and liver histology of meagre (*Argyrosomus regius*). *Aquaculture*, *468*, 175–183. https://doi.org/10.1016/j.aquaculture.2016.10.006.

Roo, J., Hernández-Cruz, C. M., Borrero, C., Fernández-Palacios, H., & Shuchardt, D. (2007). Efecto de la densidad larvaria y secuencia alimentaria en el cultivo larvario de corvina (*Argyrosomus regius* Asso, 1801) Durante el primer mes de vida. In A. Cerviño, A. Guerra, & C. Pérez (Eds.), *Actas XI Congreso Nacional de Acuicultura*, *24–28 Septiembre*, *Vigo* (pp. 743–746). Pontevedra.

Ruiz, M. A., Betancor, M. B., Robaina, L., Montero, D., Hernández-Cruz, C. M., Izquierdo, M., Rosenlund, G., Fontanillas, R., & Caballero, M. J. (2018). Dietary combination of vitamin E, C and K affects growth, antioxidant activity, and the incidence of systemic granulomatosis in meagre (*Argyrosomus regius*). *Aquaculture*, *498*, 606–620. https://doi.org/10.1016/j.aquaculture.2018.08.078.

Ruiz, M. A., Hernández-Cruz, C. M., Caballero, M. J., Fernández-Palacios, H., Saleh, R., Izquierdo, M. S., & Betancor, M. B. (2019a). Incidence of systemic granulomatosis is modulated by the feeding sequence and type of enrichment in meagre (*Argyrosomus regius*) larvae. *Aquaculture Research*, *50*, 284–295. https://doi.org/10.1111/are.13896.

Ruiz, M. A., Hernández-Cruz, C. M., Caballero, M. J., Fernández-Palacios, H., Saleh, R., Izquierdo, M. S., & Betancor, M. B. (2019b). Appearance of systemic granulomatosis is modulated by the dietary supplementation of vitamin E and C in meagre (*Argyrosomus regius*) larvae fed inert microdiets. *Aquaculture*, *506*, 139–147. https://doi.org/10.1016/j.aquaculture.2019.03.032

Sadhu, N., Sharma, S. K., Joseph, S., Dube, P., & Philipose, K. (2014). Chronic stress due to high stocking density in open sea cage farming induces variation in biochemical and immunological functions in Asian seabass (*Lates calcarifer*, Bloch). *Fish Physiology and Biochemistry*, *40*, 1105–1113. https://doi.org/10.1007/s10695-014-9909-8.

Sahin, K., Yazlak, H., Orhan, C., Tuzcu, M., Akdemir, F., & Sahin, N. (2014). The effect of lycopene on antioxidant status in rainbow trout (*Oncorhynchus mykiss*) reared under high stocking density. *Aquaculture*, *418*, 132–138. https://doi.org/10.1016/j.aquaculture.2013.10.009.

Sakai, T., Murata, H., Endo, M., Yamauchi, K., Tabata, N., & Fukudome, M. (1989). 2-Thiobarbituric acid values and contents of α-tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola quinqueradiata*. *Agricultural and Biological Chemistry*, *53*, 1739–1740. https://doi.org/10.1080/00021369.1989.10869531.

Sayeed, I., Parvez, S., Pandey, S., Bin-Hafeez, B., Haque, R., & Raisuddin, S. (2003). Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, *Channa punctatus* Bloch. *Ecotoxicology and Environmental Safety*, *56*, 295–301. https://doi.org/10.1016/S0147-6513(03)00009-5.

Schram, E., Van der Heur, J. W., Kamstra, A., & Verdegen, M. C. J. (2006). Stocking density-dependent growth of Dover sole (*Solea solea*). *Aquaculture*, *252*, 339–347. https://doi.org/10.1016/j.aquaculture.2005.07.011.

Shubha, M., & Reddy, S. R. (2011). Effect of stocking density on growth, maturity, fecundity, reproductive behaviour and fry production in the mouth brooding cichlid *Oreochromis mossambicus* (Peters). *African Journal of Biotechnology*, *10*, 9922–9930. https://doi.org/10.5897/AJB11.697.

Silva-Brito, F., Magnoni, L. J., Fonseca, S. B., Peixoto, M. J., Castro, L. F. C., Cunha, I., de Almeida Ozório, R. O., Magalhães, F. A., & Gonçalves, J. F. M. (2016). Dietary oil source and selenium supplementation modulate Fads2 and Elovl5 transcriptional levels in liver and brain of meagre (*Argyrosomus regius*). *Lipids*, *51*, 729–741. https://doi.org/10.1007/s11745-016-4157-6.

Turnbull, J., Bell, A., Adams, C., Bron, J., & Huntingford, F. (2005). Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. *Aquaculture*, *243*, 121–132. https://doi.org/10.1016/j.aquaculture.2004.09.022.

Vijayan, M., Ballantyne, J., & Leatherland, J. (1990). High stocking density alters the energy metabolism of brook charr, *Salvelinus fontinalis*. *Aquaculture*, *88*, 371–381. https://doi.org/10.1016/0044-8486(90)90162-G.

Wan, J. J., Liu, B., Ge, X. P., Xie, J., Cui, S. L., & Zhou, M. (2013). Effects of dietary vitamin C on growth performance, hematology and muscle physiochemical indexes of juvenile Wuchang bream (*Megalobrama amblycephala*). *Journal of Shanghai Ocean University*, *22*, 112–119.

Watanabe, T., Izquierdo, M. S., Takeuchi, T., Satoh, S., & Kitajima, C. (1989). Comparison between eicosapentaenoic and docosahexaenoic acids in terms of essential fatty acids efficacy in larval red seabream. *Nippon Suissan Gakk.*, *5*, 1635–1640. https://doi.org/10.2331/suisan.55.1635.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiological Reviews*, *77*, 591–625. https://doi.org/10.1152/physrev.1997.77.3.591.

Wuertz, S., Lutz, I., Gessner, J., Loeschau, P., Hogans, B., Kirschbaum, F., & Kloas, W. (2006). The influence of rearing density as environmental stressor on cortisol response of Shortnose sturgeon (*Acipenser brevirostrum*). *Journal of Applied Ichthyology*, *22*, 269–273. https://doi.org/10.1111/j.1439-0426.2007.00966.x.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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