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

Running title: Supplementation of vitamin E and C prevent granulomatosis in meagre larvae.

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Abstract

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

Keywords: meagre larvae, antioxidant vitamins, granulomatosis

1. Introduction

The whole life cycle of meagre (*Argyrosomus regius*) has been successfully closed, however there are still some challenges in meagre farming, being one of the more predominant ones the systemic granulomatosis. Systemic granulomatosis is a disease of unknown aetiology, although it has recently been evidenced that nutritional imbalances can promote its appearance (Ruiz et al., 2018a; Cotou et al., 2016). It is a non-infectious disease that affects internal organs, mainly liver, kidney and heart, where granulomas composed by a necrotic centre and surrounded by a layer of epithelial cells and macrophages are observed in the final stages (Ruiz et al., 2018a). It must be noted that the prevalence of systemic granulomatosis is so high in adult meagre that it can affect almost 100 % of population (Ghittino et al., 2004), being this stage too late to try to avoid the appearance of the disease. Nevertheless, granulomas have not only been detected in adult fish, but meagre larvae have also been found to show this histological alteration at very early stages (Ruiz et al., 2018b). In the afore mentioned study, granulomas were first described in liver and kidney at 20 days post hatching (dph) albeit differences were found among larvae fed the different dietary treatments/feeding sequences. In this sense, a co-feeding with rotifers (*Brachionus plicatilis*) and *Artemia* prior to weaning on an inert commercial microdiet proved to prevent the appearance of granulomas. On the other hand, when *Artemia* was not included in the feeding sequence, granulomas were detected from 20 dph although the incidence varied depending on the enrichment media used what again strengthens the hypothesis of a nutritional origin of the pathology. Therefore, a balanced nutrition during the first life stages of meagre could potentially prevent the development of systemic granulomatosis.

Imbalances in vitamins, particularly antioxidant vitamins such as vitamin E and C, have long been speculated to play a pivotal role in the appearance of systemic granulomatosis. Appearance of granulomas in gilthead sea bream (*Sparus aurata*) and turbot (*Scophthalmus maximus*) has been associated to a dietary deficiency of vitamin C (Paperna et a., 1980; Baudin-Laurencin et al., 1989, Coustans et al., 1990; Alexis et al., 1997).A deficiency in this nutrient causes an impairment of tyrosine catabolism, which leads to its precipitation in tissues and thereby the development of the granulomas

(Goldsmith, 1978). In previous studies, the combination of high dietary content of antioxidant vitamin E, C and K (15, 450 and 230 mg kg^{-1}, respectively) reduced the incidence of granulomas in juvenile meagre (Ruiz et al., 2018a). However, a high prevalence of granulomas was observed at the beginning of the experimental trial what prompted to evaluate the combination of vitamins at earlier life stages. If vitamins are to be blamed for the appearance of systemic granulomatosis, meagre larvae might be then at a higher risk of suffering the pathology as their higher growth and metabolic rates mean that vitamin requirements might be higher for larvae than juveniles or adult fish (Dabrowski, 1992). Additionally, limited information is available about the requirements of vitamin E and C in meagre larvae almost of the studies have been mainly focused on adults or juvenile fish. A recent study by El Kertaoui et al. (2017) showed that high levels of both vitamin E and C (1,500 and 1,800 mg kg^{-1}, respectively) improved growth and protection against oxidative stress in meagre larvae, but the effect of these antioxidant vitamins on the appearance of granulomas was not evaluated. Recently, the appearance of systemic granulomatosis has been observed to be affected by the fatty acid profile of the diet in meagre larvae, where the lowest supplementation of n-3 LC-PUFA (0.8 %) lead to a higher incidence of granulomas in liver (Carvalho et al., 2018). Docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are considered essential fatty acids in marine fish and are involved in the maintenance of structural and functional integrity of cell membranes (Izquierdo and Koven, 2011), normal growth (Rodríguez *et al*., 1994; Salhi et al., 1997) and immune-deficiency (Izquierdo, 1996).The different fatty acid profile between fish oil and krill oil could have an impact on the appearance of granulomas, moreover, an absence of adequate levels of antioxidants, may lead to lipid oxidation as long as PUFA are available for oxidation (Hamre, 2011). Krill oil is higher in some fatty acids, such as EPA and DHA, compared to fish oil (Tou et al., 2007). Moreover, the phospholipid composition is different in both oils, in fish oil fatty acids are mainly stored as triglycerides, whereas in krill 30–65 % of the fatty acids are incorporated into phospholipids (Tou et al., 2007), which have higher bioavailability and are involved in regulation of more metabolic pathways (Ulven and Holven, 2015).

The overall aim of the present study was to evaluate the role of vitamin E and C in the appearance of systemic granulomatosis in weaned larvae (30 dph). Prior to the start of the study larvae were co-fed with rotifer and *Artemia* enriched with Easy DHA Selco as larvae fed this dietary regime did not show any granulomas at 30 dph in a previous trial (Ruiz et al., 2018b). Following this feeding sequence meagre will be fed four microdiets formulated to contain graded levels of inclusion of vitamin E and C. Additionally a fifth diet was formulated to contain krill oil as the single lipid source. Fish larvae growth and survival, histopathological evaluation and biochemical analysis were determined.

2. Materials and methods

2.1. Fish

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

Rotifers were cultured at a density of 400 rotifers mL^{-1} in 500 L enrichment troncoconical-tanks, with 80 % seawater and 20 % freshwater. Rotifers were enriched with Easy DHA Selco (INVE, Dendermonde, Belgium) (0.6 g L^{-1}) for 24 h. Meagre larvae were fed with enriched rotifers twice daily from 3 to 21 dph, before each feeding, rotifers were counted and added to maintained at a density of 10 rotifers L^{-1} in the experimental tanks. *Artemia* cyst were hatched at 27 °C and 0.030 mg L^{-1} salinity until 100 % hatch was achieved. Then, they were rinsed with seawater and transferred to a culture tank at 24 °C. *Artemia* was enriched with Easy DHA Selco (0.6 g million *Artemia*$^{-1}$) for 24 h before being fed to the larvae. Meagre larvae were fed with enriched *Artemia* from 12 to 30 dph following the protocol established by Ruiz et al., (2018b). Before each feeding, *Artemia* were counted and added to maintained at a density of 1.3-1.5 *Artemia* L^{-1} in the experimental tanks. From 20 to 30 dph larvae were co-fed with *Artemia* and microdiet and fed microdiet only from 30 to 44 dph.

Larvae of 30 dph (total length 8.83± 0.65 mm, dry body weight 1.1 ± 0.01 mg) were randomly distributed in light grey colour cylindrical fibreglass experimental tanks (15 tanks; triplicate treatment) of 170 L capacity at a density of 3000 larvae $tank^{-1}$ and fed one of the five experimental diets for 14 days. All tanks were equipped with continuous

aeration and supplied with filtered UV-sterilized seawater at an increasing rate from 35% h^{-1} to a 100% h^{-1}, to guarantee good water quality during the trial. Water entered the tanks at the bottom and exited at the surface. Oxygen (4.5-6.5 g L^{-1}), salinity (34 g L^{-1}) salinity and temperature (21.8 to 22.3º C) was daily measured. Photoperiod was kept at 12 h light: 12 h dark by fluorescent lights.

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

2.2. Diets

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

The microdiet was prepared according to Liu et al. (2002) as follows: the krill meal was mixed with the water-soluble ingredients (attractants, minerals and water-soluble vitamins). Oil and fat-soluble vitamins were mixed and blended with the dry ingredients. Finally, gelatine dissolved in warm water was added to the mix. The paste was pelleted and dried at 38° C for 24 h. The final pellets were ground and sieved in two different particle

sizes (120-250 and 250-500 µm). Diets were kept at 4° C during the feeding period. Proximate composition and fatty acids levels were analysed for each diet prior to the start of the trial (Table 1 and 2). Fatty acid profile was similar in all the experimental diets excepting for diet "KRILL", which showed higher amounts of EPA (16.0 %) and DHA (8 %) than the other diets, what in turn increased total n-3 PUFA (Table 2). On the other hand, total n-6 PUFA was lower in KRILL, mainly due to the higher amount of linoleic acid in the diets with fish oil (7.2 %versus 4.1 %). Fish larvae were fed each 45 min daily from 8:00 to 20:00 with 3, 3.5 and 4 g $tank^{-1}$, during the first, second and third week respectively.

2.3. Sample collection

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

2.4. Growth and survival

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

2.5. Histopathology

Formalin fixed samples were dehydrated in a series of different concentrations of ethanol and embedded in a paraffin block. The samples were cut at 4 μm on a microtome, fixed to the microscope slide and finally stained with haematoxylin and eosin (H&E), Ziehl-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco method (Fite et al., 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological evaluation, analysing all tissues and focusing especially, in liver, kidney and heart, given that these organs are the main affected by granulomas (Ruiz et al., 2018a).

2.6. Biochemical analysis

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

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

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

expressed as μmol MDA per g of tissue and was calculated using the extinction coefficient 0.156 μM^{-1} cm^{-1}.

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

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

2.7. Statistical analysis

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

3. Results

3.1. Growth and survival

All experimental diets were well accepted by larvae. Final total length, dry weight and survival were not significant different among larvae fed the different experimental feeds at the end of the feeding trial (44 dph). The average final total length was 25.8 ± 0.4 mm, dry weight 17.5 ± 1.3 mg, survival 20.1 ± 0.5 % and SGR 17.1 ± 1.2 % (Table 3).

3.2. Histopathology

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

There was a strong and significant negative correlation between the percentage of larvae with granulomas and dietary concentration of vitamin E ($y = -0.0098x + 11.174$, $R^2 = 0.8766$; Pearson's correlation coefficient (r) = -0.93) and vitamin C ($y = -0.0022x + 6.4777$, $R^2 = 0.9278$; Pearson's correlation coefficient (r) = -0.96) (Figure 3). The TBARs content was highly correlated with the appearance of granulomas ($R^2=0.892$, $y=0.0446x+0.0756$).

All the specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stain) were negative, discarding a possible infectious origin of the granulomas (Supplementary Figure 1).

The histopathological evaluation revealed granulomas in different stages of development (Supplementary Figure 2) as described by Ruiz et al. (2018a) in ongrowing meagre. At initial stages, granulomas were observed as isolated and irregular aggregated of macrophages (Supplementary Figure 2a) that later were forming concentric layers

295 (Supplementary Figure 2b). These aggregated progressively lead to a necrotic centre with
296 external layers of fibrocytes (Supplementary Figure 2c). However, final stages of
297 development, in which the granuloma is completely composed of laminar material, were
298 not observed.

299 **3.3. Biochemical analysis**

300 **3.3.1. Whole larvae proximal composition and fatty acid profile**

301 Dietary treatment did not affect larvae whole body proximate composition after 14
302 days of feeding, with a protein content averaging 11 % and lipid exceeding 2 % among
303 larvae fed the different dietary treatments (Table 4). The substitution of krill oil by fish oil
304 significantly increased de levels of eicosapentaenoic acid (EPA, 16.6 vs 13.7 %) and
305 docosahexaenoic acid (DHA, 17.63 vs 16.05 %) in meagre larvae at the end of the feeding
306 trial, compared with the larvae fed with the other diets (EPA ~ 13.7 % and DHA ~ 16.1 %)
307 (Table 5). Furthermore, the addition of krill oil significantly increased the peroxidation
308 index in the larvae (275.3 vs 245.7) (Table 5) and the concentration of saturated fatty acids
309 (31.2 vs 29.0 %), n-3 PUFA (38.2 vs 34.3 %) and n-3 LC-PUFA (35.6 vs 31.6 %) (Table
310 5). Larvae fed fish oil diets showed significant higher concentration of oleic acid, linoleic
311 acid, monosaturated fatty acids, n-6 and n-9 PUFA regardless dietary levels of vitamin E
312 and C (Table 5).

313 **3.3.2. TBARs content**

314 The level of lipid peroxides, as indicated by TBARs content (μmol g^{-1} larval
315 tissues), was significantly lower in those larvae fed diets with the highest levels of vitamin
316 E and C (Table 4).

317 **4. Discussion**

318 It has previously been shown that the co-feeding with rotifer and *Artemia* enriched
319 with Easy DHA Selco prior to eating an inert commercial microdiet prevented the
320 appearance of granulomas in meagre larvae (Ruiz et al., 2018b). Consistently, no
321 granulomas were observed at 30 dph in the present trial after following the same feeding
322 sequencing and enrichment protocol what seems to reinforce the role of nutrition as the
323 main trigger in the appearance of systemic granulomatosis. The results of the present trial

showed that the dietary addition of different levels of vitamin E (40, 200, 400 and 800 mg kg^{-1}) and C (100, 500, 1,000 and 2,000 mg kg^{-1}) did not affect meagre larvae performance in terms of growth, length, survival and SGR at 44 dph. However, granulomas were observed in larvae fed with low levels of vitamin E and C (from 40/100 to 400/1,000 mg kg^{-1}, vitamin E/C). The results suggest that low levels of vitamin E and C (40 and 100 mg kg^{-1}, respectively) probably fulfilled the requirement for normal growth what explains the lack of differences in terms of fish performance among larvae fed the different dietary treatments but were not enough to prevent systemic granulomatosis. On this matter, a strong negative correlation was observed between the dietary levels of vitamin E ($y = -0.0098x + 11.174$, $R^2 = 0.8766$) and vitamin C ($y = -0.0022x + 6.4777$, $R^2 = 0.9278$) and the incidence of granulomas. Little is known about requirements of vitamin E and C in meagre larvae. Only El Kertaoui et al. (2017) observed that high levels (1,500 and 1,800 mg kg^{-1} of vitamin E and C, respectively) were required to improve growth and antioxidant defenses in meagre larvae at 28 dph. It is well known that the requirement for antioxidant vitamins is conditioned by the dietary fatty acids content. In this regard, all the experimental microdiets contained a sufficient amount of essential fatty acids for most marine fish species, which require at least 2 % EPA and DHA (NRC, 2011). Nevertheless, those larvae fed with higher amounts of DHA and EPA together with low dietary vitamin E and C (diet Krill) presented high incidence of granulomas, suggesting an imbalance between prooxidant and antioxidant nutrients. Accordingly, TBARs content, an indicator of lipid oxidation, was affected by the dietary inclusion of vitamin E and C, with the high supplementation of vitamin E (800 mg kg^{-1}) and C (2,000 mg kg^{-1}) significantly reducing TBARs values. Indeed, TBARs contents were highly correlated with the appearance of granulomas ($R^2=0.892$, $y=0.0446x+0.0756$). Therefore, adequate dietary levels of vitamins E and C seem to mitigate the appearance of systemic granulomatosis in meagre larvae, probably due to the decrease of the oxidation rate.

Vitamin E together with vitamin C are strong antioxidants in tissues, being able to neutralize reactive oxygen species (ROS) (Montero et al., 1999; Ai et al., 2006; Betancor et al., 2012; Gao et al., 2014) and increase the protection against lipid peroxidation (Lee and Dabrowski, 2003). The oxidative stress has been related with some diseases (Kawatsu, 1969; Cowey et al., 1984; Sakai et al., 1989; Watanabe et al., 1989; Sies et al., 1992;

Padayatty and Levine, 2001; Lewis-McCrea and Lall, 2007), therefore it is feasible to think that granulomas could also be originated by an oxidative imbalance. Lipid peroxidation contributes to the inflammatory response (Morita el al., 2016). Granuloma formation is an inflammatory response, and is composed basically by macrophages, lymphocytes and fibrocytes, being its appearance not necessarily associated with infectious diseases. This inflammation can occur in blood vessels (Petersen and Smith, 2013; Hilhorst et al., 2014). In this sense, in the present and previous studies (Ruiz et al., 2018a) irregular aggregates of cells and granulomas have been observed surrounding blood vessels, which suggests that granulomas could have a vascular origin. Vitamin C has been related with the synthesis of collagen, an important protein involved in the generation of blood vessels (Lim and Lovell, 1978; Nusgen et al., 2001). Besides, vitamins C and E are involved in the prevention of endothelial dysfunction and the prevention of oxidative stress (Riitta et al., 2003; Engler et al., 2003). In this sense, an imbalance between ROS and antioxidants could be happening in larvae fed with low addition of vitamin E and C, as indicated by TBARs values, which could lead to inflammatory response in blood vessel with the subsequent macrophages infiltration and formation of granulomas. Limited information is available on the effect of antioxidant vitamins in the formation of granulomas. In other fish species vitamin C deficiency has been related to precipitation of tyrosine in tissues, being the origin of granulomas, in species such as sea bream and turbot (Baudin-Laurencin et al., 1989; Coustans et al., 1990; Alexis et al., 1997). In agreement, a previous study showed that the dietary increase of vitamins E and C lead to a reduction in the percentage of granulomas in liver and heart of juvenile meagre together with a decrease in TBARs contents (Ruiz et al., 2018ab), what indicates less lipid peroxidation.

The substitution of fish oil by krill oil significantly increased de levels of eicosapentaenoic acid (EPA, 16.6 %) and docosahexaenoic acid (DHA, 17.6 %) in meagre larvae with 44 dph, compared with the larvae fed the other diets (EPA ~ 13.7 and DHA ~ 16.1 %). This difference in the levels of n-3 LC-PUFA seemed to have an impact on the TBARs content which in turn translated into a higher incidence of granulomas compared to larvae fed fish oil in combination with the same dietary levels of antioxidant vitamins (Diet C+, 400 and 1,000 mg kg^{-1} vitamin E and C, respectively). Apart from being an excellent source of EPA and DHA, krill oil is rich in phospholipids and particularly

386 phosphatidylcholine (Winther et al., 2011). Phospholipids have been described to have a
387 stronger biological effect than triglycerides, because they can be more rapidly digested and
388 are more effectively incorporated to the tissues than triglycerides (Ackman and Ratnayake
389 1989), can act as ligands for nuclear receptor (Li et al., 2005; Chakravarthy et al., 2009),
390 are involved in the steroidogenesis and cholesterol metabolism, and have been shown to
391 augment the bioavailability of DHA and EPA (Amate et al., 2001; Cansell et al., 2003;
392 Cansell et al., 2009). Despite of the high phospholipid level provided by the krill oil, it
393 could not prevent the appearance of granulomas, needing supplementation with higher
394 levels of vitamin E and C (over 400 and 1,000 mg kg^{-1}, respectively) in order to inhibit its
395 appearance. However, the percentage of granulomas was significantly higher in larvae fed
396 diet "krill" than those larvae fed diet "C+", although both diets contained the same levels of
397 vitamin E (844 and 859 mg kg^{-1}, respectively) and C (1,460 and 1,450 mg kg^{-1},
398 respectively) were roughly the same. This could be related to the higher EPA and DHA
399 contents (therefore, higher peroxidation index) found in larvae fed diet "krill", what
400 suggests that the balance between prooxidant and antioxidant nutrients is disturbed in
401 favour of prooxidants. In this point, it should be noted that the higher peroxidation index
402 should be correlated to higher TBARs values. Nevertheless, larvae fed diet "krill" were not
403 different to those of fish fed fish oil (C+). This could be due the fact that EPA and DHA are
404 in phospholipid forms and were more protected in the krill diet, while in the diet with fish
405 oil they were in triglycerides, being more susceptible to oxidation. Moreover, although krill
406 oil contains antioxidants, mainly astaxanthin (Tou et al., 2007), these were no able to
407 prevent the appearance of granulomas. These results suggest that the appearance of
408 granulomas is more related to the supplementation of different levels of vitamin E and C
409 more than to the source of dietary fatty acids. In fact, in a previous study the appearance of
410 granulomas in juvenile meagre was modulated by the inclusion of different levels of the
411 antioxidants vitamins E and C (Ruiz et al., 2018a).

412 Concluding, the supplementation of vitamin E and C at 40 and 100 mg kg^{-1}
413 respectively is adequate to ensure good meagre larvae performance. However, these
414 vitamin levels might not be enough to prevent the appearance of systemic granulomatosis,
415 as indicated by the strong negative correlation between dietary vitamin E and C contents
416 and the prevalence of granulomas and TBARs values. Levels of dietary vitamin E and C of

1,082 and 2,910 mg kg^{-1} (Diet EECC) completely prevented the appearance of granulomas. The substitution of fish oil by krill oil was enough to the correct growth of meagre larvae but increased the percentage of granulomas and the peroxidation index. Therefore, it has been demonstrated in the present and previous studies (Ruiz et al., 2018b) that systemic granulomatosis can be completely mitigated in meagre larvae by controlling feeding sequence as well as levels of antioxidant nutrients.

5. Acknowledgements

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Figure legends

Figure 1. Incidence of granulomas (%) in meagre larvae at the end of the dietary trial (44 dph). Each value represents mean ± SD (n= 120).

Figure 2. Percentage of affected organs with granulomas in meagre larvae of 44 dph fed different levels of vitamin E and C.

Figure 3. Effect of dietary vitamin E and C on percentage of affected meagre larvae with granulomas at 44 dph.

Supplementary Figure 1. Negative results in granulomas for specific stains. A) Ziehl-Neelsen, B) Gram stain and C) Fite-Faraco stain in kidney.

Supplementary Figure 2. Granulomas at different stages of development in kidney of meagre larvae (44 dph) at the end of the experimental trial. **A)** Irregular aggregated of macrophages. **B)** Concentric layers of macrophages and some lymphocytes. **C)** Necrotic center surrounded by layers of macrophages and an outer layer of fibrocytes.

Tables

Table 1. Formulation and analysed proximate composition of diets fed to meagre larvae from 30 to 44 dph, containing different levels of vitamin E and C and either fish or krill oil as the lipid source.

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

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

664 **Table 2**. Diets fatty acid composition (percentage of fatty acids) used for feeding meagre
665 larvae fed from 30 to 44 days post hatching (dph) in the present trial.

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

666 Data expressed as means of three technical replicates per batch of diet.[1]Includes 15:0 and
667 17:0.[2]Includes 14:1n-7. 14:1n-5. 15:1n-5. 16:1n-5. 18:1n-5. 20:1n-9. and 20:1n-5.[3]Includes.
668 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3. [5]LC- PUFA, long-chain
669 polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

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672 **Table 3**. Growth performance of meagre larvae fed the experimental feeds at 30 (initial)
673 and 44 days post hatching (dph).

	Diets					
	Initial	C+	C-	EC	EECC	KRILL
Total length (mm)	8.8 ± 1.4	26.3 ± 4.2	25.6 ± 3.9	24.9 ± 4.3	26.0 ± 4.1	26.2 ± 4.3
Dry weight (mg)	1.1 ± 0.2	16.2 ± 4.0	16.6 ± 1.8	16.4 ± 4.7	19.2 ± 3.5	17.3 ± 3.7
SGR (% d^{-1})	-	16.8 ± 4.0	17.5 ± 1.8	16.9 ± 4.7	18.5 ± 3.5	17.2 ± 3.7
Survival (%)	-	19.5 ± 1.3	20.1 ± 1.2	20.9 ± 2.3	20.3 ± 1.0	19.7 ± 0.9

674 Data are means ± SD. dph, days post hatching; SGR, specific growth rate.
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Table 4. Proximate composition and TBARs content in meagre larvae (44 dph) fed with the experimental diets.

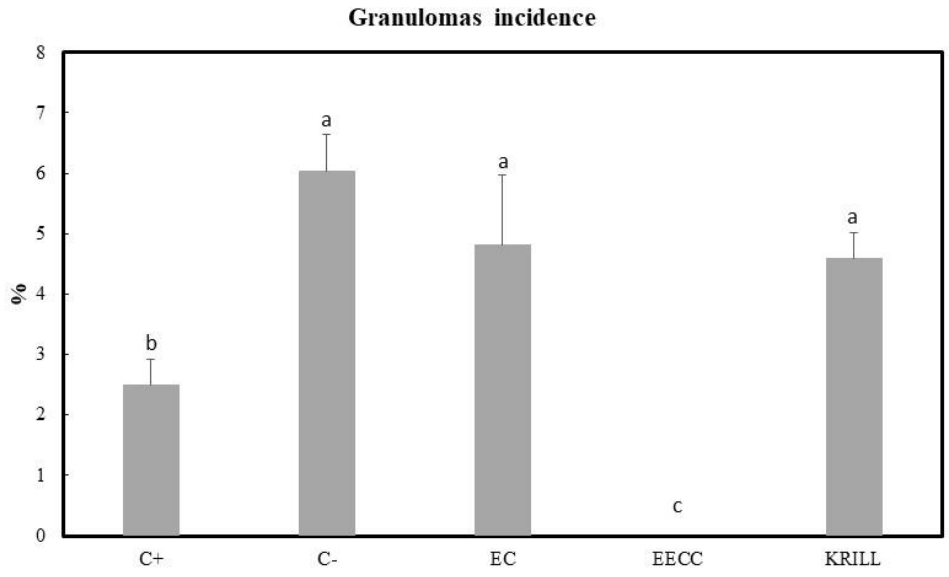
Diets	C+	C-	EC	EECC	KRILL
Proximate composition (%)					
Protein	11.0 ± 1.1	10.4 ± 2.1	10.6 ± 1.4	10.9 ± 1.8	11.8 ± 2.0
Lipid	2.8 ± 0.4	2.5 ± 0.2	2.3 ± 0.3	2.4 ± 0.4	2.4 ± 0.1
Moisture	82.9 ± 2.8	83.9 ± 2.1	83.7 ± 2.1	82.7 ± 0.9	82.9 ± 2.2
Ash	2.5 ± 0.6	2.8 ± 0.1	2.9 ± 0.2	3.1 ± 0.2	2.3 ± 0.1
TBARs content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

Data expressed as means of three technical replicates per batch of larvae (n = 3). Different superscript letters denote differences among treatments identified by one-way ANOVA ($P<0.05$).

712 **Table 5**. Fatty acid composition (percentage of fatty acids) of meagre larvae fed with
713 experimental diets at the end of the dietary trial (44 days post hatching).

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

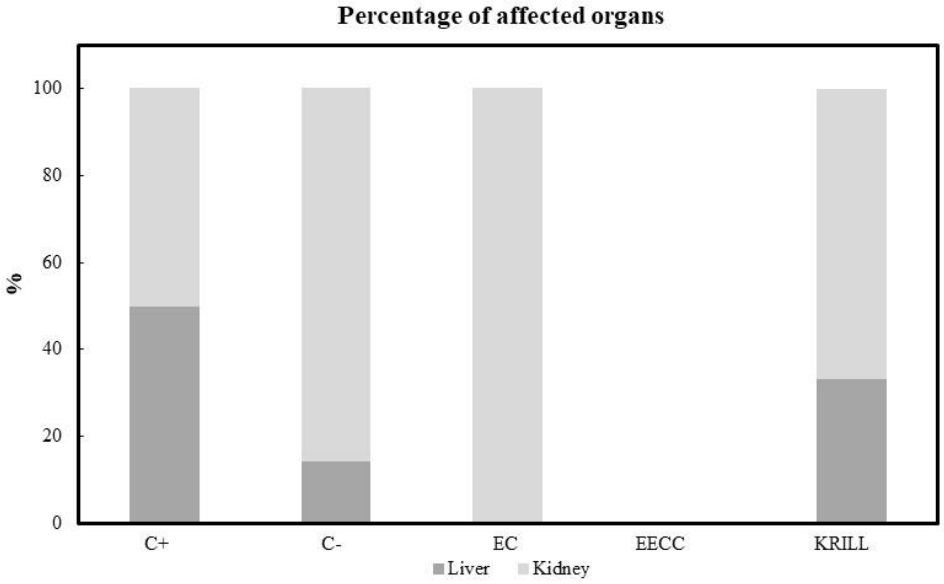
714 Data expressed as means of three technical replicates per batch of larvae (n = 3). Different
715 superscript letters denote differences among treatments identified by one-way ANOVA
716 ($P<0.05$). [1]Includes 15:0 and 17:0.[2]Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5,
717 20:1n-9 and 20:1n-5.[3]Includes 22:5n-6 and 22:4n-6. [4]Includes 16:3n-3 and 16:4n-3.
718 [5]Includes. 22:1n-9. 20:3n-9. 20:2n-9. 20:1n-9. 18:2n-9. 18:1n-9. [6] LC-PUFA, long-chain
719 polyunsaturated fatty acid (sum of 20:4n-3. 20:5n-3 22:5n-3 and 22:6n-3). PIn,
720 peroxidation index.
721



722

723 Figure 1

724

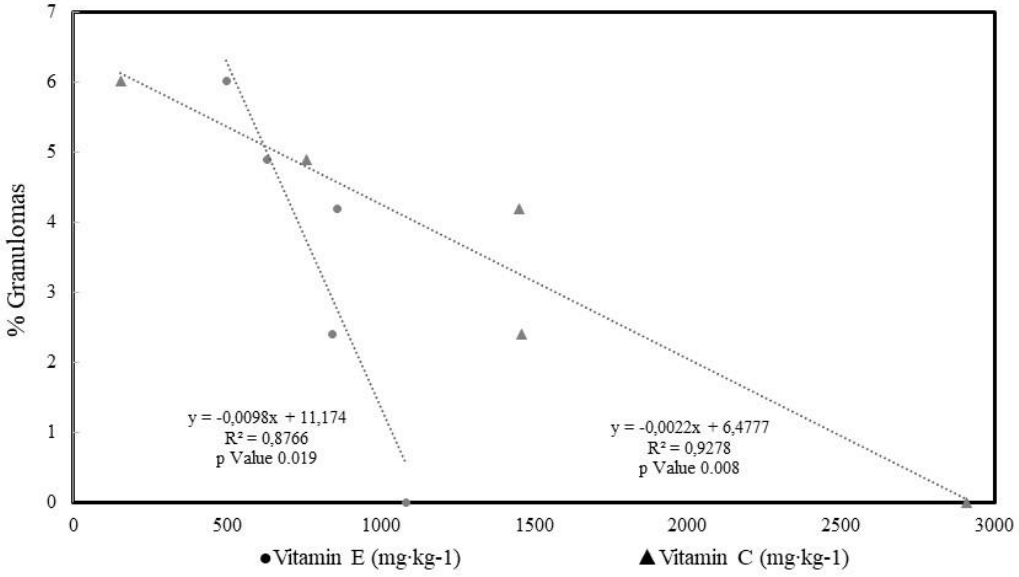


725

726

727 Figure 2

728



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730 Figure 3

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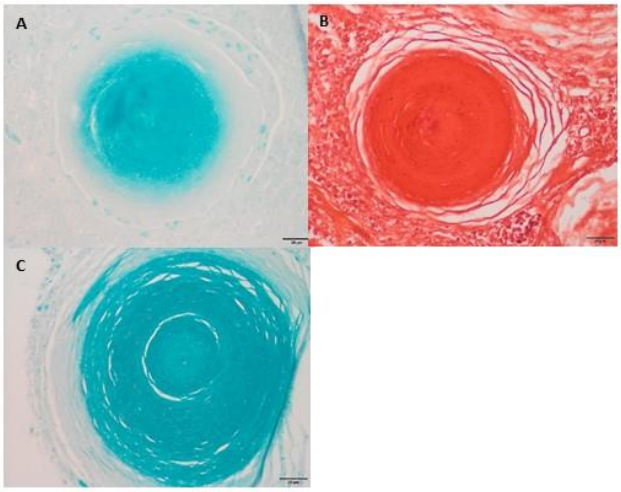
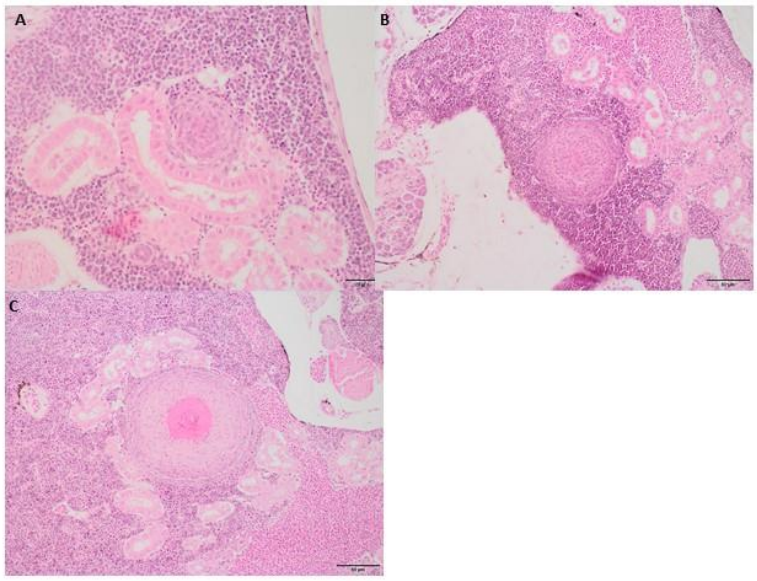


Supplementary Figure 1



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738 Supplementary Figure 2