

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.

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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<sup>-1</sup> E, 100 mg kg<sup>-1</sup> C), C+ (400 mg kg<sup>-1</sup> E, 1,000 mg kg<sup>-1</sup> C), Krill (400 mg kg<sup>-1</sup> E, 1,000  
38 mg kg<sup>-1</sup> C and substitution of fish oil by krill oil), EC (200 mg kg<sup>-1</sup> E, 500 mg kg<sup>-1</sup> C) and  
39 EECC (800 mg kg<sup>-1</sup> E, 2,000 mg kg<sup>-1</sup> 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<sup>-1</sup> E, 100  
45 mg kg<sup>-1</sup> 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<sup>-1</sup> E and 2,000 mg kg<sup>-1</sup> 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<sup>-1</sup>, 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<sup>-1</sup>, 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<sup>-1</sup>, 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<sup>-1</sup> 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<sup>-1</sup>) 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<sup>-1</sup> in the experimental tanks.  
139 *Artemia* cyst were hatched at 27 °C and 0.030 mg L<sup>-1</sup> 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*<sup>-1</sup>) 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<sup>-1</sup> 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<sup>-1</sup> 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<sup>-1</sup>  
152 to a 100% h<sup>-1</sup>, 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<sup>-1</sup>), salinity (34 g L<sup>-1</sup>) 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<sup>-1</sup>) and C (1,000 mg kg<sup>-1</sup>) 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<sup>-1</sup> 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  $\mu\text{m}$ ). 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<sup>-1</sup>, 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<sub>n</sub>) 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  $\mu\text{m}$  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  $1.0 \text{ ml min}^{-1}$  at ambient temperature. Samples were injected (50  $\mu\text{l}$ )  
245 in a high performance liquid chromatograph (HPLC) with UV detection at a wavelength of  
246 293 nm to determine the vitamin E using (+)- $\alpha$ -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  $\mu\text{m}$   
252 filter and stored at  $4^\circ \text{C}$  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  $25.8 \pm 0.4$   
271 mm, dry weight  $17.5 \pm 1.3$  mg, survival  $20.1 \pm 0.5$  % and SGR  $17.1 \pm 1.2$  % (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<sup>-1</sup> of vitamin E and C,  
277 respectively) followed by diet C+ (400/1,000 mg kg<sup>-1</sup> 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<sup>-1</sup>). 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<sup>-1</sup>) and C (100, 500, 1,000 and 2,000 mg kg<sup>-1</sup>) 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<sup>-1</sup>, vitamin E/C). The results suggest that low levels of vitamin E and C (40 and 100 mg  
329 kg<sup>-1</sup>, 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<sup>-1</sup> 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<sup>-1</sup>) and C (2,000 mg kg<sup>-1</sup>) 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 de 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<sup>-1</sup> 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<sup>-1</sup>, 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<sup>-1</sup>, respectively) and C (1,460 and 1,450 mg kg<sup>-1</sup>,  
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<sup>-1</sup>  
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<sup>-1</sup> (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.

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619 **Figure legends**

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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.

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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.

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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 <sup>1</sup>	3.00	3.00	3.00	3.00	3.00
Fish oil	7.00	7.00	7.00	7.00	-
Soy lecithin <sup>2</sup>	2.00	2.00	2.00	2.00	2.00
Vitamin E <sup>3</sup>	0.04	0.004	0.02	0.08	0.04
Vitamin C <sup>3</sup>	0.10	0.01	0.05	0.20	0.10
Mineral Premix <sup>4</sup>	4.70	4.70	4.70	4.70	4.70
Vitamin Premix <sup>5</sup>	5.69	5.69	5.69	5.69	5.69
Attractant <sup>6</sup>	3.00	3.00	3.00	3.00	3.00
Proximate composition					
Vitamin E (mg kg <sup>-1</sup> )	844.3	497.1	632.7	1082.3	859.8
Vitamin C (mg kg <sup>-1</sup> )	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 <sup>1</sup>Panreac, Barcelona, Spain. <sup>2</sup>Acrofarma, Barcelona, Spain. <sup>3</sup> g · 100<sup>-1</sup>, Vitamin E: α-  
 651 tocopheryl acetate (Sigma-Aldrich, Madrid, Spain), Ascorbyl monophosphate ROVIMIX  
 652 Stay-C-35 (Roche, Paris, France). <sup>4</sup>Mineral premix supplied g per 100 g diet: NaCl 215.133  
 653 mg, MgSO<sub>4</sub> 7H<sub>2</sub>O 677.545 mg, NaH<sub>2</sub>PO<sub>4</sub> H<sub>2</sub>O 381.453 mg, Ca(H<sub>2</sub>PO<sub>4</sub>) 2H<sub>2</sub>O 671.610 mg,  
 654 FeC<sub>6</sub>H<sub>5</sub>O<sub>7</sub> 146.884 mg, C<sub>3</sub>H<sub>5</sub>O<sub>3</sub> 1/2Ca 1,617.210 mg, Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> 6H<sub>2</sub>O 0.693 mg, ZnSO<sub>4</sub>  
 655 7H<sub>2</sub>O 14.837 mg, CuSO<sub>4</sub> 5H<sub>2</sub>O 1.247 mg, MnSO<sub>4</sub> H<sub>2</sub>O 2.998 mg, CoSO<sub>4</sub> 7H<sub>2</sub>O 10.706 mg.  
 656 <sup>5</sup>Vitamin 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. <sup>6</sup>Attractant 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 (%)

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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>	<b>Diets</b>				
	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
<b>Σ Saturated<sup>1</sup></b>	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
<b>Σ Monosaturated<sup>2</sup></b>	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
<b>Σ n-6PUFA<sup>3</sup></b>	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
<b>Σ n-3PUFA<sup>4</sup></b>	25.0	24.8	24.6	24.7	28.8
<b>(n-3+n-6) PUFA</b>	33.2	32.9	32.7	32.8	33.4
<b>Total n-3 LC-PUFA<sup>5</sup></b>	20.5	20.4	20.2	20.3	24.7
<b>PI<sub>n</sub></b>	166.4	165.1	163.6	163.9	190.3

666 Data expressed as means of three technical replicates per batch of diet.<sup>1</sup>Includes 15:0 and  
 667 17:0.<sup>2</sup>Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5, 20:1n-9, and 20:1n-5.<sup>3</sup>Includes.  
 668 22:5n-6 and 22:4n-6. <sup>4</sup>Includes 16:3n-3 and 16:4n-3. <sup>5</sup>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 <sup>-1</sup> )	-	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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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
<b>Proximate composition (%)</b>					
<b>Protein</b>	11.0 ± 1.1	10.4 ± 2.1	10.6 ± 1.4	10.9 ± 1.8	11.8 ± 2.0
<b>Lipid</b>	2.8 ± 0.4	2.5 ± 0.2	2.3 ± 0.3	2.4 ± 0.4	2.4 ± 0.1
<b>Moisture</b>	82.9 ± 2.8	83.9 ± 2.1	83.7 ± 2.1	82.7 ± 0.9	82.9 ± 2.2
<b>Ash</b>	2.5 ± 0.6	2.8 ± 0.1	2.9 ± 0.2	3.1 ± 0.2	2.3 ± 0.1
<b>TBARs content (µmol g<sup>-1</sup> dry mass)</b>	769.2 ± 110.5 <sup>a</sup>	1028.8 ± 159.3 <sup>a</sup>	862.2 ± 136.3 <sup>a</sup>	138.5 ± 45.7 <sup>b</sup>	974.6 ± 118.9 <sup>a</sup>

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).

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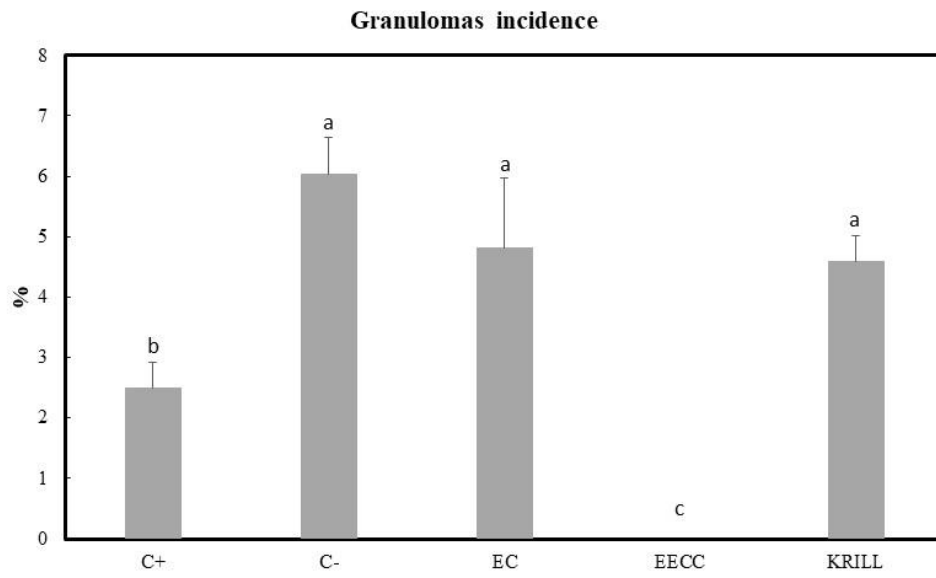


712 **Table 5.** Fatty acid composition (percentage of fatty acids) of meagre larvae fed with  
 713 experimental diets at the end of the dietary trial (44 days post hatching).

<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 <sup>a</sup>	20.9	± 0.3 <sup>a</sup>	21.3	± 0.6 <sup>a</sup>	21.0	± 0.2 <sup>a</sup>	22.5	± 0.2 <sup>b</sup>
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 <sup>ab</sup>	0.2	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>a</sup>	0.0	± 0.0 <sup>ab</sup>	0.1	± 0.0 <sup>a</sup>
<b>Σ Saturated<sup>1</sup></b>	<b>28.8</b>	<b>± 0.5<sup>a</sup></b>	<b>28.9</b>	<b>± 0.2<sup>a</sup></b>	<b>29.3</b>	<b>± 0.7<sup>a</sup></b>	<b>29.1</b>	<b>± 0.3<sup>a</sup></b>	<b>31.2</b>	<b>± 0.3<sup>b</sup></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 <sup>b</sup>	15.5	± 0.3 <sup>b</sup>	15.1	± 1.0 <sup>b</sup>	14.7	± 0.2 <sup>b</sup>	11.0	± 0.7 <sup>a</sup>
18:1n-7	5.3	± 0.1 <sup>a</sup>	5.5	± 0.0 <sup>a</sup>	5.4	± 0.0 <sup>a</sup>	5.3	± 0.1 <sup>a</sup>	5.9	± 0.1 <sup>b</sup>
20:1n-7	1.0	± 0.0 <sup>b</sup>	1.1	± 0.0 <sup>b</sup>	1.0	± 0.1 <sup>b</sup>	1.0	± 0.0 <sup>b</sup>	0.7	± 0.0 <sup>a</sup>
22:1n-11	0.3	± 0.0 <sup>b</sup>	0.3	± 0.0 <sup>b</sup>	0.3	± 0.1 <sup>b</sup>	0.2	± 0.1 <sup>b</sup>	0.0	± 0.0 <sup>a</sup>
<b>Σ Monosaturated<sup>2</sup></b>	<b>26.6</b>	<b>± 0.31<sup>b</sup></b>	<b>27.1</b>	<b>± 0.6<sup>b</sup></b>	<b>26.6</b>	<b>± 1.5<sup>b</sup></b>	<b>26.0</b>	<b>± 0.3<sup>b</sup></b>	<b>22.8</b>	<b>± 1.1<sup>a</sup></b>
18:2n-6	6.6	± 0.1 <sup>b</sup>	6.5	± 0.2 <sup>b</sup>	6.6	± 0.0 <sup>b</sup>	6.6	± 0.1 <sup>b</sup>	4.5	± 0.1 <sup>a</sup>
18:3n-6	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>a</sup>
20:2n-6	0.2	± 0.0 <sup>b</sup>	0.2	± 0.0 <sup>b</sup>	0.2	± 0.0 <sup>b</sup>	0.2	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>a</sup>
20:3n-6	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>a</sup>
20:4n-6	0.9	± 0.2	0.9	± 0.1	0.9	± 0.1	1.0	± 0.3	0.9	± 0.1
<b>Σ n-6 PUFA<sup>3</sup></b>	<b>8.2</b>	<b>± 0.2<sup>b</sup></b>	<b>8.0</b>	<b>± 0.1<sup>b</sup></b>	<b>8.1</b>	<b>± 0.1<sup>b</sup></b>	<b>8.3</b>	<b>± 0.4<sup>b</sup></b>	<b>5.8</b>	<b>± 0.2<sup>a</sup></b>
18:3n-3	1.4	± 0.0 <sup>b</sup>	1.4	± 0.0 <sup>b</sup>	1.3	± 0.1 <sup>b</sup>	1.4	± 0.2 <sup>b</sup>	0.9	± 0.0 <sup>a</sup>
18:4n-3	1.4	± 0.1 <sup>a</sup>	1.4	± 0.0 <sup>a</sup>	1.3	± 0.0 <sup>a</sup>	1.3	± 0.1 <sup>a</sup>	1.6	± 0.1 <sup>b</sup>
20:3n-3	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>b</sup>	0.1	± 0.0 <sup>a</sup>
20:4n-3	0.3	± 0.0 <sup>b</sup>	0.3	± 0.0 <sup>b</sup>	0.3	± 0.0 <sup>b</sup>	0.3	± 0.0 <sup>b</sup>	0.3	± 0.0 <sup>a</sup>
20:5n-3	13.7	± 0.8 <sup>a</sup>	13.7	± 0.3 <sup>a</sup>	13.7	± 0.6 <sup>a</sup>	13.7	± 1.2 <sup>a</sup>	16.6	± 1.4 <sup>b</sup>
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 <sup>a</sup>	15.9	± 0.8 <sup>a</sup>	16.0	± 0.6 <sup>a</sup>	16.3	± 0.4 <sup>a</sup>	17.6	± 0.2 <sup>b</sup>
<b>Σ n-3PUFA<sup>4</sup></b>	<b>34.4</b>	<b>± 0.4<sup>a</sup></b>	<b>34.1</b>	<b>± 0.9<sup>a</sup></b>	<b>34.1</b>	<b>± 1.1<sup>a</sup></b>	<b>34.6</b>	<b>± 0.8<sup>a</sup></b>	<b>38.3</b>	<b>± 1.5<sup>b</sup></b>
<b>Σ n-9PUFA<sup>5</sup></b>	<b>15.9</b>	<b>± 0.1<sup>b</sup></b>	<b>16.3</b>	<b>± 0.3<sup>b</sup></b>	<b>15.9</b>	<b>± 1.1<sup>b</sup></b>	<b>15.5</b>	<b>± 0.2<sup>b</sup></b>	<b>11.8</b>	<b>± 0.6<sup>a</sup></b>
<b>(n-3+n-6) PUFA</b>	<b>8.2</b>	<b>± 0.2<sup>b</sup></b>	<b>8.0</b>	<b>± 0.1<sup>b</sup></b>	<b>8.1</b>	<b>± 0.1<sup>b</sup></b>	<b>8.3</b>	<b>± 0.4<sup>b</sup></b>	<b>5.8</b>	<b>± 0.2<sup>a</sup></b>
<b>Total n-3 LC-PUFA<sup>6</sup></b>	<b>31.5</b>	<b>± 0.3<sup>a</sup></b>	<b>31.2</b>	<b>± 1.0<sup>a</sup></b>	<b>31.3</b>	<b>± 1.2<sup>a</sup></b>	<b>32.5</b>	<b>± 2.1<sup>a</sup></b>	<b>35.7</b>	<b>± 1.5<sup>b</sup></b>
<b>PIn</b>	<b>246.3</b>	<b>± 2.1<sup>a</sup></b>	<b>243.7</b>	<b>± 7.7<sup>a</sup></b>	<b>244.4</b>	<b>± 8.2<sup>a</sup></b>	<b>248.5</b>	<b>± 3.9<sup>a</sup></b>	<b>275.3</b>	<b>± 3.0<sup>b</sup></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). <sup>1</sup>Includes 15:0 and 17:0. <sup>2</sup>Includes 14:1n-7, 14:1n-5, 15:1n-5, 16:1n-5, 18:1n-5,  
 717 20:1n-9 and 20:1n-5. <sup>3</sup>Includes 22:5n-6 and 22:4n-6. <sup>4</sup>Includes 16:3n-3 and 16:4n-3.  
 718 <sup>5</sup>Includes. 22:1n-9. 20:3n-9. 20:2n-9. 20:1n-9. 18:2n-9. 18:1n-9. <sup>6</sup> 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.

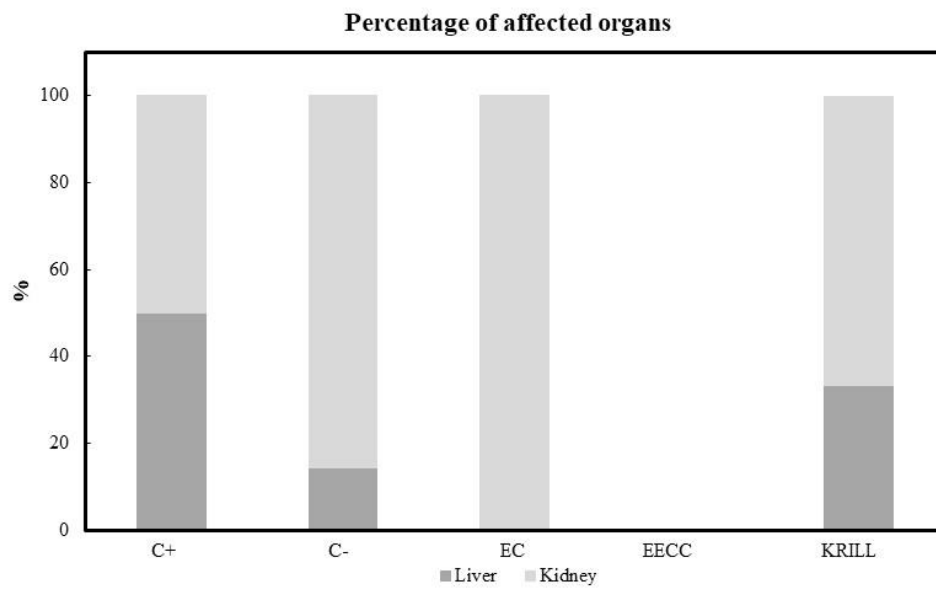
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723 Figure 1

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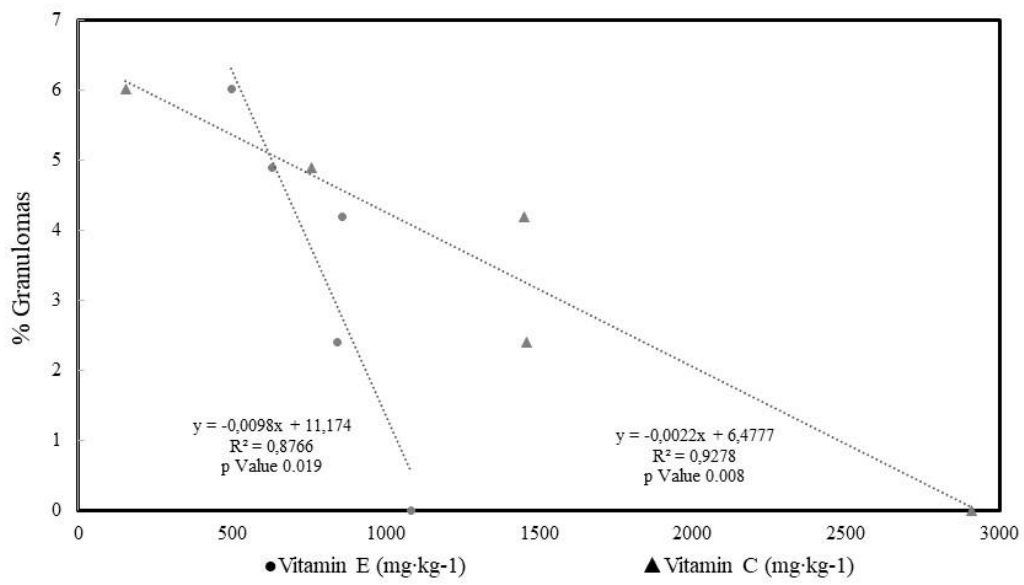


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727 Figure 2

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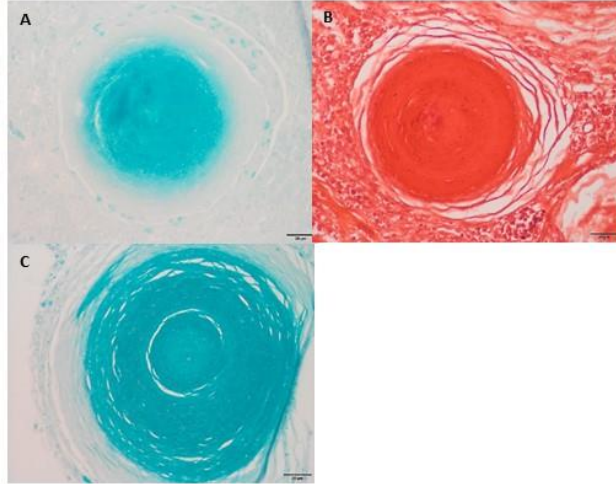


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Figure 3

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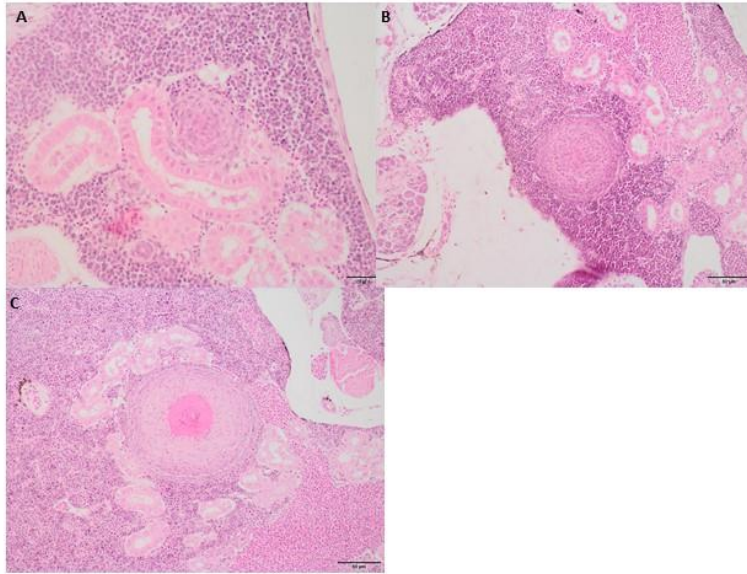
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Supplementary Figure 1

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Supplementary Figure 2