

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

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

6 Ruiz, M.A.^{1*}, Hernández-Cruz, C.M.¹, Caballero M.J.¹, Fernández-Palacios, H.¹, Saleh,
7 R.^{1,2}, Izquierdo, M.S.¹, Betancor, M.B.³

8
9
10 ¹Aquaculture Research Group (GIA), Instituto Ecoaqua, Universidad de Las Palmas de
11 Gran Canaria, PCTM, Crta. Taliarte s/n, 35214, Telde, Spain.

12 ²

13 ³Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling
14 FK9 4LA, United Kingdom.

15
16 ***Corresponding author:** miguel.ruiz106@alu.ulpgc.es

17 Accepted refereed manuscript of:

18 Ruiz MA, Hernandez-Ruiz CM, Caballero MJ, Fernandez-Palacios H, Saleh R, Izquierdo MS & Betancor MB (2019)
19 Appearance of systemic granulomatosis is modulated by the dietary supplementation of vitamin E and C in meagre
(*Argyrosomus regius*) larvae fed inert microdiets [Supplementation of vitamin E and C prevent granulomatosis in meagre
larvae]. *Aquaculture*, 506, pp. 139-147.

20 DOI: <https://doi.org/10.1016/j.aquaculture.2019.03.032>

21 © 2019, Elsevier. Licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International
<http://creativecommons.org/licenses/by-nc-nd/4.0/>

29 **Abstract**

30

31 Systemic granulomatosis has already been reported in meagre larvae with an adequate
32 feeding protocol and enrichment media preventing its appearance in the first weeks of life.
33 Afterwards, the control of this disease could be prevented through nutritional components
34 of the inert food, being the antioxidants the key to success. For this reason, in the present
35 study, meagre larvae were reared from 30 days post hatching (dph) with five isonitrogenous
36 and isolipidic experimental microdiets with different levels of vitamin E and C: C- (40 mg
37 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
38 mg kg⁻¹ C and substitution of fish oil by krill oil), EC (200 mg kg⁻¹ E, 500 mg kg⁻¹ C) and
39 EECC (800 mg kg⁻¹ E, 2,000 mg kg⁻¹ C). Prior to this, larvae were co-fed with rotifers and
40 *Artemia* following a protocol which prevented the appearance of granulomas, as previously
41 demonstrated. The substitution of fish oil by krill oil significantly increased levels of
42 eicosapentaenoic acid (EPA, 16.6 %) and docosahexaenoic acid (DHA, 17.6 %) in meagre,
43 consequently increasing the peroxidation index, which in turn translated into a higher
44 incidence of granulomas. Although even low levels of vitamin E and C (40 mg kg⁻¹ E, 100
45 mg kg⁻¹ C; C-) allowed the adequate growth of larvae, these levels were not enough to
46 prevent the appearance of granulomas, requiring superior levels of both antioxidant
47 vitamins (800 mg kg⁻¹ E and 2,000 mg kg⁻¹ C) to mitigate systemic granulomatosis. This
48 mitigation was simultaneous with the reduction of thiobarbituric acid reactive substances
49 TBARs content in larvae, which were highly correlated with the appearance of granulomas
50 ($R^2=0.892$, $y=0.0446x+0.0756$). A strong negative correlation was observed between the
51 dietary levels of vitamin E ($y = -0.0098x + 11.174$, $R^2 = 0.8766$, $p \text{ value} = 0.019$, $r = -0.93$)
52 and vitamin C ($y = -0.0022x + 6.4777$, $R^2 = 0.9278$, $p \text{ value} = 0.003$, $r = -0.96$) and the
53 percentage of larvae with granulomas. The results showed that the occurrence of systemic
54 granulomatosis seems to be associated to the larvae peroxidation status, so that high dietary
55 levels of vitamin E and C (800 and 2,000 mg kg⁻¹, respectively; Diet EECC), reduced lipid
56 peroxidation and completely prevented the appearance of granulomas in meagre larvae at
57 44 dph.

58

59 **Keywords:** meagre larvae, antioxidant vitamins, granulomatosis

60 1. Introduction

61

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

84 Imbalances in vitamins, particularly antioxidant vitamins such as vitamin E and C,
85 have long been speculated to play a pivotal role in the appearance of systemic
86 granulomatosis. Appearance of granulomas in gilthead sea bream (*Sparus aurata*) and
87 turbot (*Scophthalmus maximus*) has been associated to a dietary deficiency of vitamin C
88 (Paperna et al., 1980; Baudin-Laurencin et al., 1989, Coustans et al., 1990; Alexis et al.,
89 1997). A deficiency in this nutrient causes an impairment of tyrosine catabolism, which
90 leads to its precipitation in tissues and thereby the development of the granulomas

91 (Goldsmith, 1978). In previous studies, the combination of high dietary content of
92 antioxidant vitamin E, C and K (15, 450 and 230 mg kg⁻¹, respectively) reduced the
93 incidence of granulomas in juvenile meagre (Ruiz et al., 2018a). However, a high
94 prevalence of granulomas was observed at the beginning of the experimental trial what
95 prompted to evaluate the combination of vitamins at earlier life stages. If vitamins are to be
96 blamed for the appearance of systemic granulomatosis, meagre larvae might be then at a
97 higher risk of suffering the pathology as their higher growth and metabolic rates mean that
98 vitamin requirements might be higher for larvae than juveniles or adult fish (Dabrowski,
99 1992). Additionally, limited information is available about the requirements of vitamin E
100 and C in meagre larvae almost of the studies have been mainly focused on adults or
101 juvenile fish. A recent study by El Kertaoui et al. (2017) showed that high levels of both
102 vitamin E and C (1,500 and 1,800 mg kg⁻¹, respectively) improved growth and protection
103 against oxidative stress in meagre larvae, but the effect of these antioxidant vitamins on the
104 appearance of granulomas was not evaluated. Recently, the appearance of systemic
105 granulomatosis has been observed to be affected by the fatty acid profile of the diet in
106 meagre larvae, where the lowest supplementation of n-3 LC-PUFA (0.8 %) lead to a higher
107 incidence of granulomas in liver (Carvalho et al., 2018). Docosahexaenoic acid (DHA,
108 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are considered essential fatty acids in
109 marine fish and are involved in the maintenance of structural and functional integrity of cell
110 membranes (Izquierdo and Koven, 2011), normal growth (Rodríguez *et al.*, 1994; Salhi et
111 al., 1997) and immune-deficiency (Izquierdo, 1996). The different fatty acid profile between
112 fish oil and krill oil could have an impact on the appearance of granulomas, moreover, an
113 absence of adequate levels of antioxidants, may lead to lipid oxidation as long as PUFA are
114 available for oxidation (Hamre, 2011). Krill oil is higher in some fatty acids, such as EPA
115 and DHA, compared to fish oil (Tou et al., 2007). Moreover, the phospholipid composition
116 is different in both oils, in fish oil fatty acids are mainly stored as triglycerides, whereas in
117 krill 30–65 % of the fatty acids are incorporated into phospholipids (Tou et al., 2007),
118 which have higher bioavailability and are involved in regulation of more metabolic
119 pathways (Ulven and Holven, 2015).

120

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

129 **2. Materials and methods**

130 **2.1. Fish**

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

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

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

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

156 All procedures were conducted in accordance with the regulations set forward by the
157 Spanish RD 53/2013 (BOE 8th February 2013) and the Directive 2010/63/EU of the
158 European Parliament and of the Council of 22 September 2010 on the protection of animals
159 used for scientific purposes. The experiment was subjected to ethical review by the Animal
160 Welfare and Bioethical Committee at the University of Las Palmas de Gran Canaria (Ref
161 06/2018 OEBA ULPGC).

162 **2.2. Diets**

163 Five isonitrogenous and isolipidic experimental microdiets (pellet size 120-250 &
164 250-500 µm) were formulated (Tables 1 and 2). Krill meal was the source of protein
165 whereas fish oil was the source of lipid, excepting for the diet labelled “Krill” in which krill
166 oil was the single lipid source. Prior to preparing the feeds, the krill meal was defatted
167 (three consecutive times with a chloroform: krill meal ratio of 3:1) to allow a better control
168 of the fatty acid profile of the microdiet. A positive control diet (C+) was formulated based
169 on the vitamin E (400 mg kg⁻¹) and C (1,000 mg kg⁻¹) levels found in a commercial
170 microdiet (Gemma Micro 150 and 300 µm; Skretting, France). Based on this level of
171 vitamins, other three diets with higher and lower levels of vitamin E and C was formulated,
172 diet C-, Krill, EC and EECC (40/100, 400/1,000, 200/500 and 800/2,000 mg kg⁻¹ vitamin E
173 and C respectively). Krill oil diet was formulated using krill oil as the only lipid source.
174 Soy lecithin was used as a source of phospholipids, excepting in diet “krill” were
175 phospholipid were provided from the krill oil used.

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

181 sizes (120-250 and 250-500 μm). Diets were kept at 4° C during the feeding period.
182 Proximate composition and fatty acids levels were analysed for each diet prior to the start
183 of the trial (Table 1 and 2). Fatty acid profile was similar in all the experimental diets
184 excepting for diet “KRILL”, which showed higher amounts of EPA (16.0 %) and DHA (8
185 %) than the other diets, what in turn increased total n-3 PUFA (Table 2). On the other hand,
186 total n-6 PUFA was lower in KRILL, mainly due to the higher amount of linoleic acid in
187 the diets with fish oil (7.2 % versus 4.1 %). Fish larvae were fed each 45 min daily from
188 8:00 to 20:00 with 3, 3.5 and 4 g tank⁻¹, during the first, second and third week
189 respectively.

190 **2.3. Sample collection**

191 Samplings were performed at 30 and 44 dph. At the beginning of the experiment (30
192 dph) 100 larvae were sacrificed with an overdose of anaesthetic (clove oil; Guinama,
193 Valencia, Spain) and fixed in 4 % buffered formalin for histological analysis. After two
194 weeks (44 dph) 70 larvae per tank were sacrificed with clove oil and kept in ice during the
195 sampling. 40 larvae were measured for total length (TL) using a profile projector (Mitutoyo
196 PJ- 3000A, Kanagawa, Japan) and fixed in 4 % buffered formalin for histological analysis
197 (120 larvae per diet). The remaining 30 larvae were collected to determine dry weight at
198 each sampling point. At 44 dph all remaining larvae were collected for biochemical and
199 TBARs analysis and stored at -80 °C until analysis.

200 **2.4. Growth and survival**

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

207

208

209 **2.5. Histopathology**

210 Formalin fixed samples were dehydrated in a series of different concentrations of
211 ethanol and embedded in a paraffin block. The samples were cut at 4 µm on a microtome,
212 fixed to the microscope slide and finally stained with haematoxylin and eosin (H&E),
213 Ziehl-Neelsen (ZN) (Martoja and Martoja-Pearson, 1970), Fite-Faraco method (Fite et al.,
214 1947) and Gram stain (Gregersen, 1978). Then, the samples were used for histopathological
215 evaluation, analysing all tissues and focusing especially, in liver, kidney and heart, given
216 that these organs are the main affected by granulomas (Ruiz et al., 2018a).

217 **2.6. Biochemical analysis**

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

222 Fatty acids from total lipids were prepared by transmethylation as described by
223 Christie (1982). Fatty acid methyl esters (FAMES) were separated and quantified by gas-
224 liquid chromatography following the conditions described by Izquierdo et al. (1992). Lipid
225 susceptibility to oxidation was estimated using the peroxidation index (PI_n) with following
226 formula: $PI_n = 0.025 \times (\text{percentage of monoenoics}) + 1 \times (\text{percentage of dienoics}) + 2 \times$
227 $(\text{percentage of trienoics}) + 4 \times (\text{percentage of tetraenoics}) + 6 \times (\text{percentage of pentaenoics})$
228 $+ 8 \times (\text{percentage of hexaenoics})$ (Witting and Horwitt, 1964).

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

238 expressed as $\mu\text{mol MDA per g of tissue}$ and was calculated using the extinction coefficient
239 $0.156 \mu\text{M}^{-1} \text{cm}^{-1}$.

240 The concentration of vitamin E was determined in diets. Samples were weighed,
241 homogenized in ethanolic pyrogallol and saponified as described McMurray et al., 1980.
242 HPLC analysis was performed using 150 x 4.60 mm, 5 μm reverse-phase Luna and C18
243 column (Phenomenox, CA, USA). The mobile phase was methanol:ultrapure water (98:2
244 v/v) with a flow rate of 1.0 ml min^{-1} at ambient temperature. Samples were injected (50 μl)
245 in a high performance liquid chromatograph (HPLC) with UV detection at a wavelength of
246 293 nm to determine the vitamin E using (+)- α -tocopherol (Sigma-Aldrich) as the external
247 standard.

248 The concentration of vitamin C was determined in the experimental feeds as
249 described by Betancor et al. (2012). Samples were weighed, homogenised and dissolved in
250 0.4 M phosphate buffer (adjusted to pH 3.0 with phosphoric acid). The samples were
251 centrifuged at 3.000 rpm, supernatants removed and filtered through a disposable 0.45 μm
252 filter and stored at 4°C until the measurement in a HPLC with UV detection. The
253 determination of vitamin C concentration was achieved by comparison with tris
254 (cyclohexylammonium) ascorbic acid-2-phosphate (Sigma-Aldrich) as the external
255 standard.

256 **2.7. Statistical analysis**

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

264

265

266 3. Results

267 3.1. Growth and survival

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

272 3.2. Histopathology

273 At the beginning of the experiment (30 dph) no granulomas were observed at the
274 microscopic evaluation. Nevertheless, after 14 days (44 dph) significant differences were
275 found in the percentage of larvae with granulomas among diets, being higher in larvae fed
276 diets C-, Krill and EC (40/100, 400/1,000 and 200/500 mg kg⁻¹ of vitamin E and C,
277 respectively) followed by diet C+ (400/1,000 mg kg⁻¹ of vitamin E and C, respectively)
278 (Figure 1). No granulomas were observed in any larvae fed with the highest levels of
279 vitamin E and C (800/2,000 mg kg⁻¹). Kidney was the main affected tissue with granulomas
280 (86.7 % of fish with granulomas), followed by liver (13.3 % of fish with granulomas)
281 (Figure 2).

282 There was a strong and significant negative correlation between the percentage of
283 larvae with granulomas and dietary concentration of vitamin E ($y = -0.0098x + 11.174$, R^2
284 $= 0.8766$; Pearson's correlation coefficient (r) = -0.93) and vitamin C ($y = -0.0022x +$
285 6.4777 , $R^2 = 0.9278$; Pearson's correlation coefficient (r) = -0.96) (Figure 3). The TBARs
286 content was highly correlated with the appearance of granulomas ($R^2=0.892$,
287 $y=0.0446x+0.0756$).

288 All the specific stainings (Ziehl-Neelsen, Fite-Faraco and Gram stain) were
289 negative, discarding a possible infectious origin of the granulomas (Supplementary Figure
290 1).

291 The histopathological evaluation revealed granulomas in different stages of
292 development (Supplementary Figure 2) as described by Ruiz et al. (2018a) in ongrowing
293 meagre. At initial stages, granulomas were observed as isolated and irregular aggregated of
294 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 the 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 ($\mu\text{mol g}^{-1}$ 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

324 showed that the dietary addition of different levels of vitamin E (40, 200, 400 and 800 mg
325 kg⁻¹) and C (100, 500, 1,000 and 2,000 mg kg⁻¹) did not affect meagre larvae performance
326 in terms of growth, length, survival and SGR at 44 dph. However, granulomas were
327 observed in larvae fed with low levels of vitamin E and C (from 40/100 to 400/1,000 mg
328 kg⁻¹, vitamin E/C). The results suggest that low levels of vitamin E and C (40 and 100 mg
329 kg⁻¹, respectively) probably fulfilled the requirement for normal growth what explains the
330 lack of differences in terms of fish performance among larvae fed the different dietary
331 treatments but were not enough to prevent systemic granulomatosis. On this matter, a
332 strong negative correlation was observed between the dietary levels of vitamin E ($y = -$
333 $0.0098x + 11.174$, $R^2 = 0.8766$) and vitamin C ($y = -0.0022x + 6.4777$, $R^2 = 0.9278$) and
334 the incidence of granulomas. Little is known about requirements of vitamin E and C in
335 meagre larvae. Only El Kertaoui et al. (2017) observed that high levels (1,500 and 1,800
336 mg kg⁻¹ of vitamin E and C, respectively) were required to improve growth and antioxidant
337 defenses in meagre larvae at 28 dph. It is well known that the requirement for antioxidant
338 vitamins is conditioned by the dietary fatty acids content. In this regard, all the
339 experimental microdiets contained a sufficient amount of essential fatty acids for most
340 marine fish species, which require at least 2 % EPA and DHA (NRC, 2011). Nevertheless,
341 those larvae fed with higher amounts of DHA and EPA together with low dietary vitamin E
342 and C (diet Krill) presented high incidence of granulomas, suggesting an imbalance
343 between prooxidant and antioxidant nutrients. Accordingly, TBARs content, an indicator of
344 lipid oxidation, was affected by the dietary inclusion of vitamin E and C, with the high
345 supplementation of vitamin E (800 mg kg⁻¹) and C (2,000 mg kg⁻¹) significantly reducing
346 TBARs values. Indeed, TBARs contents were highly correlated with the appearance of
347 granulomas ($R^2=0.892$, $y=0.0446x+0.0756$). Therefore, adequate dietary levels of vitamins
348 E and C seem to mitigate the appearance of systemic granulomatosis in meagre larvae,
349 probably due to the decrease of the oxidation rate.

350 Vitamin E together with vitamin C are strong antioxidants in tissues, being able to
351 neutralize reactive oxygen species (ROS) (Montero et al., 1999; Ai et al., 2006; Betancor et
352 al., 2012; Gao et al., 2014) and increase the protection against lipid peroxidation (Lee and
353 Dabrowski, 2003). The oxidative stress has been related with some diseases (Kawatsu,
354 1969; Cowey et al., 1984; Sakai et al., 1989; Watanabe et al., 1989; Sies et al., 1992;

355 Padayatty and Levine, 2001; Lewis-McCrea and Lall, 2007), therefore it is feasible to think
356 that granulomas could also be originated by an oxidative imbalance. Lipid peroxidation
357 contributes to the inflammatory response (Morita et al., 2016). Granuloma formation is an
358 inflammatory response, and is composed basically by macrophages, lymphocytes and
359 fibrocytes, being its appearance not necessarily associated with infectious diseases. This
360 inflammation can occur in blood vessels (Petersen and Smith, 2013; Hilhorst et al., 2014).
361 In this sense, in the present and previous studies (Ruiz et al., 2018a) irregular aggregates of
362 cells and granulomas have been observed surrounding blood vessels, which suggests that
363 granulomas could have a vascular origin. Vitamin C has been related with the synthesis of
364 collagen, an important protein involved in the generation of blood vessels (Lim and Lovell,
365 1978; Nusgen et al., 2001). Besides, vitamins C and E are involved in the prevention of
366 endothelial dysfunction and the prevention of oxidative stress (Riitta et al., 2003; Engler et
367 al., 2003). In this sense, an imbalance between ROS and antioxidants could be happening in
368 larvae fed with low addition of vitamin E and C, as indicated by TBARs values, which
369 could lead to inflammatory response in blood vessel with the subsequent macrophages
370 infiltration and formation of granulomas. Limited information is available on the effect of
371 antioxidant vitamins in the formation of granulomas. In other fish species vitamin C
372 deficiency has been related to precipitation of tyrosine in tissues, being the origin of
373 granulomas, in species such as sea bream and turbot (Baudin-Laurencin et al., 1989;
374 Coustans et al., 1990; Alexis et al., 1997). In agreement, a previous study showed that the
375 dietary increase of vitamins E and C lead to a reduction in the percentage of granulomas in
376 liver and heart of juvenile meagre together with a decrease in TBARs contents (Ruiz et al.,
377 2018ab), what indicates less lipid peroxidation.

378 The substitution of fish oil by krill oil significantly increased the levels of
379 eicosapentaenoic acid (EPA, 16.6 %) and docosahexaenoic acid (DHA, 17.6 %) in meagre
380 larvae with 44 dph, compared with the larvae fed the other diets (EPA ~ 13.7 and DHA ~
381 16.1 %). This difference in the levels of n-3 LC-PUFA seemed to have an impact on the
382 TBARs content which in turn translated into a higher incidence of granulomas compared to
383 larvae fed fish oil in combination with the same dietary levels of antioxidant vitamins (Diet
384 C+, 400 and 1,000 mg kg⁻¹ vitamin E and C, respectively). Apart from being an excellent
385 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

417 1,082 and 2,910 mg kg⁻¹ (Diet EECC) completely prevented the appearance of granulomas.
418 The substitution of fish oil by krill oil was enough to the correct growth of meagre larvae
419 but increased the percentage of granulomas and the peroxidation index. Therefore, it has
420 been demonstrated in the present and previous studies (Ruiz et al., 2018b) that systemic
421 granulomatosis can be completely mitigated in meagre larvae by controlling feeding
422 sequence as well as levels of antioxidant nutrients.

423 5. Acknowledgements

424 This study was funded by the project “Exploring the biological and socio-economic
425 potential of new/emerging candidate fish species for expansion of the European aquaculture
426 industry (DIVERSIFY)” of the European Commission; Directorate-general for research and
427 Innovation, project no. FP7-KBBE-2013-7, GRANT AGREEMENT NUMBER 603121.

428

429 6. References

- 430 Ackman, R.G., Ratnayake, W.M.N., 1989. Fish oils, seal oils, esters and acids -are all forms of T-3 intake equal? In:
431 Health Effect of Fish and Fish Oils (Chandra, R.K. Eds.), ARTS Biomedical, Newfoundland, Canada, pp. 373-393.
- 432 Ai, Q., Mai, K., Tan, B., Xu, W., Zhang, W., Ma, H., Liufu, Z., 2006. Effects of dietary vitamin C on survival, growth,
433 and immunity of large yellow croaker, *Pseudosciaena crocea*. *Aquaculture*. 261, 327–336.
434 <https://doi.org/10.1016/j.aquaculture.2006.07.027>
- 435 Alexis, M.N., Karanikolas, K.K., Richards, R.H., 1997. Pathological findings owing to the lack of ascorbic
436 acid in cultured gilthead bream (*Sparus aurata* L.). *Aquaculture*. 151, 209-218.
437 [https://doi.org/10.1016/S0044-8486\(96\)01475-5](https://doi.org/10.1016/S0044-8486(96)01475-5)
- 438 Amate, L., Gil, A., Ramirez, M., 2001. Feeding infant piglets formula with long-chain polyunsaturated fatty acids as
439 triacylglycerols or phospholipids influences the distribution of these fatty acids in plasma lipoprotein fractions. *J. Nutr.*
440 131, 1250-1255. <https://doi.org/10.1093/jn/131.4.1250>
- 441 AOAC, 2010. *Official Methods of Analysis*, 17th Ed. Association of Official Analytical Chemists, Washington, D. C.,
442 U.S.A.
- 443 Baudin-Laurencin, F., Messenger, J.L., Stephan, G., 1989. Two examples of nutritional pathology related to vitamin E and
444 C deficiencies, in: *Advances in tropical aquaculture*, Tahiti, 171-181.
- 445 Betancor, M.B., Caballero, M.J., Terova, G., Corà, S., Saleh, R., Benítez-Santana, T., Bell, J.G., Hernández-Cruz, C.M.,
446 Izquierdo, M., 2012. Vitamin C Enhances Vitamin E Status and Reduces Oxidative Stress Indicators in Sea Bass Larvae
447 Fed High DHA Microdiets. *Lipids*. 47, 1193–1207. <https://doi.org/10.1007/s11745-012-3730-x>
- 448 Burk, R.F., Trumble, M.J., Lawrence, R.A., 1980. Rat hepatic cytosolic GSH-dependent enzyme protection against lipid
449 peroxidation in the NADPH microsomal lipid peroxidation system. *Biochim. Biophys. Acta*. 618, 35-41.
450 [https://doi.org/10.1016/0005-2760\(80\)90051-X](https://doi.org/10.1016/0005-2760(80)90051-X)

451 Cansell, M., Nacka, F., Combe, N., 2003. Marine lipid-based liposomes increase in vivo FA bioavailability. *Lipids*. 38,
452 551-559. <https://doi.org/10.1007/s11745-003-1341-0>

453 Cansell, M.S., Battin, A., Degrace, P., Gresti, J., Clouet, P., 2009. Early dissimilar fates of liver eicosapentaenoic Acid in
454 rats fed liposomes or fish oil and gene expression related to lipid metabolism. *Lipids*. 44, 237-247.
455 <https://doi.org/10.1007/s11745-008-3279-x>

456 Carvalho, M., Castro, P., Peres, H., Fontanillas, R., Rosenlund, G., Monter, D., Acosta, F., Robaina, L., Izquierdo, M.,
457 2018. Effect of increasing dietary levels of n-3 long-chain polyunsaturated fatty acids on liver composition and
458 histopathology of meagre (*Argyrosomus regius*, Asso 1801) fingerlings. 18th International Symposium on Fish Nutrition
459 and Feeding, 3-7 June 2018, Las Palmas de Gran Canaria, Spain.

460 Chakravarthy, M.V., Lodhi, I.J., Yin, L., Malapaka, R.R.V., Xu, H.E., 2009. Identification of a physiologically relevant
461 endogenous ligand for PPAR α in liver. *Cell*. 138, 476-488. <https://doi.org/10.1016/j.cell.2009.05.036>

462 Christie, W.W., 1982 . *Lipid Analysis*, 2nd Ed. Pergamon, Oxford.

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

467 Coustans, M.F., Guillaume, J., Metailler, R., Dugornay, O., Messenger, J.L., 1990. An ascorbic acid deficiency on
468 tyrosinemia and renal granulomatous disease in turbot (*Scophthalmus maximus*) interaction with a slight
469 polyhypovitaminosis. *Comparative Biochemistry and Physiology Part A: Physiology*. 97, 145-152.
470 [https://doi.org/10.1016/0300-9629\(90\)90161-K](https://doi.org/10.1016/0300-9629(90)90161-K)

471 Cowey, C.B., Degener, E., Tacon, A.G.J., Youngson, A., Bell, J.G., 1984. The effects of vitamin E and oxidised fish oil
472 on the nutrition of rainbow trout (*Salmo gairdneri*) grown at natural, varying water temperature. *Br. J. Nutr.* 51, 443-451.
473 <https://doi.org/10.1079/BJN19840050>

474 Dabrowski, K., 1992. Ascorbate concentration in fish ontogeny. *J. Fish Biol.* 40, 273–279. <https://doi.org/10.1111/j.1095-8649.1992.tb02572.x>

476 El Kertaoui, N., Hernández- Cruz, C.M., Montero, D., Caballero, M.J., Saleh, R., Afonso, J.M., Izquierdo, M., 2017. The
477 importance of dietary HUFA for meagre larvae (*Argyrosomus regius*; Asso, 1801) and its relation with antioxidant
478 vitamins E and C. *Aquac. Res.* 48, 419-433. <https://doi.org/10.1111/are.12890>

479 Fite, G.L., Cambre, F.J., Turner, M.H., 1947. Procedures for demonstrating lepra bacilli in paraffin sections. *Arch. Pathol.*
480 43, 624-625.

481 Folch, J.M., Lees M., Stanley Sloane, G.H., 1957. A simple method for the isolation and purification of total lipids from
482 the animal tissues. *J. Biol. Chem.* 226, 497-509.

483 Gao J., Koshio, S., Ishikawa, M., Yokoyama, S., Edward, R., 2014. Interactive effects of vitamin C and E
484 supplementation on growth performance, fatty acid composition and reduction of oxidative stress in juvenile Japanese
485 flounder *Paralichthys olivaceus* fed dietary oxidized fish oil. *Aquaculture*. 422-423, 84-90.
486 <https://doi.org/10.1016/j.aquaculture.2013.11.031>

487 Ghittino, C., Manuali, E., Latini, M., Agnetti, F., Rogato, F., Agonini, R., Colussi, S., Prearo, M., 2004. Caso di
488 granulomatosi sistemica in ombrina boccardoro (*Argyrosomus regius*) e raffronto con le lesioni istologiche presenti
489 nell'orata. *Ittiopatologia*. 1, 59-67.
490

491 Goldsmith, L.A., 1978. Molecular biology and molecular pathology of a newly described molecular disease. Tyrosinemia
492 II (the Richner - Hanhart syndrome). *Experimental cell biology*. 46, 96-113.

493 Gregersen, T., 1978. Rapid method for distinction of gram-negative from gram-positive bacteria. *Eur. J. Appl. Microbiol. Biotechnol.* 5, 123-127. <https://doi.org/10.1007/BF00498806>

494

495 Hamre, K., 2011. Metabolism, interactions, requirements and functions of vitamin E in fish. *Aquacult. Nutr.* 17, 98-115.

496 Hilhorst, M., Shirai, T., Berry, G., Goronzy, J.J., Weyand, C.M., 2014. T Cell–Macrophage Interactions and Granuloma Formation in Vasculitis. *Front. Immunol.* 5, 432. <https://doi.org/10.3389/fimmu.2014.00432>

497

498 Izquierdo, M.S., 1996. Essential fatty acid requirements of cultured marine fish species. *Aquacult. Nutr.* 2, 193-191. <https://doi.org/10.1111/j.1365-2095.1996.tb00058.x>

499

500

501 Izquierdo, M.S., Koven, W., 2011. Lipids, in: Holt J. (Eds.), *Larval Fish Nutrition.*, Wiley-Blackwell, Chichester, UK, pp. 47-84.

502

503

504 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](https://doi.org/10.1016/0044-8486(92)90163-F)

505

506

507

508 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.

509

510

511 Lee, K., Dabrowski, K., 2003. Interaction between vitamins C and E affects their tissue concentrations, growth, lipid oxidation, and deficiency symptoms in yellow perch (*Perca flavescens*). *Br. J. Nutr.* 89, 589-596. <https://doi.org/10.1079/BJN2003819>

512

513

514 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>

515

516

517

518 Li, Y., Choi, M., Cavey, G., Daugherty, J., Suino, K., 2005. Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. *Mol. Cell.* 17, 491-502. <https://doi.org/10.1016/j.molcel.2005.02.002>

519

520

521

522 Lim, C., Lovell, R.T., 1978. Pathology of the vitamin C deficiency syndrome in channel catfish (*Ictalurus punctatus*). *J. Nutr.* 108, 1137-1146. <https://doi.org/10.1093/jn/108.7.1137>

523

524

525 Liu, J., Caballero, M.J., Izquierdo, M.S., El-Sayed Ali, T., Hernández-Cruz, C.M., Valencia, A., Fernández-Palacios, H., 2002. Necessity of dietary lecithin and eicosapentaenoic acid for growth, survival, stress resistance and lipoprotein formation in gilthead sea bream *Sparus aurata*. *Fisheries Sci.* 6, 1165-1172. <https://doi.org/10.1046/j.1444-2906.2002.00551.x>

526

527

528

529

530 Engler, M.M., Engler, M.B., Mallor, M.J., Chiu, E.Y., Schloetter, M.C., Paul, S.M., Stuehlinger, M., Lin, K.Y., Cooke, J.P., Morrow, J.D., Ridker, P.M., Rifai, N., Miller, E., Witztum, J.L., Mietus-Snyder, M., 2003. Antioxidant vitamins C and E improve endothelial function in children with hyperlipidemia. *Circulation.* 108, 1059-1063. <https://doi.org/10.1161/01.CIR.0000086345.09861.A0>

531

532

533

534 Martoja, R., Martoja-Pearson, M., 1970. *Técnicas de Histología Animal*, 1° Ed. Toray-Masson S.A., Barcelona.

535

536 McMurray, C.H., Blanchflower, W.J., Rice, D.A., 1980. Influence of extraction techniques on determination of α -tocopherol in animal feedstuffs. *J. AOAC Int.* 63, 1258–1261.

537

538

539 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 aurata*) juveniles subjected to crowding stress. *Aquaculture.* 171, 269-78. [https://doi.org/10.1016/S0044-8486\(98\)00387-1](https://doi.org/10.1016/S0044-8486(98)00387-1)

540

541

- 542 Morita, M., Naito, Y., Yoshikawa, T., Niki, E., 2016. Plasma lipid oxidation induced by peroxyxynitrite, hypochlorite,
543 lipoyxygenase and peroxy radicals and its inhibition by antioxidants as assessed by diphenyl-1-pyrenylphosphine. *Redox*
544 *Biol.* 8, 127–135. <https://doi.org/10.1016/j.redox.2016.01.005>
- 545 NRC (2011) *Nutrient Requirements of Fish and Shrimp*. National Academic Press, Washington D.C.
546 <https://doi.org/10.17226/13039>
- 547 Nusgens, B.V., Colige, A.C., Lambert, C.A., Lapière, C.M., Humbert, P., Rougier, A., Haftek, M., Richard, A., Creidi, P.,
548 2001. Topically Applied Vitamin C Enhances the mRNA Level of Collagens I and III, Their Processing Enzymes and
549 Tissue Inhibitor of Matrix Metalloproteinase 1 in the Human Dermis. *J. Invest. Dermatol.* 116, 853-859.
550 <https://doi.org/10.1046/j.0022-202x.2001.01362.x>
- 551 Padayatty, S.J., Levine, M., 2001. New insights into the physiology and pharmacology of vitamin C. *CMAJ* 164, 353–
552 355.
- 553 Paperna, I., Harrison, J.G., Kissil, G.W., 1980. Pathology and histopathology of a systemic granuloma in *Sparus aurata*
554 (L.) cultured in the Gulf of Aqaba. *J. Fish Dis.* 3, 213-221.
- 555 Petersen, H., Smith, A., 2013. The role of the innate immune system in granulomatous disorders. *Front. Immunol.*
556 <https://doi.org/10.3389/fimmu.2013.00120>
- 557 Riitta, M.S., Kristiina, N., Jari, K., Elina, P., Sari, V., Tiina, H.R., Tomi-Pekka, T., Veli-Pekka, V., Ulla, R., Hanna-Maari,
558 L., Meri, V., Jukka, T.S., Henrik, E.P., 2003. Six-Year Effect of Combined Vitamin C and E Supplementation on
559 Atherosclerotic Progression. *Circulation.* 107, 947-953. <https://doi.org/10.1161/01.CIR.0000050626.25057.51>
- 560 Rodríguez, C., Pérez, J.A., Lorenzo, A., Izquierdo, M.S., Cejas, J.R., 1994. n-3 HUFA requirement of larval gilthead
561 seabream *Sparus aurata* when using high levels of eicosapentaenoic acid. *Comparative Biochemistry and Physiology Part*
562 *A: Physiology.* 4, 693-698. [https://doi.org/10.1016/0300-9629\(94\)90371-9](https://doi.org/10.1016/0300-9629(94)90371-9)
- 563 Ruiz, M.A., Betancor, M.B., Robaina, L., Montero, D., Hernández-Cruz, C.M., Izquierdo, M., Rosenlund, G., Fontanillas,
564 R., Caballero, M.J., 2018a. Dietary combination of vitamin E, C and K affects growth, antioxidant activity, and the
565 incidence of systemic granulomatosis in meagre (*Argyrosomus regius*). *Aquaculture.* 498, 606-620.
566 <https://doi.org/10.1016/j.aquaculture.2018.08.078>
567
- 568 Ruiz, M.A., Hernández-Cruz, C.M., Caballero M.J., Fernández-Palacios, H., Saleh, R., Izquierdo, M.S., Betancor, M.B.,
569 2018b. Incidence of systemic granulomatosis is modulated by the feeding sequence and type of enrichment in meagre
570 (*Argyrosomus regius*). *Aquacult. Res.* 50, 284-295. <https://doi.org/10.1111/are.13896>
- 571 Sakai, T., Murata, H., Endo, M., Yamauchi, K., Tabata, N., Fukudome, M., 1989. 2-Thiobarbituric acid values and
572 contents of α -tocopherol and bile pigments in the liver and muscle of jaundiced yellowtail, *Seriola quinqueradiata*. *Agric.*
573 *Biol. Chem.* 53, 1739-1740. <https://doi.org/10.1080/00021369.1989.10869531>
- 574 Salhi, M., Izquierdo, M.S., Hernández-Cruz, C.M., Socorro, J., Fernández-Palacios, H., 1997. The improved incorporation
575 of polyunsaturated fatty acids and changes in liver structure in larval gilthead seabream fed on microdiets. *J. Fish Biol.* 51,
576 869-879. <https://doi.org/10.1111/j.1095-8649.1997.tb01526.x>
577
578
- 579 Sies, H., Stahl, W., Sundquist, A.R., 1992. Antioxidant functions of vitamins. Vitamins E and C, beta-carotene and other
580 carotenoids. *Ann. NY Acad. Sci.* 30, 7-20. <https://doi.org/10.1111/j.1749-6632.1992.tb17085.x>
- 581 Tou, J.C., Jaczynski, J., Chen, Y.C., 2007. Krill for human consumption: nutritional value and potential health benefits.
582 *Nutr. Rev.* 65, 63-77. <https://doi.org/10.1111/j.1753-4887.2007.tb00283.x>
- 583 Ulven, S.M., Holven, K.B., 2015. Comparison of bioavailability of krill oil versus fish oil and health effect. *Vasc. Health*
584 *Risk. Manag.* 11, 511-524. doi:10.2147/VHRM.S85165

585 Watanabe, T., Izquierdo, M.S., Takeuchi, T., Satoh, S., Kitajima, C., 1989. Comparison between eicosapentaenoic and
586 docosahexaenoic acids in terms of essential fatty acids efficacy in larval red seabream. *Nippon Suissan Gakk.* 5, 1635-
587 1640. <https://doi.org/10.2331/suisan.55.1635>

588 Winther, B., Hoem, N., Berge, K., Reubsæet, L., 2011. Elucidation of phosphatidylcholine composition in krill oil
589 extracted from *Euphausia superba*. *Lipids.* 46, 25–36. <https://doi.org/10.1007/s11745-010-3472-6>

590
591 Witting, L.A., Horwitt, M.K., 1964. Effect of degree of fatty acid unsaturation in tocopherol deficiency-induced
592 creatinuria. *J. Nutr.* 82, 19–33. <https://doi.org/10.1093/jn/82.1.19>
593

594

595

596

597

598

599

600

601

602

603

604

605

606

607

608

609

610

611

612

613

614

615

616

617

618

619 **Figure legends**

620

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

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

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

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

629

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

634

635

636

637

638

639

640

641

642

643

644

645

646 **Tables**

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

| Ingredient (%) | Diets | | | | |
|----------------------------------|--------|-------|-------|--------|--------|
| | 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 |

650 ¹Panreac, Barcelona, Spain. ²Acrofarma, Barcelona, Spain. ³ g · 100⁻¹, Vitamin E: α-
 651 tocopheryl acetate (Sigma-Aldrich, Madrid, Spain), Ascorbyl monophosphate ROVIMIX
 652 Stay-C-35 (Roche, Paris, France). ⁴Mineral premix supplied g per 100 g diet: NaCl 215.133
 653 mg, MgSO₄ 7H₂O 677.545 mg, NaH₂PO₄ H₂O 381.453 mg, Ca(H₂PO₄) 2H₂O 671.610 mg,
 654 FeC₆H₅O₇ 146.884 mg, C₃H₅O₃ 1/2Ca 1,617.210 mg, Al₂(SO₄)₃ 6H₂O 0.693 mg, ZnSO₄
 655 7H₂O 14.837 mg, CuSO₄ 5H₂O 1.247 mg, MnSO₄ H₂O 2.998 mg, CoSO₄ 7H₂O 10.706 mg.
 656 ⁵Vitamin premix supplied per 100 g diet: cyanocobalamine 0.03 mg, astaxanthin 5.0 mg,
 657 folic acid 5.4 mg, pyridoxine-HCl 17.3 mg, thiamine 21.7 mg, riboflavin 72.5 mg, calcium-
 658 pantothenate 101.5 mg, p-aminobenzoic acid 145.0 mg, nicotinic acid 290.1 mg, myo-
 659 inositol 1450.9 mg, menadione 17.3 mg. ⁶Attractant premix supplied per 100 g diet:
 660 inosine-5-monophosphate 500.0 mg, betaine 660.0 mg, L-serine 170.0 mg, L-phenylalanine
 661 250.0 mg, DL-alanine 500.0 mg, L-sodium aspartate 330.0 mg, L-valine 250.0 mg, glycine
 662 170.0 mg. Proximate composition (%)

663

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.

| <i>Fatty acids (%)</i> | Diets | | | | |
|--------------------------------------|--------------|-------|-------|-------|-------|
| | 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 Saturated¹ | 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 Monosaturated² | 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-6PUFA³ | 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-3PUFA⁴ | 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-PUFA⁵ | 20.5 | 20.4 | 20.2 | 20.3 | 24.7 |
| PI_n | 166.4 | 165.1 | 163.6 | 163.9 | 190.3 |

666 Data expressed as means of three technical replicates per batch of diet.¹Includes 15:0 and
 667 17:0.²Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, and 20:1n-5.³Includes.
 668 22:5n-6 and 22:4n-6. ⁴Includes 16:3n-3 and 16:4n-3. ⁵LC- PUFA, long-chain
 669 polyunsaturated fatty acid (sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3).

670

671

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.

675

676

677

678

679

680

681

682

683

684

685

686

687

688

689

690

691 **Table 4.** Proximate composition and TBARs content in meagre larvae (44 dph) fed with the
 692 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 |

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

696

697

698

699

700

701

702

703

704

705

706

707

708

709

710

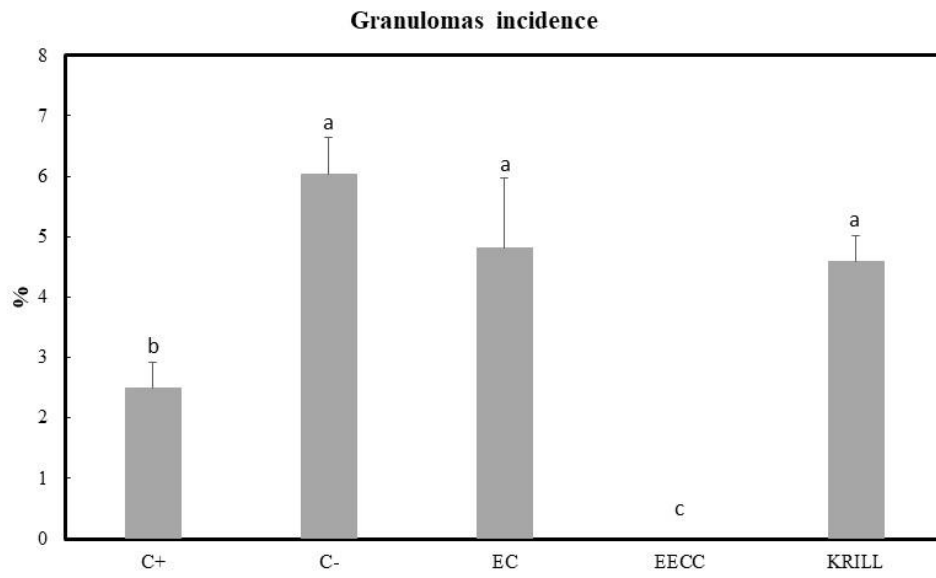
711

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).

| <i>Fatty acids (%)</i> | 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 Saturated¹ | 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 Monosaturated² | 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 PUFA³ | 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-3PUFA⁴ | 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-9PUFA⁵ | 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-PUFA⁶ | 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). ¹Includes 15:0 and 17:0. ²Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5,
 717 20:1n-9 and 20:1n-5. ³Includes 22:5n-6 and 22:4n-6. ⁴Includes 16:3n-3 and 16:4n-3.
 718 ⁵Includes. 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.

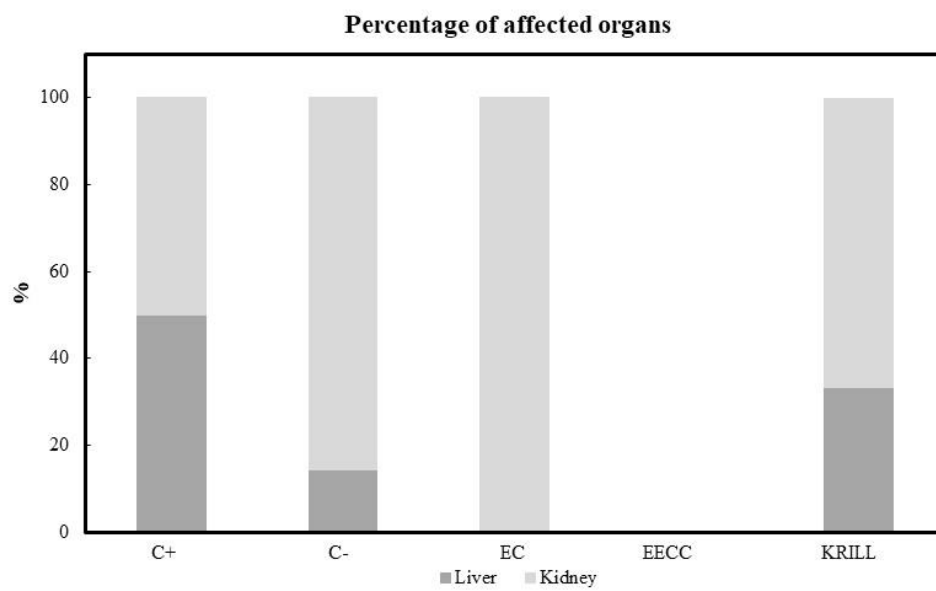
721



722

723 Figure 1

724

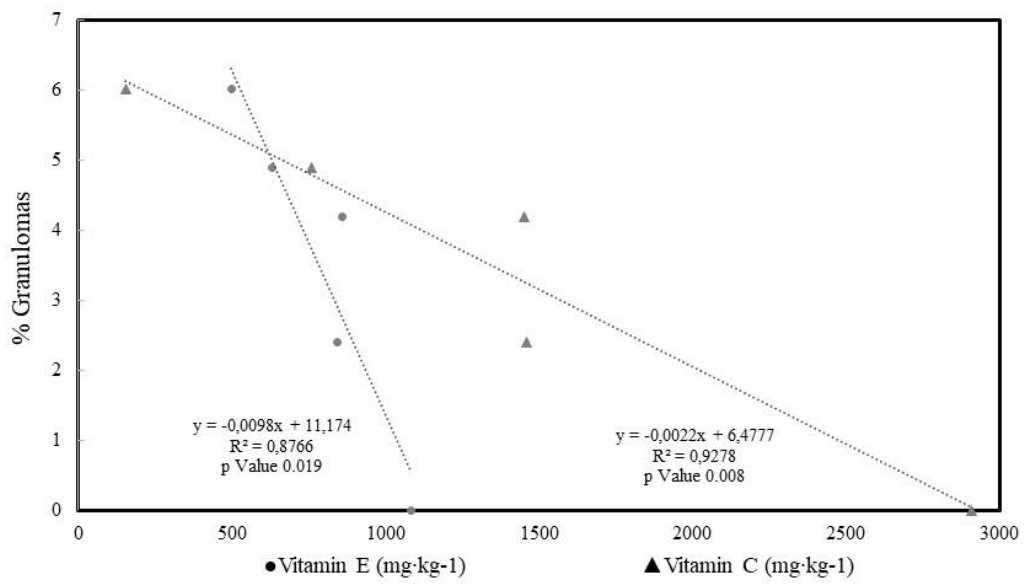


725

726

727 Figure 2

728

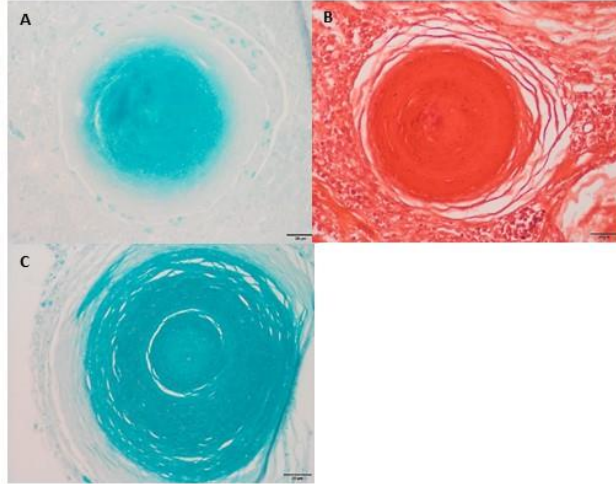


729

730

Figure 3

731



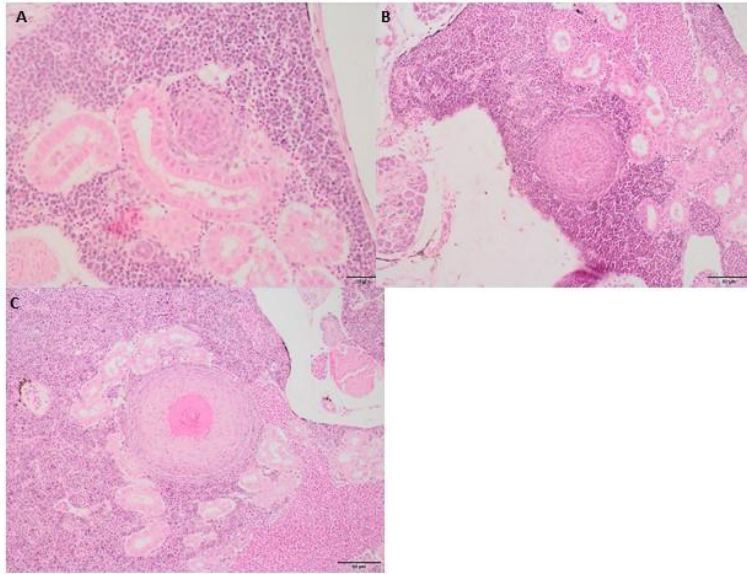
732

733

734

Supplementary Figure 1

735



736

737

738

Supplementary Figure 2