

1 **EFFECTS OF DIETARY ARACHIDONIC ACID IN**
2 **EUROPEAN SEABASS (*DICENTRARCHUS LABRAX*)**
3 **DISTAL INTESTINE LIPID CLASSES AND GUT HEALTH**

4 **Rivero-Ramírez F.¹, Torrecillas S.¹, Betancor M.B.², Izquierdo M.S.¹, Caballero**
5 **M.J.¹, Montero D.^{1*}**

6 ¹ Grupo de Investigación en Acuicultura (GIA), Instituto Universitario Ecoaqua, University of Las
7 Palmas de Gran Canaria, ULPGC, Crta. Taliarte s/n, 35214 Telde, Las Palmas, Canary Islands, Spain

8 ² Institute of Aquaculture, School of Natural Sciences, University of Stirling, Stirling FK9 4LA
9 Scotland, United Kingdom

10 * Corresponding author. Daniel Montero. E-mail address: daniel.montero@ulpgc.es

11 **ABSTRACT**

12 The use of low fishmeal/fish oil in marine fish diets affects dietary essential fatty acids
13 (EFAs) composition and concentration and subsequently, may produce a marginal
14 deficiency of those fatty acids with a direct impact on the fish intestinal physiology.
15 Supplementation of essential fatty acids is necessary to cover the requirements of the
16 different EFAs, including the ones belonging to the n-6 series, such as arachidonic acid
17 (ARA). ARA, besides its structural role in the configuration of the lipid classes of
18 intestine, plays an important role on the functionality of the gut associated immune
19 tissue (GALT).

20 The present study aimed to test five levels of dietary ARA (ARA0.5 (0.5%), ARA1
21 (1%), ARA2 (2%), ARA4 (4%) and ARA6 (6%) for European seabass (*Dicentrarchus*
22 *labrax*) juveniles in order to: (a) determine its effect in selected distal intestine (DI) lipid
23 classes composition; and (b) how these changes affected gut bacterial translocation rates
24 and selected GALT-related genes expression pre and post challenge.

25 No differences were found between distal intestines of fish fed the graded ARA levels
26 in total neutral lipids and total polar lipids. However, DI of fish fed the ARA6 diet
27 presented higher ($P<0.05$) level of phosphatidylethanolamine (PE) and sphingomyelin
28 (SM) than those DI of fish fed the ARA0.5 diet. In general terms, fatty acid profiles of
29 DI lipid classes mirrored those of the diet dietary. Nevertheless, a selective retention of

30 ARA could be observed in glycerophospholipids when dietary levels are low (Diet
31 ARA0.5), as reflected in the higher glycerophospholipids-ARA/dietary-ARA ratio for
32 those animals. Increased ARA dietary supplementation was inversely correlated with
33 eicosapentaenoic acid (EPA) content in lipid classes, when data from fish fed the diets
34 with the same basal composition (Diets ARA1 to ARA6). ARA supplementation did not
35 affect intestinal morphometry, goblet cells number or fish survival, in terms of gut
36 bacterial translocation, along the challenge test. However, after the experimental
37 infection with *Vibrio anguillarum*, the relative expression of *cox-2* and *il-1 β* were up-
38 regulated ($P < 0.05$) in DI of fish fed the diets ARA0.5 and ARA2 compared to fish fed
39 the rest of the experimental diets. Although dietary ARA did not affect fish survival, it
40 altered the fatty acids composition of glycerophospholipids and the expression of pro-
41 inflammatory genes after infection when included at the lowest concentration, which
42 could be compromising the physical and the immune functionality of the DI, denoting
43 the importance of ARA supplementation when low FO diets are used for marine fish.

44 **Keywords**

45 Aquaculture. *Dicentrarchus labrax*. Arachidonic acid. Gut Polar lipids. Distal intestine.
46 Gut health.

47

48 **1. INTRODUCTION**

49 Nowadays, due to economic and environmental reasons aquafeeds include
50 important levels of vegetable oil (VO), rich in 18:C polyunsaturated fatty acids
51 (PUFAs) (Hardy et al. 2010). In marine finfish, contrarily to freshwater species, in some
52 cases these substitutions are critical, since they have a limited capacity of elongate and
53 desaturate PUFAs into their long chain families (Tocher 2003). Thus, presenting dietary
54 requirements of long chain PUFA (LC-PUFAs), in particular for eicosapentaenoic acid
55 (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3), and arachidonic acid (ARA,
56 20:4 n-6) (Tocher 2015), due to their important role into growth performance, nervous
57 system or immune system development and functioning, for what they are recognized as
58 essential fatty acids (EFA) for marine fish (Tocher et al. 2008).

59 LC-PUFAs are selectively esterified into cell surface glycerophospholipids
60 (GPs) by fatty acyltransferase enzymes, affecting signaling processes as regulation of
61 nuclear receptors and transcription (Crowder et al. 2017), membrane stability and

62 fluidity, and, eventually, cell functions (Tocher 2003; Fernandez and West 2005;
63 Yaqoob and Calder 2007). These functions can be exerted directly by GPs as
64 phosphatidylcholine (PC) and phosphatidylserine (PS) which are activators of protein
65 kinase C (Tocher et al. 2008), or through derivatives as phosphoinositides, diacylglycerol,
66 lysophosphatidic acid or oxidized PC, to bind and activate receptors as, for instance,
67 peroxisome proliferator activated receptor (Davies et al. 2001). Similarly, GPs
68 constitute a reservoir of fatty acids (FA) that are released by phospholipase A2 (Pla2) to
69 be used by cyclooxygenase (Cox) and lipoxygenase (Lox) enzymes for eicosanoid
70 production (Tocher 2003) as prostaglandins (PGs), thromboxanes or leukotrienes,
71 among others. Eicosanoids are a group of highly active hormone-like molecules that
72 exert their biological effects in a paracrine manner in many physiological processes as
73 the inflammatory response (Tocher 2003; Yaqoob and Calder 2007).

74 Given the fact that dietary oils and fats affect FA profile in fish tissues,
75 especially in marine species (Tocher 2015), the organ function will be also influenced
76 by dietary lipids (Tocher 2003). For instance, reductions of dietary EFA for gilthead
77 seabream (*Sparus aurata*) together with changes on other FAs by the different dietary
78 lipid sources are responsible for alterations in the morphology of intestine (Caballero et
79 al. 2003 and 2004). The digestive tract of teleosts is one of the main entrances for
80 pathogens (Zapata & Cooper, 1990), and particularly the gut-associated immune system
81 (GALT) has a great importance in maintaining its health status (Rombout et al., 2011;
82 Torrecillas et al. 2012). Fish gut houses a regional immune specialization and it is
83 considered an important place for antigen uptaking, playing a key role achieving oral
84 immune-protection (Rombout et al. 2011). In distal intestine (DI), lymphocytes,
85 granulocytes and leukocytes, are spread on the epithelium and constitute the GALT, a
86 local immune system that reacts to disturbances of homeostasis as those that occur
87 during an infectious process or inclusion of terrestrial sources in diet (Torrecillas et al.
88 2014; Salinas 2015). These immune cells can produce eicosanoids to induce immune-
89 cell proliferation, cytokine-release or to chemo-attract other immune cells (Zou and
90 Secombes 2016). Hence, dietary imbalances of EFAs can lead to modifications on cell
91 membranes composition and, therefore, alter gut morphology, growth performance and
92 fish health (Tocher 2003; Montero et al. 2001, 2003, 2005, 2008, 2010).

93 Recent studies are demonstrating that ARA plays an important role on fish growth
94 performance (Bessonart et al. 1999; Carrier et al. 2011; Koven et al. 2003; Lund et al.

95 2007; Bae et al. 2010; Luo et al. 2012; Torrecillas et al. 2018a), lipid metabolism (Luo
96 et al. 2012; Xu et al. 2018), or fish health and disease resistance (Xu et al. 2010;
97 Torrecillas et al. 2017c), among others. Besides, the essential role of ARA and its
98 relative low levels compared to n-3 LC-PUFAs in the marine environment and in fish
99 tissues, have probably led to the strong preference of enzymes involved in eicosanoid
100 synthesis, at the expense of EPA (Liu et al. 2006; Yaqoob and Calder 2007; Furne et al.
101 2013). Indeed, the ratio ARA/EPA on the target organ, affects the synthesis of
102 eicosanoids (Ganga et al. 2005,2006; Xu et al. 2018). Similarly, ARA-derived
103 eicosanoids compete with those from EPA for the same cell membrane receptors
104 (Sargent et al. 1999a; Ganga et al., 2005; Adam et al., 2017; Tian et al., 2017) although
105 those originated from ARA seem to be more biologically active (Leslie 2004). Beyond
106 eicosanoid production, the ARA role on immunity covers a great number of other
107 mechanisms in cells as the activation of the NADPH oxidase enzyme in leukocytes to
108 trigger the respiratory burst (Brash et al. 2001).

109 Farmed European seabass presents reduced ARA tissue levels when compared with
110 wild specimens (Alasalvar et al. 2002; Bell et al. 2007; Fuentes et al. 2010 Lenas et al.
111 2011) indicating a necessary increase of dietary ARA. Indeed, studies of optimum
112 levels of ARA have been made in larval stages of these species (Koven 2001, 2003;
113 Atalah et al. 2011; Montero et al. 2015c) but scarce information exists in juveniles
114 regarding ARA content in GPs and its influence in the intestinal immune response
115 (Torrecillas et al. 2017c,d).

116 Therefore, an experiment was conducted out using graded levels of dietary
117 ARA- for European seabass juveniles to determine the influence and the content of this
118 EFA in lipid classes of DI and the related effects on gut morphology, expression of
119 intestinal immune-related genes, survival and resistance to intestinal infection.

120 **2. MATERIAL AND METHODS**

121 **2.1. Experimental diets**

122 Five isolipidic and isoproteic experimental dry pelleted diets based on a
123 commercial formulation were prepared to contain graded levels of ARA (total FA in
124 diet, %) as follows: ARA0.5 (0.5%), ARA1 (1%), ARA2 (2%), ARA4 (4%) and ARA6
125 (6%). Diet ingredients, proximate composition, and FA) profiles are reported in Table 1

126 and 2. This basal diet was supplemented to achieve desired ARA content in diets
127 ARA2, ARA4, and ARA6 with increasing quantities of Vevodar[®] (DSM Food
128 Specialties, the Netherlands), a commercial fungal-oil rich in ARA obtained from
129 *Mortierella alpine* (authorized in European Union by Commission Decision
130 2008/968/CE). Diet ARA0.5, was formulated with defatted fish meal (FM) and without
131 fish oil (FO) to reduce the presence of ARA and supplemented with vegetable oils to
132 reach requirements. When necessary, supplementation of DHA and EPA was done
133 using DHA50 and EPA50 (CRODA, East Yorkshire, UK).

134 **2.2. Fish and experimental conditions**

135 For this feeding trial, eight hundred and forty European seabass juveniles reared
136 in a commercial farm were maintained in quarantine in the facilities of Marine Science-
137 Technology Park (PCTM) of University of Las Palmas de Gran Canaria (ULPGC), for 4
138 weeks before the experience, and fed a commercial diet. Tanks were supplied with
139 seawater at a natural temperature of 22.8–24.9 °C in a flow-through system and kept at a
140 natural photoperiod (12L:12D). Dissolved oxygen ranged between 5-8 ppm. Fish were
141 fed the experimental diets for 70 days and, at the end of this feeding trial, fish were
142 submitted to a challenge test against *Vibrio anguillarum* via intestinal inoculation.

143 All animal manipulation in this trial complied European Union Council
144 guidelines (86/609/EU) and Spanish legislation (RD 53/2013) and had been approved
145 by Bioethical Committee of the ULPGC (Ref. 007/2012 CEBA ULPGC).

146 **2.3. Feeding trial**

147 With an average weight and length of 13.4 ± 0.3 g and 9.9 ± 0.1 cm respectively
148 (mean \pm SD), animals were randomly allocated in 15 fiberglass 200 L tanks (55
149 fish/tank; 4 kg m^{-3} of stocking density). Diets were assayed in triplicate and animals
150 were fed by hand for 70 days until apparent satiation, three times a day, 6 days a week.
151 After 70 days, samples of DI were taken for biochemical, histological and gene-
152 expression analyses. Survival was recorded during the whole period of the feeding trial.

153 **2.4. Challenge trial**

154 After 70 days of experiment, fish were transferred to the Biosecurity Facilities of
155 ULPGC in PCTM (Telde, Las Palmas, Canary Island, Spain). After 2 weeks of
156 adaptation to the new experimental conditions, fish were inoculated with a sublethal
157 dose (10^7 CFU ml⁻¹ per fish) of *V. anguillarum* using the method of anal cannulation
158 assayed previously in similar experimental conditions (Torrecillas et al. 2007). Fish
159 were fed their corresponding experimental diets for 7 days, as frequent than before. At 2
160 days after the infection, samples of DI were taken for immune-related genes analyses.
161 Survival was recorded along this trial.

162 **2.5. Lipid class and fatty acid content of selected glycerophospholipids of distal** 163 **intestine**

164 At day 70, eight fish per tank (N= 24 fish/diet), were used for biochemical
165 analysis. The intestine was extracted out for analysis and distal section was separated as
166 previously described by Torrecillas et al. (2013). Fish tissues were kept at -80° C until
167 the analysis. Biochemical composition of distal intestine and diets were conducted
168 following standard procedures from Association of Official Analytical Chemists
169 (AOAC, 2016). The analysis of lipid class and fatty acid composition of selected
170 glycerophospholipids (GPs) was conducted in the Institute of Aquaculture, Stirling
171 University (UK). Separation of main lipid classes was realized in 10 × 10 cm plates
172 (VWR, Lutterworth, UK) by double development high-performance thin-layer
173 chromatography (HPTLC) using the technics described by Tocher and Harvie (1988),
174 and Olsen and Henderson (1989). Firstly, plates were pre-run in diethyl ether and then
175 activated at 120° C for 1 hour. The lipid classes were visualized after spraying with 3%
176 (w/v) copper acetate, containing 8% (v/v) phosphoric acid by charring at 160° C for 20
177 min. Quantification was made by densitometry using a CAMAG-3 TLC scanner
178 (Version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS Planar
179 Chromatography Manager. Samples and authentic standards run alongside, in the same
180 conditions, on high-performance thin layer chromatography (HPTLC) plates, as the way
181 to determine the identities of individual lipid classes by contrasting Rf values. Total
182 GPs, including PC, PS, phosphatidylethanolamine (PE), and phosphatidylinositol (PI)
183 were isolated from HPTLC plates and subjected to acid-catalyzed transesterification
184 according to the method of Tocher and Harvie (1988). Afterwards, extraction and
185 purification were performed as described by Christie (1982). To separate and quantify
186 fatty acid methyl esters (FAMES) of selected GPs, a gas-liquid chromatography was

187 executed using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) with the conditions
188 determined previously (Izquierdo et al. 1992).

189 **2.6. Histological studies**

190 Samples from DI (N= 6 fish/diet) obtained after 70 days of feeding and taken as
191 described by Torrecillas et al., (2013) were fixed in neutral-buffered formalin (4%).
192 After 48 hours, tissues were dehydrated with an increased graded series of ethanol,
193 submerged in xylene and embedded in paraffin blocks. Sections of 4µm were cut and
194 stained with hematoxylin and eosin (H&E) and Alcian Blue-PAS (pH= 2.5) (Martoja
195 and Martoja-Pierson 1970), for optical examinations and to differentiate mucus-
196 secreting cells, respectively. Micrographs analyzed were obtained with a Nikon
197 Microphot- FXA microscope (objective lens 20X plus eyepiece 10X) equipped with an
198 Olympus DP50 camera. Cell count and measures of DI were made according to
199 Torrecillas et al. (2007), using Image-Pro Plus v5 software (Media Cybernetics Inc.,
200 Rockville, MD, USA). Structural measures of DI were studied with a light microscope
201 (N=72; 12 sections per fish × 6 fish per tank × 3 tanks per diet) and using individual fish
202 weight as co-variable. Following measures were calculated: fold area, FA; fold
203 perimeter, FP; fold length, FL; fold width, FW; submucosa width, SW. To estimate
204 mucus production, the number of mucus-secreting cells by unit of area was counted (N=
205 288; 48 folds per fish × 2 fish per tank × 3 tanks per diet).

206 **2.7. RNA extraction, cDNA synthesis and Quantitative Real-Time PCR analysis**

207 After 70 days of feeding and during challenge trial (2 days), DI (N= 9 fish/diet)
208 samples were collected in order to realize real time (RT) qPCR analyses. Tissues were
209 submerged into Invitrogen™ RNeasy™ Stabilization Solution (Thermo Fisher
210 Scientific Inc., USA) and conserved at -20°C. Then, using TRI-Reagent (Sigma-
211 Aldrich, Saint Louis, MO, USA) and RNeasy® mini Kit (QUIAGEN, Germany), total
212 RNA was extracted from 100 mg of pooled tissues, (N=3 fish/tank). RNA was
213 quantified by spectrophotometry using Nanodrop 1000 (Thermo Fisher Scientific Inc.,
214 USA) and integrity was evaluated on a 1.4% agarose gel with Gel Red™ (Biotium Inc.,
215 Hayward, CA). The synthesis of cDNA was realized from 1µg RNA with iScript™
216 cDNA Synthesis Kit (Bio-Rad Hercules, California) in 20µl final volume. Selected
217 genes related to GALT functioning and eicosanoid production were as follows and

218 respectively: interleukin 10 (*il-10*), interleukin-1beta (*il-1 β*), tumor necrosis factor alpha
219 (*tnfa*), and cyclooxygenase 2 (*cox-2*). RT-qPCR reactions were performed by triplicate
220 and conditions were 1X (95°C, 10min), 35x (95°C, 45s/corresponding annealing
221 temperature, 45s/72°C, 45s) 1X (72°C, 30s). Conditions, sequences and references are
222 registered in Table 3. Two genes, elongation factor 1 (*ef-1*) and β -*actin*, were tested as
223 housekeeping but *ef-1* was found to be more stable to make calculations. Reactions
224 were performed in an iCycler Optical Module (Bio-Rad, USA), the final volume used
225 was 15 μ l, containing 2 μ l of cDNA (diluted 1/10), 0.6 μ l of each primer (10 mM) and
226 7.5 μ l of Brilliant SYBR Green QPCR Master Mix (Bio-Rad Hercules, CA, USA).
227 Blank samples, with 2 μ l of water replacing cDNA, were included in each assay as a
228 contamination control. The Livak & Schmittgen (2001) method was used to calculate
229 relative expression of each gene.

230 **2.8. Statistical analysis**

231 All statistical analyses were performed using SPSS 21 software package for
232 Windows (IBM, Chicago, IL, USA). All data, presented as mean \pm SD, were tested for
233 normality and homoscedasticity. Statistical analyses followed methods outlined by
234 Sokal and Rolf (1995). Data were submitted to a One-way analysis of variance
235 (ANOVA). When F values showed significance, individual means were compared using
236 post hoc tests for multiple means comparison. When data were not normally distributed,
237 data analysis was made by non-parametric test (Kruskal-Wallis and U Mann-Whitney).
238 When Levene's test showed P<0.05, but ANOVA and Wells test showed P<0.05, post
239 hoc test used was Games-Howell. Pearson coefficient was used for correlations and
240 statistical significance was set at P<0.05. Survival curves were performed and analyzed
241 using the method described by Kaplan-Meier (Kaplan and Meier 1958).

242 **3. RESULTS**

243 **3.1 Growth parameters**

244 The growth study has been previously reported (Torrecillas et al., 2018a) but it is
245 important to point out that fish growth presented differences at the end of feeding trial.
246 Briefly, fish fed the lowest dietary ARA levels showed significantly lower (P<0.05)
247 weight (g) (ARA0.5 =33.0 \pm 1.1) than those from the other diets, that are those diets in
248 which ARA was supplemented on the same base diet (ARA1= 44.4 \pm 1.1; ARA2= 43.8

249 ± 1.0 ; ARA4= 43.9 ± 3.7 ; ARA6= 42.8 ± 2.5) (mean \pm SD). Dietary ARA levels did not
250 affect ($P>0.05$) cumulative survival percentages for European sea bass fed the
251 experimental diets for 70 days (over 95% for all diets).

252 **3.2 Lipid class composition of distal intestine**

253 No differences were found between diets in the Σ neutral lipids or the Σ polar
254 lipids of DI (Table 4). Regarding polar lipids, PC, followed by PE, were in higher
255 proportion than the rest of lipid class (Table 4). Lysophosphatidylcholine (LPC)
256 presented the lowest proportion (Table 4). Among polar lipids, SM and PE were the
257 only lipid class affected by dietary ARA ($P=0.041$ and $P=0.049$; respectively) (Table 4).
258 Fish fed diet ARA6 had significant ($P<0.05$) higher level of PE than control diet
259 (ARA0.5) (Table 4). Similarly, SM was more abundant in ARA6 than in ARA0.5,
260 ARA1 and ARA2 (Table 4). Besides, significant correlations between dietary ARA and
261 lipid classes in DI were found for PE ($0.743/P=0.001$), PC ($0.640/P=0.010$) and SM
262 ($0.700/P=0.004$), (Pearson coefficient/P value).

263 **3.3 Fatty acid composition of selected glycerophospholipids in distal intestine.**

264 The FA composition of four main GPs (PC, PE, PS and PI) was analyzed in DI
265 (Table 5 a, b, c & d). Increasing dietary ARA levels mirrored in the content of ARA in
266 GPs (GPsARA). However, the lowest dietary ARA level (ARA0.5) induced a selective
267 incorporation of ARA in all the GPs, reflected in the content of ARA ($P<0.05$; Tables
268 5a to 5d). The higher GPsARA/dietary ARA ratio ($P<0.05$) found for PC, PE and PS in
269 fish fed ARA0.5 diet in comparison to the values obtained for the animals feeding either
270 of the rest of the diets, was also reflecting the selective incorporation of ARA (Tables 5a
271 to 5c). For PI, no differences ($P>0.05$) were found in the GPsARA/dietary ARA ratio
272 between fish fed ARA0.5 and ARA1 diets (Table 5d). The GPsARA/dietary ARA ratio
273 in all GPs analyzed in DI, reflected that content of ARA was higher than dietary ARA.
274 Significant ($P<0.05$) correlations were found in DI between dietary ARA levels and the
275 GPsARA in all analyzed polar lipids: PC ($0.992/P<0.001$), PS ($0.872/P<0.001$), PE
276 ($0.969/P<0.001$), PI ($0.750/P=0.001$) (Pearson coefficient/P value) (Tables 5a to 5d).

277 Fish fed to ARA 0.5 diet presented high content of $\Sigma n-6$ PUFA and $\Sigma n-3$ PUFA due to
278 the higher content of 18:2n-6 and 18:3n-3 from the diet, respectively. For the rest of the
279 experimental diets, where ARA was supplemented on the same basal diet from diet

280 ARA1 to ARA6), all GPs analyzed in DI, increasing dietary ARA induced an
281 accumulation of Σ n-6 PUFA ($P<0.05$), mainly due to the increased GPsARA in the
282 different GPs, (Tables 5a to 5d). Moreover, in PC, PE and PS, dietary ARA induced a
283 significant ($P<0.05$) reduction of Σ n-3 PUFA (Tables 5a to 5c). The increment of
284 dietary levels of ARA was inversely correlated with the EPA content in GPs, although
285 negative correlations were not significant ($P>0.05$), except for PE (data not shown), due
286 to reduced dietary EPA level in diet ARA0.5 compared to the other diets (Table 2).
287 Negative and significant ($P<0.05$) correlations between dietary ARA level and EPA
288 content were found for all GPs when ARA0.5 diet was excluded from the statistical
289 analysis: PC (-0.904/ $P<0.001$), PS (-0.777/ $P=0.003$), PE (-0.941/ $P<0.001$), and PI (-
290 0.807/ $P=0.002$) (Pearson coefficient/ P value) (Tables 5a to 5d). Besides, differences of
291 Σ saturated and Σ PUFA were found in PC, with the higher ($P<0.05$) Σ PUFA level and
292 the lower ($P<0.05$) level of Σ saturated in those fish fed ARA0.5 diet, due to significant
293 increases of oleic, linoleic and alpha-linolenic acids, (Table 5a). Differences in DHA
294 content were found in PS and PE among fish fed the different dietary treatments (Tables
295 5b and 5c). In PS, lower ($P<0.05$) level of DHA was found in fish fed ARA0.5 diet than
296 ARA1, ARA2, and ARA4 (Table 5b). In PE, lower ($P<0.05$) level of DHA was found in
297 fish fed ARA0.5 and ARA6 diets when compared with the rest of experimental diets
298 (Table 5c).

299 **3.4 Histological studies**

300 Morphometric analysis of DI showed no significant ($P>0.05$) differences in any
301 intestinal measure (Table 6) when related to fish real weight. Similarly, no effect of
302 dietary ARA was observed in the density of goblet cells by unit of area in relation to the
303 real fish weight (Table 6).

304 **3.5 Relative expression of selected genes after feeding trial and challenge test** 305 **against *Vibrio anguillarum*.**

306 The cumulative mortality after challenge test against *V. anguillarum* was not
307 affected by dietary ARA ($P>0.05$). Despite the differences in the survival percentages
308 were not significant, there was a trend to lower mortality in fish fed diet ARA6, which
309 did not present mortality along the experimental intestinal infection, whereas the

310 survival percentage of fish fed the experimental diets ranged between 76.5 and 88.2%,
311 for diets ARA0.5 and ARA4 respectively).

312 The relative expression of immune related genes, including *il-1 β* , *tnfa*, *il-10* and
313 *cox-2*, were analyzed in DI at both basal and 2 days post infection (Fig.1). No effect was
314 found on *tnfa* relative gene expression (Fig. 1a). After the feeding period (basal level),
315 increased expression of pro-inflammatory *il-1 β* (P=0.030) was found in fish fed
316 ARA0.5 diet in comparison to fish fed ARA1 and ARA2 (Fig.1b). After 2 days post
317 infection, there was an up-regulation of *il-1 β* relative gene expression in fish fed
318 ARA0.5 and ARA2 diets when compared with those fish fed the rest of the diets
319 (P<0.001) (Fig.1b). An increment of *il-10* relative expression was found in fish fed
320 ARA1 and ARA6 (P=0.002) at basal level compared to fish fed the other diets, whereas
321 after infection a reduction was found in fish fed ARA2 compared to those fed the rest of
322 the diets (P<0.001) (Fig.1c). No differences (P>0.05) were found at basal level for *cox-2*
323 relative expression (Fig.1d). At 2 DPI, *cox-2* gene expression was up-regulated
324 (P<0.05) in fish fed ARA2 (Fig.1d) when comparing to fish fed the rest of the dietary
325 treatments.

326 4. DISCUSSION

327 Fish have dietary requirements of GPs for normal growth, homeostasis
328 maintenance, survival, or immune system function (Tocher et al. 2008; Adam et al.,
329 2017; Tian et al., 2017). Among other functions, GPs are related with lipid transport and
330 plasticity of the cell membranes (Tocher et al. 2008). Besides, GPs, act as precursors of
331 metabolism mediators as diacylglycerol or phosphoinositides, these last related with cell
332 polarity to keep cytoarchitecture, which is determinant in epithelial barrier and transport
333 functions allocated in the enterocyte-mucose layer (Shewan et al. 2011). GPs have
334 described to be affected by the dietary fatty acid profile, both the amount of each GP
335 and also the fatty acid composition of each lipid class (Olsen et al. 2003).

336 In this study, levels of dietary ARA were correlated with the concentration of the
337 different lipid class levels in DI of European seabass. Although increased dietary ARA
338 seemed to be related with increased the concentration of PE and SM in DI, with values
339 higher in the diets supplemented with high (ARA4 or ARA6) content of ARA, it was
340 also correlated to PC level, a lipid class that is required for SM synthesis (Patel and Witt

341 2017) and is related to PE through remodeling pathways (Tocher et al. 2008). Previous
342 studies have demonstrated the importance of SM in epithelial barriers of fish and other
343 vertebrates, despite the structural differences between marine and terrestrial epithelia
344 (Feingold 2007; Pullmannová et al. 2014; Cheng et al. 2018). In fact, this polar lipid,
345 disposed in the outer leaflet of the cell membrane with another choline-container lipid
346 as PC (Tocher et al. 2008), is more abundant in membranes of temperate-water fish
347 suggesting its role in the membrane fluidity (Storelli et al. 1998; Palmerini et al. 2009).
348 In Atlantic salmon, reductions in dietary EPA and DHA increased skin SM levels,
349 denoting alterations of the barrier function of the skin with reductions of these EFAs
350 (Cheng et al. 2018). Besides, SM has been linked with the regulation of the release of
351 ARA, by the inhibition of the c-Pla2 α bind to the GPs (Nakamura and Murayama 2014).
352 In the present experiment, SM in DI increased when ARA increased in diet, with the
353 subsequent decrease of the n-3 LC-PUFA/ARA ratio. The increase of SM in the gut of
354 fish fed high dietary ARA could be ameliorating a possible increase of cPla2 activity
355 induced by the high amount of ARA in the GPs of those fish fed the higher levels of
356 ARA in diet.

357 It is known that high LC-PUFA content induces the decarboxylation of PS to PE
358 at membrane level of different organelles as mitochondria or Golgi (Kainu et al. 2013).
359 In the present study, PE levels in DI were increased by dietary ARA, with the highest
360 level corresponding to those fish fed the highest dietary ARA level. This could be
361 related to the fact that the generation of PE through the PS decarboxylation pathway
362 generated preferentially PE species with a PUFA at the sn-2 position (Bleijerveld et al.
363 2007). However, the synthesis of PE through decarboxylation of PS has been shown to
364 be promoted by DHA and not by ARA (Ikemoto et al. 1999), and thus, other metabolic
365 pathways different than PS decarboxylation cannot be rejected to explain the increases
366 of PE in the DI of the fish fed high ARA in diet.

367 Dietary ARA also influenced fatty acid profiles of lipid classes in the distal
368 section of the intestine. Olsen et al. (2003) showed that the effect of the type of dietary
369 lipid is reflected in the fatty acid profile of the intestine and it is dependent of the
370 section of intestine studied. In this study, correlations were found between dietary ARA
371 and content of ARA for the four GPs studied in DI.

372 As described for other species, PI was the lipid class with the highest content of
373 ARA (Bell and Sargent 2003). Moreover, due to the abundance of PC and PE in the

374 tissue studied, higher ARA content was found in those GPs in agreement with previous
375 studies (Bell et al. 1995). Besides, the increased content of ARA in studied GPs with
376 respect to the dietary level occurred in all diets and GPs analyzed, although with more
377 intensity in fish fed the lowest ARA level as reflected in the higher ratio GPs-
378 ARA/dietary for those animals. This selective retention can be considered as a way to
379 keep functionality during EFA deficiencies (Skalli et al. 2006) as negative effects of
380 EFA deficiencies can be magnified at chronic stressful situations. Indeed, ARA
381 reductions were found in liver polar lipids when gilthead sea bream were subjected to
382 high stocking densities probably due to its selective utilization in that stressful situation
383 (Montero et al. 2001). Moreover, DHA concentration was also higher than dietary DHA
384 levels in all studied GPs, particularly in PE and PS, although it must be taken into
385 account that DHA is preferentially esterified to PE and PS (Kim et al., 2004), and thus
386 DHA concentration in polar lipids depends not only on the DHA level in diets but also
387 on the esterification within those lipid classes. The relatively high levels of ARA and/or
388 DHA despite their dietary inclusion were in agreement to their preferential
389 incorporation previously found by other authors in European sea bass tissues (Farndale
390 et al. 1999; Eroldoğan et al. 2013; Torrecillas et al. 2015a) including in polar lipids
391 (Torrecillas et al. 2013) and in other species (Bell et al. 2001; Montero et al. 2001,
392 2003; Fountoulaki et al. 2003; Dantagnan et al. 2017). Furthermore, results from the
393 present study indicate that inclusion of EPA in GPs was negatively correlated by the
394 supplementation of ARA in diet (excluding from this correlation the results from diet
395 0.5 formulated with different ingredients and different fatty acid profile), suggesting
396 competition between EPA and ARA during phospholipid esterification, in agreement
397 with previous studies (Bell et al. 1991, Bessonart et al. 1999; Fountoulaki et al. 2003;
398 Atalah et al. 2011). Competition between both fatty acids as substrate for different
399 enzymes is of especial relevance during eicosanoid synthesis, as both fatty acids are
400 substrates for eicosanoid production, affecting different fish functions, including
401 immune system (Bell et al. 1996b, Montero et al., 2015c; Adam et al., 2017).

402 The graded dietary levels of ARA used in the present study did not affect
403 survival, in agreement with previous studies using graded dietary ARA levels in
404 European sea bass larvae (Atalah et al. 2011) or in other marine species such as gilthead
405 seabream, Senegal sole (*Solea senegalensis*) or Japanese sea bass (*Lateolabrax*
406 *japonicus*) (Fountoulaki et al. 2003; Villalta et al. 2005; Xu et al. 2010). Other studies in

407 gilthead seabream have found positive effects (Bessonart et al. 1999) related to stress
408 resistance (Koven et al. 2001; Willey et al. 2003). Besides, low or too high dietary ARA
409 has been described to induce a reduction of fish survival during a bacterial challenge in
410 Atlantic salmon (*Salmo salar*) (Dantagnan et al. 2017). In the present experiment, the
411 graded levels of dietary ARA did not affect survival after challenge test, but induced
412 changes in the expression of GALT-related genes, as described for other species such as
413 Atlantic salmon (Dantagnan et al. 2017) or guppy (*Poecilia reticulata*) (Khozing-
414 Goldberg et al. 2006). Indeed, a previous study has related dietary ARA with
415 mechanisms of protection against damage in the intestine (Tarnawski et al. 1989). In
416 this sense, intestine is an organ subjected to injury, intestinal barrier being highly
417 compromised and subsequently acting as one of the main entrances for pathogens (Ellis
418 2001; Campos-Pérez et al. 2000).

419 The relation between intestine and eicosanoid synthesis has been widely studied
420 in different fish species (Sargent et al. 1999a; Tocher 2003; Calduch-Giner et al. 2016).
421 Although ARA and EPA are substrates for COX and LOX enzymes to produce
422 eicosanoids (Bell & Sargent 2003, Tocher et al. 2008), these enzymes seem to have
423 stronger preference for released-ARA than for EPA at least in freshwater fish and
424 salmonids (Bell and Sargent 2003; Tocher et al. 2008; Furne et al. 2013). In this trial,
425 the supplementation of dietary ARA did not influence directly basal levels of *cox-2*
426 relative expression in gut, suggesting no effect on PGE2 production in intestine as
427 described for other vertebrates (Tateishi et al. 2014) which is also supported by the
428 absence of significant differences in PI levels, the main pool of ARA for eicosanoids
429 production (Yaqoob and Calder 2007). However, after infection with *V. anguillarum*, in
430 the present study European seabass juveniles fed 2% of ARA in diet increased *cox-2*
431 relative expression, which has been related with protection to gastric mucosal defenses
432 including stimulation of mucus secretion and maintenance of mucosal blood flow
433 (Wallace and Devchan 2005). The gastro-protective properties of Cox-2-derived PGs
434 have been demonstrated in eel (*Anguilla anguilla*) gastric mucosa (Faggio et al. 2000),
435 and *cox-2* expression in the intestine has been also associated to a response of Atlantic
436 salmon to acute stress, mainly in DI (Oxley et al. 2010).

437 The up-regulation of *cox-2* levels found in the present study after bacterial
438 infection was coincident with the increased *il-1 β* gene relative expression. The Cox-2
439 enzyme and pro-inflammatory cytokines such as $\text{Il-1}\beta$ seems to be linked through the

440 p38 mitogen-activated protein kinase (P38 mapk) (Camacho-Barquero et al. 2007),
441 which is known to be present in fish (Ribeiro et al. 2010; Yang et al. 2014b). The Mapk
442 can be activated by ARA metabolites in a dose-dependent manner (Alexander et al.
443 2001), which in turn can activate *cox-2* expression (Sui et al. 2014). Besides, Mapk
444 constitutes a signaling pathway involved in regulation of multiple cell functions
445 including autophagy, a cell process of self-degradation to maintain homeostasis in
446 which proinflammatory cytokines are implicated (Sui et al. 2014). PE plays an
447 important role in autophagy because it is utilized by proteins required for the formation
448 of autophagosomes to attach to cell membranes (Ichimura et al. 2000; Iula et al. 2018),
449 and, besides, these autophagic vesicles are utilized for secretion of cytosolic Il-1 β (Iula
450 et al. 2018). At the same time, Il-1 β has been suggested to be involved in the PE
451 synthesis via Mapk (Sluzalska et al. 2017). In this way, the modification of PE levels in
452 DI can be related with the secretion of Il-1 β . In the present study, increased *il-1 β*
453 relative expression at basal time in diet ARA0.5 could be related to PE reduction in that
454 diet, although other factors influencing the PE reduction cannot be rejected, as this diet
455 had lower amount of DHA and EPA. Besides, other authors have shown that increased
456 levels of Il-1 β can reduce SM synthesis without affecting other choline-GPs as PC
457 (Kronqvist et al. 1999). In this experiment, and when considering only the diets with
458 same basal composition and graded ARA (diets from ARA1 to ARA6), the reduced
459 levels found in SM levels could be related to increments in ARA release in those fish
460 fed lower ARA level or to regulation of its synthesis, both mechanisms affected by Il-1 β
461 release.

462 In conclusion, ARA is selectively retained in the GPs of DI of European seabass,
463 supporting its important physiological role in this tissue. This ARA selective retention is
464 especially evident when low dietary ARA levels are fed (Diet ARA0.5), as reflected in
465 the higher glycerophospholipids-ARA/dietary-ARA ratio found. However, these
466 variations were not enough to alter DI morphology or/and bacterial translocation rates,
467 regardless of the ARA-deficiency related up-regulation of DI pro-inflammatory genes.
468 Altogether pointing to a long-term compromised physical barrier integrity and immune
469 functionality of the DI, denoting the importance of ARA supplementation when low FO
470 diets are used for marine fish.

471 **Funding information:** This research was financed by the Spanish Ministry of Economy
472 and Competitiveness (MINECO) in the Project AGL2012-39919 (PROINMUNOIL).

473 Additionally, Rivero-Ramírez F. was financed with a predoctoral fellowship (ULPGC-
474 2013) and Torrecillas S. received a ULPGC postdoctoral fellowship (PICULPGC-2013-
475 CIENCIAS, concept 643.00.06).

476 **Compliance with ethical standards:** The handling of animals at this experiment
477 complied the guidelines of the European Union Council (86/609/EU) and Spanish
478 legislation (RD 53/2013) and was approved by the Bioethical Committee of the ULPGC
479 (Ref. 007/2012 CEBA ULPGC).

480 5. REFERENCES

481 Adam, A.C., Lie, K.K., Moren, M., Skjærven, K.H. High dietary arachidonic acid levels
482 induce changes in complex lipids and immune-related eicosanoids and increase
483 levels of oxidised metabolites in zebrafish (*Danio rerio*). British J. Nutr. Vol 117,
484 2017, 1075-1085. doi:10.1017/S000711451700215X

485 Alasalvar C., Taylor K.D.A., Zubcov E., Shahidi F., Alexis M. Differentiation of
486 cultured and wild sea bass (*Dicentrarchus labrax*): total lipid content, fatty acid
487 and trace mineral composition. Food Chem. Vol. 79 (2). 2002 145-150. DOI:
488 10.1016/S0308-8146(02)00122-X

489 Alexander L. D., Cui X. L., Falck J. R., Douglas J. G. Arachidonic acid directly
490 activates members of the mitogen-activated protein kinase superfamily in rabbit
491 proximal tubule cells. Kidney Int. Vol. 59(6). 2001 Jun. 2039-2053. DOI:
492 10.1046/j.1523-1755.2001.00718.x

493 AOAC, Official Methods of Analysis of Analytical Chemistry, twentieth ed., AOAC
494 INTERNATIONAL, Arlington, VA, USA, 2016

495 Atalah E., Hernández-Cruz C. M., Ganuza E., Benítez-Santana T., Ganga R., Roo J.,
496 Montero D. & Izquierdo M. S. Importance of dietary arachidonic acid for the
497 growth, survival and stress resistance of larval European sea bass (*Dicentrarchus*
498 *labrax*) fed high dietary docosahexaenoic and eicosapentaenoic acids. Aquac. Res.
499 Vol. 42 (9). 2011 Aug. 1261-1268. DOI: 10.1111/j.1365-2109.2010.02714.x

500 Bae J.-Y., Kim D.-J., Yoo K.-Y., Kim S.-G., Lee J.-Y. and Bai. S. C. Effects of Dietary
501 Arachidonic Acid (20:4n-6) Levels on Growth Performance and Fatty Acid
502 Composition of Juvenile Eel, *Anguilla japonica*. Asian-Australas. J. Anim. Sci.
503 Vol. 23 (4). 2010 Feb. 508-514. DOI: 10.5713/ajas.2010.90491

504 Bell J. G., McVicar A. H., Park M. T., Sargent J.R. High Dietary Linoleic Acid Affects
505 the Fatty Acid Compositions of Individual Phospholipids from Tissues of Atlantic
506 Salmon (*Salmo salar*): Association with Stress Susceptibility and Cardiac Lesion.
507 J. Nutr. Vol. 121(8). 1991 Aug. 1163-1172. DOI: 10.1093/jn/121.8.1163

508 Bell J. G., Castell J. D., Tocher D. R., MacDonald F. M. and Sargent J. R. Effects of
509 different dietary arachidonic acid: docosahexaenoic acid ratios on phospholipid

- 510 fatty acid compositions and prostaglandin production in juvenile turbot
511 (*Scophthalmus maximus*). *Fish Physiol. Biochem.* Vol. 14(2). 1995 Apr. 139-151.
512 DOI: 10.1007/BF00002457
- 513 Bell J. G., Ashton I., Secombes C.J., Weitzel B. R., Dick J. R., Sargent J. R. Dietary
514 lipid affects phospholipid fatty acid compositions, eicosanoid production and
515 immune function in Atlantic salmon (*Salmo salar*). *Prostaglandins Leukot. Essent.*
516 *Fatty Acids.* Vol. 54(3). 1996 Mar. 173-182. DOI: 10.1016/S0952-
517 3278(96)90013-7
- 518 Bell J. G., McEvoy J., Tocher D. R., McGhee F., Campbell P.J., Sargent J. R.
519 Replacement of Fish Oil with Rapeseed Oil in Diets of Atlantic Salmon (*Salmo*
520 *salar*) Affects Tissue Lipid Compositions and Hepatocyte Fatty Acid Metabolism.
521 *J. Nutr.* Vol. 131(5). 2001 May. 1535-1543. DOI: 10.1093/jn/131.5.1535
- 522 Bell J. G., Sargent J. R. Arachidonic acid in aquaculture feeds: current status and future
523 opportunities. *Aquac.* Vol. 218 (1-4). 2003 Mar. 491-499. DOI: 10.1016/S0044-
524 8486(02)00370-8
- 525 Bell J. G., Preston T., Henderson R. J., Strachan F., Bron J. E., Cooper K., and Morrison
526 D. J. Discrimination of Wild and Cultured European Sea Bass (*Dicentrarchus*
527 *labrax*) Using Chemical and Isotopic Analyses. *J. Agric. Food Chem.* Vol. 55
528 (15). 2007 Jun. 5934-5941. DOI: 10.1021/jf0704561
- 529 Bessonart M., Izquierdo M. S., Salhi M., Hernández-Cruz C.M., González M.M.,
530 Fernández-Palacios H. Effect of dietary arachidonic acid levels on growth and
531 survival of gilthead sea bream (*Sparus aurata* L.) larvae. *Aquac.* Vol. 179 (1-4).
532 1999 Sep. 265-275. DOI: 10.1016/S0044-8486(99)00164-7
- 533 Bleijerveld O.B., Brouwers J.F., Vaandrager A.B., Helms J.B., Houweling M. The
534 CDP-ethanolamine pathway and phosphatidylserine decarboxylation generate
535 different phosphatidylethanolamine molecular species. *J. Biol. Chem.* Vol. 282
536 (39). 2007 Sep. 28362-28372. DOI: 10.1074/jbc.M703786200
- 537 Brash. A. R. Arachidonic acid as a bioactive molecule. *J. Clin. Invest.* Vol. 107(11).
538 2001 Jun. 1339-1345. DOI: 10.1172/JCI13210
- 539 Buonocore F., Randelli E., Bird S., Secombes C. J., Facchiano A., Costantini S.,
540 Scapigliati G. Interleukin-10 expression by real-time PCR and homology
541 modelling analysis in the European sea bass (*Dicentrarchus Labrax* L.). *Aquac.*
542 Vol. 270 (1-4): 512-522 (2007). DOI: 10.1016/j.aquaculture.2007.05.040
- 543 Caballero M.J., Izquierdo M. S., Kjørsvik E., Montero D., Socorro J., Fernández A.J.,
544 Rosenlund G. Morphological aspects of intestinal cells from gilthead seabream
545 (*Sparus aurata*) fed diets containing different lipid sources. *Aquac.* Vol. 225 (1-4),
546 2003 Jul. 325-340. DOI: 10.1016/S0044-8486(03)00299-0
- 547 Caballero M.J., Izquierdo M. S., Kjørsvik E., Fernández A. J. and Rosenlund G.
548 Histological alterations in the liver of sea bream, *Sparus aurata* L., caused by
549 short- or long-term feeding with vegetable oils. Recovery of normal morphology

- 550 after feeding fish oil as the sole lipid source. *J. Fish Dis.* Vol. 27 (9). 2004 Sep.
551 531-41. DOI: 10.1111/j.1365-2761.2004.00572.x
- 552 Calduch-Giner J. A., Sitjà-Bobadilla A. and Pérez-Sánchez J. Gene Expression Profiling
553 Reveals Functional Specialization along the Intestinal Tract of a Carnivorous
554 Teleostean Fish (*Dicentrarchus labrax*). *Front. Physiol.* Vol. 7. 2016 Aug; 359-
555 376. DOI: 10.3389/fphys.2016.00359
- 556 Camacho-Barquero L., Villegas I., Sánchez-Calvo J. M., Talero E., Sánchez-Fidalgo S.,
557 Motilva V., Alarcón de la Lastra C. Curcumin, a Curcuma longa constituent, acts
558 on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic
559 experimental colitis. *Int. Immunopharmacol.* Vol. 7 (3). 2007 Mar. 333-342. DOI:
560 10.1016/j.intimp.2006.11.006
- 561 Campos-Perez J.J., Ward M., Grabowski P.S., Ellis A.E., Secombes C.J. The gills are
562 an important site of iNOS expression in rainbow trout *Oncorhynchus mykiss* after
563 challenge with the Gram-positive pathogen *Renibacterium salmoninarum*.
564 *Immunology.* Vol. 99 (1). 2000 Jan. 153-161. DOI: 10.1046/j.1365-
565 2567.2000.00914.x
- 566 Carrier III J. K., Watanabe W. O., Harel M., Rezek T. C., Seaton P. J., Shafer T. H.
567 Effects of dietary arachidonic acid on larval performance, fatty acid profiles,
568 stress resistance, and expression of Na⁺/K⁺ ATPase mRNA in black sea bass
569 *Centropristis striata*. *Aquac.* Vol. 319 (1–2). 2011 Sep. 111-121. DOI:
570 10.1016/j.aquaculture.2011.06.027
- 571 Cheng K., Bou M., Ruyter B., Pickova J., Ehtesham E., Du L., Venegas C., Moazzami
572 A. A. Reduced dietary levels of EPA and DHA have a major impact on
573 the composition of skin membrane lipids in Atlantic salmon (*Salmo salar* L.). *J.*
574 *Agric. Food Chem.* Vol. 66 (33). 2018 Aug. 8876-8884. DOI:
575 10.1021/acs.jafc.8b02886
- 576 Christie, (1982). *Lipid Analysis*. Second revised ed. Oxford: Pergamon Press (1982).
577 201 p.
- 578 Crowder M. K., Seacrist C. D., Blind. R. D. Phospholipid regulation of the nuclear
579 receptor superfamily. *Adv. in Biological Regul.* Vol. 63 (special issue). 2017 Jan.
580 6-14. DOI: 10.1016/j.jbior.2016.10.006
- 581 Dantagnan P., Gonzalez K., Hevia M., Betancor M.B., Hernández A. J., Borquez A.,
582 Montero D. Effect of the arachidonic acid/vitamin E interaction on the immune
583 response of juvenile Atlantic salmon (*Salmo salar*) challenged against
584 *Piscirickettsia salmonis*. *Aquac. Nutrition* Vol. 23 (4). 2017 Aug. 710-720. DOI:
585 10.1111/anu.12438
- 586 Davies S. S., Pontsler A. V., Marathe G. K., Harrison K. A., Murphy R. C., Hinshaw J.
587 C., Prestwich G. D., Hilaire A. St., Prescottti S. M., Zimmerman G. A., and
588 McIntyre T. M. Oxidized Alkyl Phospholipids Are Specific, High
589 Affinity Peroxisome Proliferator-activated Receptor γ Ligands and Agonists. *The*
590 *J. of Biological Chem.* Vol. 276. 2001 Feb. 16015-16023. DOI:
591 10.1074/jbc.M100878200

- 592 Ellis. A.E. Innate host defense mechanisms of fish against viruses and bacteria. Dev.
593 Comp. Immunol. Vol. 25 (8-9). 2001 Oct-Dec. 827-839. DOI: 10.1016/S0145-
594 305X(01)00038-6
- 595 Eroldoğan T.O., Yılmaz A.H., Turchini G.M., Arslan M., Sirkecioğlu N.A., Engin K.,
596 Özşahinoğlu I., Mumoğullarında F. Fatty acid metabolism in European sea bass
597 (*Dicentrarchus labrax*): effects of n-6 PUFA and MUFA in fish oil replaced diets.
598 Fish Physiol. Biochem. Vol. 39 (4). 2013 Aug. 941-955. DOI: 10.1007/s10695-
599 012-9753-7
- 600 Faggio C., Denaro M.G., Lionetto M.G., Trischitta F. Protective effects of
601 prostaglandins in the isolated gastric mucosa of the eel, *Anguilla anguilla*. J.
602 Comp. Physiol. B. Vol. 170 (5-6). 2000 Sep. 357-363. DOI:
603 10.1007/s003600000111
- 604 Farndale B. M., Bell J.G., Bruce M. P., Bromage N. R., Oyen F., Zanuy S., Sargent J. R.
605 Dietary lipid composition affects blood leucocyte fatty acid compositions and
606 plasma eicosanoid concentrations in European sea bass (*Dicentrarchus labrax*).
607 Aquac. Vol. 179 (1-4). 1999 Sep. 335-350. DOI: 10.1016/S0044-
608 8486(99)00169-6
- 609 Feingold. K. R. The Role of Epidermal Lipids in Cutaneous Permeability Barrier
610 Homeostasis. J. Lipid Res. Vol. 48 (12). 2007 Dec. 2531-2546.
611 DOI:10.1194/jlr.R700013-JLR200
- 612 Fernandez M. L. and West K. L. Mechanisms by which Dietary Fatty Acids Modulate
613 Plasma Lipids. The J. of Nutrition Vol. 135 (9). 2005 Sep. 2075-2078. DOI:
614 10.1093/jn/135.9.2075
- 615 Fountoulaki E., Alexis M.N., Nengas I., Venou B. Effects of dietary arachidonic acid
616 (20:4n-6), on growth, body composition, and tissue fatty acid profile of gilthead
617 bream fingerlings (*Sparus aurata* L.). Aquac. Vol. 225 (1-4). 2003 Jul. 309-323.
618 DOI: 10.1016/S0044-8486(03)00298-9
- 619 Fuentes A., Fernández-Segovia I., Serra J. A., Barat J. M. Comparison of wild and
620 cultured sea bass (*Dicentrarchus labrax*) quality. Food Chem. Vol. 119 (4). 2010
621 Apr. 1514-1518. DOI: 10.1016/j.foodchem.2009.09.036
- 622 Furne M., Holen E., Araujo P., Lie K. K., Moren M. Cytokine gene expression and
623 prostaglandin production in head kidney leukocytes isolated from Atlantic cod
624 (*Gadus morhua*) added different levels of arachidonic acid and eicosapentaenoic
625 acid. Fish Shellfish Immunol. Vol. 34 (3). 2013 Mar. 770-777. DOI:
626 10.1016/j.fsi.2012.11.044
- 627 Ganga R., J.Bell J.G., Montero D., Robaina L., Caballero M. J., Izquierdo M. S. Effect
628 of dietary lipids on plasma fatty acid profiles and prostaglandin and leptin
629 production in gilthead seabream (*Sparus aurata*). Comp. Biochem. Physiol. B.
630 Biochem. Biol. Mol. Vol. 142 (4). 2005 Dic. 410 – 418. DOI:
631 10.1016/j.cbpb.2005.09.010

- 632 Ganga R., Tort L., Acerete L., Montero D. and Izquierdo M. S. Modulation of ACTH-
633 induced cortisol release by polyunsaturated fatty acids in interrenal cells from
634 gilthead seabream, *Sparus aurata*. *J. Endocrinol.* Vol. 190 (1). 2006 Ju. 39-45.
635 DOI: 10.1677/joe.1.06770
- 636 Geay F., Ferrareso S., Zambonino-Infante J.L., Bargelloni L., Quentel C., Vandeputte
637 M., Kaushik S., Cahu C.L., Mazurais D. Effects of the total replacement of fish-
638 based diet with plant-based diet on the hepatic transcriptome of two European sea
639 bass (*Dicentrarchus labrax*) half-sibfamilies showing different growth rates with
640 the plant-based diet. *BMC Genomics.* Vol. 12. 2011 Oct. 522-540. DOI:
641 10.1186/1471-2164-12-522
- 642 Hardy R. W. Utilization of plant proteins in fish diets: effects of global demand and
643 supplies of fishmeal. *Aquac. Res.* Vol. 41. 2010 Apr. 770-776.
644 DOI:10.1111/j.1365-2109.2009.02349.x
- 645 Ichimura Y., Kirisako T., Takao T., Satomi Y., Shimonishi Y., Ishihara N., Mizushima
646 N., Tanida I., Kominami E., Ohsumi M., Noda T., Ohsumi Y. A ubiquitin-like
647 system mediates protein lipidation. *Nature* Vol. 408 (6811). 2000 Nov 23. 488-
648 492. DOI: 10.1038/35044114
- 649 Ikemoto A., Kobayashi T., Emoto K., Umeda M., Watanabe S., and Okuyama H.
650 Effects of docosahexaenoic and arachidonic acids on the synthesis and
651 distribution of aminophospholipids during neuronal differentiation of PC12 cells.
652 *Arch. Biochem. Biophys.* Vol. 364 (1). 1999 Apr. 67-74. DOI:
653 10.1006/abbi.1999.1110
- 654 Iula L., Keitelman I. A., Sabbione F., Fuentes F., Guzman M., Galletti J. G., Gerber P.
655 P., Ostrowski M., Geffner J. R., Jancic C. C., and Trevani A. S. Autophagy
656 Mediates Interleukin-1 β Secretion in Human Neutrophils. *Front. Immunol.* Vol. 9.
657 2018 Feb. 269-283. DOI: 10.3389/fimmu.2018.00269
- 658 Izquierdo M. S., Arakawa T., Takeuchi T., Haroun R., Watanabe T. Effect of n-3HUFA
659 levels in artemia on growth of larval japanese flounder (*Paralichthys olivaceous*).
660 *Aquac.* Vol. 105 (1). 1992 Jul. 73-82. DOI: 10.1016/0044-8486(92)90163-F
- 661 Kainu V., Hermansson M., Hänninen S., Hokynar K., Somerharju P. Import of
662 phosphatidylserine to and export of phosphatidylethanolamine molecular species
663 from mitochondria. *Biochim. Biophys. Acta.* Vol. 1831 (2). 2013 Feb. 429-437.
664 DOI: 10.1016/j.bbalip.2012.11.003
- 665 Kaplan E. L. and Meier P. Nonparametric Estimation From Incomplete Observations. *J.*
666 *Am. Stat. Assoc.* Vol. 53. 1958. 457-481. DOI:
667 10.1080/01621459.1958.10501452
- 668 Khozin-Goldberg I., Cohen Z., Pimenta-Leibowitz M., Nechev J., Zilberg D. Feeding
669 with arachidonic acid-rich triacylglycerols from the microalga *Parietochloris incisa*
670 improved recovery of guppies from infection with *Tetrahymena* sp. *Aquac.* Vol.
671 255 (1-4). 2006 May. 142-150. DOI: 10.1016/j.aquaculture.2005.12.017

- 672 Kim, H.Y., Bigelow, J., Kevala, J.H. Substrate preference in phosphatidylserine
673 biosynthesis for docosahexaenoic acid containing species. *Biochemistry* Vol. 43.
674 2004. 1030-1036. DOI: 10.1021/bi035197x
- 675 Koven W., Barr Y., Lutzky S., Ben-Atia I., Weiss R., Harel M., Behrens P., Tandler A.
676 The effect of dietary arachidonic acid (20:4 n-6) on growth, survival and
677 resistance to handling stress in gilthead seabream (*Sparus aurata*) larvae. *Aquac.*
678 vol. 193 (1-2). 2001 Feb. 107-122. DOI: 10.1016/S0044-8486(00)00479-8
- 679 Koven W., Van Anholt R., Lutzky S., Atia I.B., Nixon O., Ron B., Tandler A. The
680 effect of dietary arachidonic acid on growth, survival, and cortisol levels in
681 different-age gilthead seabream larvae (*Sparus auratus*) exposed to handling or
682 daily salinity change. *Aquac.* Vol. 228 (1-4). 2003 Dec. 307-320. DOI:
683 10.1016/S0044-8486(03)00317-X
- 684 Kronqvist R., Leppimäki P., Mehto P., Slotte J. P. The effect of interleukin 1 β on the
685 biosynthesis of cholesterol, phosphatidylcholine, and sphingomyelin in
686 fibroblasts, and on their efflux from cells to lipid-free apolipoprotein A-I.
687 *European J. of Biochemistry* Vol. 262 (3). 1999 Jun. 939-946. DOI:
688 10.1046/j.1432-1327.1999.00484.x
- 689 Lenas D., Chatziantoniou S., Nathanailides C., Triantafillou D. Comparison of wild and
690 farmed sea bass (*Dicentrarchus labrax* L) lipid quality. *Procedia Food Sci.* Vol. 1
691 (Special issue). 2011. 1139-1145. DOI: 10.1016/j.profoo.2011.09.170
- 692 Leslie C. C. Regulation of arachidonic acid availability for eicosanoid production.
693 *Biochem. Cell Biol.* Vol. 82 (1). 2004 Feb. 1-17. DOI: 10.1139/o03-080
- 694 Liu W., Cao D., Oh S. F., Serhan C. N., and Kulmacz R. J. Divergent cyclooxygenase
695 responses to fatty acid structure and peroxide level in fish and mammalian
696 prostaglandin H synthases. *FASEB J.* Vol. 20 (8). 2006 Jun. 1097-1108. DOI:
697 10.1096/fj.05-5273com
- 698 Livak K. J. and Schmittgen T. D. Analysis of relative gene expression data using real-
699 time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* Vol. 25
700 (4). 2001. 402-408. DOI:10.1006/meth.2001.1262
- 701 Lund I., Steinfeldt S. J., Hansen B. W. Effect of dietary arachidonic acid,
702 eicosapentaenoic acid and docosahexaenoic acid on survival, growth and
703 pigmentation in larvae of common sole (*Solea solea* L.). *Aquac.* Vol. 273 (4).
704 2007 Dec. 532-544. DOI: 10.1016/j.aquaculture.2007.10.047
- 705 Luo Z., Tan X.-Y., Li X.-D., Yin G.-J. Effect of dietary arachidonic acid levels on
706 growth performance, hepatic fatty acid profile, intermediary metabolism and
707 antioxidant responses for juvenile *Synechogobius hasta*. *Aquac. Nutrition* Vol. 18
708 (3). 2012 Oct. 340-348. DOI: 10.1111/j.1365-2095.2011.00906.x
- 709 Martoja R. & Martoja-Pierson M. *Téc. de Histol. Anim.* (1970). Toray-Masson S.A,
710 Barcelona

- 711 Montero D., Robaina L.E., Socorro J., Vergara J.M., Tort L. & Izquierdo M. S.
 712 Alteration of liver and muscle fatty acid composition in gilthead seabream (*Sparus*
 713 *aurata*) juveniles held at high stocking density and fed an essential fatty acid
 714 deficient diet. *Fish physiol. and Biochem.* Vol. 24 (1). 2001 Jan. 63–72. DOI:
 715 10.1023/A:1011145426543
- 716 Montero D., T. Kalinowski, A. Obach, Robaina L., Tort L., Caballero M. J., Izquierdo
 717 M. S. Vegetable lipid sources for gilthead seabream (*Sparus aurata*): effects on
 718 fish health. *Aquac.* Vol. 225 (1-4). 2003 Jul. 353–370. DOI: 10.1016/S0044-
 719 8486(03)00301-6
- 720 Montero D., Robaina L., Caballero M. J., Ginés R., Izquierdo M. S. Growth, feed
 721 utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets
 722 containing vegetable oils: A time-course study on the effect of a re-feeding period
 723 with a 100% fish oil diet. *Aquac.* Vol. 248 (1-4). 2005 Jul. 121–134. DOI:
 724 10.1016/j.aquaculture.2005.03.003
- 725 Montero D., Grasso V., Izquierdo M.S., Ganga R., Real F., Tort L., Caballero M. J.,
 726 Acosta F. Total substitution of fish oil by vegetable oils in gilthead sea bream
 727 (*Sparus aurata*) diets: Effects on hepatic Mx expression and some immune
 728 parameters. *Fish Shellfish Immunol.* Vol. 24 (2). 2008 Feb. 147-155. DOI:
 729 10.1016/j.fsi.2007.08.002
- 730 Montero D., F. Mathlouthi, Tort L., J.M. Afonso, Torrecillas S., A. Fernández-Vaquero,
 731 D. Negrin, Izquierdo M. S. Replacement of dietary fish oil by vegetable oils
 732 affects humoral immunity and expression of pro-inflammatory cytokines genes in
 733 gilthead sea bream *Sparus aurata*. *Fish Shellfish Immunol.* Vol. 29 (6). 2010 Dec.
 734 1073-1081. DOI: 10.1016/j.fsi.2010.08.024
- 735 Montero D., Terova G., Rimoldi S., Betancor M.B., Atalah E., Torrecillas S., Caballero
 736 M.J., Zamorano M.J., Izquierdo M. Modulation of the Expression of Components
 737 of the Stress Response by Dietary Arachidonic Acid in European Sea Bass
 738 (*Dicentrarchus labrax*) Larvae. *Lipids.* Vol. 50 (10). 2015 Oct. 1029-1041. DOI:
 739 10.1007/s11745-015-4057-1
- 740 Nakamura H. and Murayama T. The Role of Sphingolipids in Arachidonic Acid
 741 Metabolism. *J. Pharmacol. Sci.* Vol. 124 (3). 2014 Mar. 307-312 DOI:
 742 10.1254/jphs.13R18CP
- 743 Olsen R.E. and Henderson R.J. The rapid analysis of neutral and polar marine lipids
 744 using double-development HPTLC and scanning densitometry. *J. of Exper.*
 745 *Marine Biol. and Ecol.* Vol. 129 (2). 1989 Aug. 189-197. DOI: 10.1016/0022-
 746 0981(89)90056-7
- 747 Olsen R. E., Dragnes B. T., Myklebust R., Ringø E. Effect of soybean oil and soybean
 748 lecithin on intestinal lipid composition and lipid droplet accumulation of rainbow
 749 trout, *Oncorhynchus mykiss* Walbaum. *Fish physiol. and Biochem.* Vol. 29 (3).
 750 2003 Jul. 181–192. DOI: 10.1023/B:FISH.0000045708.67760.43
- 751 Oxley A., Jolly C., Eide T., Jordal A.E., Svardal A., Olsen R.E. The combined impact
 752 of plant-derived dietary ingredients and acute stress on the intestinal arachidonic

- 753 acid cascade in Atlantic salmon (*Salmo salar*). *Br. J. Nutr.* Vol. 103 (6). 2010 Mar.
754 851-861. DOI: 10.1017/S0007114509992467.
- 755 Palmerini C. A., Mazzoni M., Giovinazzo G., Arienti G. Blood Lipids in Antarctic and
756 in Temperate-Water Fish Species *J. of Membr. Biol.* Vol. 230 (3). 2009 Aug. 125–
757 131 DOI: 10.1007/s00232-009-9192-2
- 758 Patel D. and Witt S. N. Ethanolamine and Phosphatidylethanolamine: Partners in Health
759 and Disease. *Oxid. Med. Cell Longev.* Vol. 2017 (4829180). 2017 Jul. DOI:
760 10.1155/2017/4829180
- 761 Picchiatti S., Fausto A. M., Randelli E., Carnevali O., Taddei A. R., Buonocore F.,
762 Scapigliati G., Abelli L. Early treatment with *Lactobacillus delbrueckii* strain
763 induces an increase in intestinal T-cells and granulocytes and modulates immune-
764 related genes of larval *Dicentrarchus labrax* (L.). *Fish Shellfish Immunol.* Vol. 26
765 (3). 2009 Mar. 368-76. DOI: 10.1016/j.fsi.2008.10.008
- 766 Pullmannová P., Staňková K., Pospíšilová M., Skolová B., Zbytovská J., Vávrová K.
767 Effects of sphingomyelin/ceramide ratio on the permeability and microstructure of
768 model stratum corneum lipid membranes. *Biochim. Biophys. Acta.* Vol. 1838 (8).
769 2014 Aug. 2115-2126. DOI: 10.1016/j.bbamem.2014.05.001
- 770 Ribeiro C.M., Hermsen T., Taverne-Thiele A.J., Savelkoul H.F., Wiegertjes G.F.
771 Evolution of recognition of ligands from Gram-positive bacteria: similarities and
772 differences in the TLR2-mediated response between mammalian vertebrates and
773 teleost fish. *J. Immunol.* Vol. 184 (5). 2010 Mar. 2355-2368. DOI:
774 10.4049/jimmunol.0900990
- 775 Román L., Real F., Padilla D., El Aamri F., Déniz S., Grasso V., Acosta F. Cytokine
776 expression in head-kidney leucocytes of European sea bass (*Dicentrarchus labrax*
777 L.) after incubation with the probiotic *Vagococcus fluvialis* L-21. *Fish Shellfish*
778 *Immunol.* Vol. 35 (4). 2013 Oct. 1329-1332. DOI: 10.1016/j.fsi.2013.07.036
- 779 Rombout J. H.W.M., Abelli L., Picchiatti S., Scapigliati G., Kiron V. Teleost intestinal
780 immunology. *Fish Shellfish Immunol.* Vol. 31 (5). 2011 Nov. 616-626.
781 DOI:10.1016/j.fsi.2010.09.001
- 782 Salinas I. The Mucosal Immune System of Teleost Fish. *Biol.* Vol. 4 (3). 2015 Aug.
783 525-539. DOI:10.3390/biology4030525
- 784 Sargent J., Bell J.G., McEvoy L., Tocher D. & Estevez A. Recent developments in the
785 essential fatty acid nutrition of fish. *Aquac.* Vol. 177 (1–4). 1999 Jul. 191-199.
786 DOI: 10.1016/S0044-8486(99)00083-6
- 787 Shewan, A., Eastburn D. J., and Mostov K. Phosphoinositides in Cell Architecture.
788 *Cold Spring Harb. Perspect. Biol.* Vol. 3(8). 2011 Aug. 3:a004796. DOI:
789 10.1101/cshperspect.a004796
- 790 Skalli A., Robin J.H., Le Bayon N., Le Delliou H., Person-Le Ruyet J. Impact of
791 essential fatty acid deficiency and temperature on tissues' fatty acid composition
792 of European sea bass (*Dicentrarchus labrax*). *Aquac.* Vol. 255 (1–4). 2006 May.
793 223-232. DOI: 10.1016/j.aquaculture.2005.12.006

- 794 Sluzalska K.D., Liebisch G., Lochnit G., Ishaque B., Hackstein H., Schmitz G., Rickert
795 M., Steinmeyer J. Interleukin-1 β affects the phospholipid biosynthesis of
796 fibroblast-like synoviocytes from human osteoarthritic knee joints. *Osteoarthr.*
797 *Cartil.* Vol. 25(11). 2017 Nov.1890-1899. DOI: 10.1016/j.joca.2017.07.011
- 798 Sokal, R.R. and Rolf S.J., 1995. *Biometry*. In: *The principles and practice of statistics in*
799 *biological research*.3rd ed. New York: Freeman. DOI: 10.2307/2343822
- 800 Storelli C., Acierno R., Maffia M. Membrane lipid and protein adaptations in Antarctic
801 fish. *Cold Ocean Physiol.* 1998. 166-189. DOI: 10.1017/CBO9780511661723.008
- 802 Sui X., N. Kong, L. Ye, W. Han, J. Zhou, Q. Zhang, C. He, H. Pan. p38 and JNK
803 MAPK pathways control the balance of apoptosis and autophagy in response to
804 chemotherapeutic agents. *Cancer Lett.* Vol. 344 (2). 2014 Mar. 174-179. DOI:
805 10.1016/j.canlet.2013.11.019
- 806 Tarnawski A., Hollander D., Stachura J., Krausez W. J. & Gergely H. Protection of the
807 rat gastric mucosa against aspirin injury by arachidonic acid: a dietary
808 prostaglandin precursor fatty acid. *Eur. J. Clin. Invest.* Vol. 19 (3). 1989 Jun. 278-
809 290. DOI: 10.1111/j.1365-2362.1989.tb00231.x
- 810 Tateishi N., Kakutani S., Kawashima H., Shibata H., and Morita I. Dietary
811 supplementation of arachidonic acid increases arachidonic acid and lipoxin A4
812 contents in colon, but does not affect severity or prostaglandin E2 content in
813 murine colitis model. *Lipids in Health and Dis.* Vol. 13 (1). 2014 Feb. 30-40.
814 DOI: 10.1186/1476-511X-13-30
- 815 Tian, J.J., Lei, C.X., Ji, H., Kaneko. G., Zhou, J.Z., Yu, H.B., Li, Y., Yu., E., Xie, J.
816 Comparative analysis of effects of dietary arachidonic acid and EPA on growth,
817 tissue fatty acid composition, antioxidant response and lipid metabolism in
818 juvenile grass carp, *Ctenopharyngodon idellus*. *British J. Nutr.* Vol 118, 2017,
819 411-422. doi:10.1017/S000711451700215X
- 820 Tocher D. R. and Harvie D. G. Fatty acid compositions of the major phosphoglycerides
821 from fish neural tissues; (n-3) and (n-6) polyunsaturated fatty acids in rainbow
822 trout (*Salmo gairdneri*) and cod (*Gadus morhua*) brains and retinas. *Fish Physiol*
823 *Biochem.* Vol. 5 (4). 1988 Oct. 229-239. DOI: 10.1007/BF01874800
- 824 Tocher D. R., Carr J. and Sargent J. R. Metabolism and Functions of Lipids and Fatty
825 Acids in Teleost Fish. *Rev. in Fisheries Sci.* Vol. 11 (2). 2003 Apr. 107-184. DOI:
826 10.1080/713610925
- 827 Tocher D. R., Bendiksen E.Å., Campbell P.J., Bell J.G. The role of phospholipids in
828 nutrition and metabolism of teleost fish. *Aquac.* Vol. 280 (1-4). 2008 Aug. 21-34.
829 DOI: 10.1016/j.aquaculture.2008.04.034
- 830 Tocher D. R., Carr J. and Sargent J. R. Omega-3 long-chain polyunsaturated fatty acids
831 and aquaculture in perspective. *Aquac.* Vol. 449. 2015 Dec. 94-107. DOI:
832 10.1016/j.aquaculture.2015.01.010
- 833 Torrecillas S., Makol A., Caballero M. J., Montero D., Robaina L., Real F., Sweetman
834 J., Tort L., Izquierdo M. S. Immune stimulation and improved infection resistance

- 835 in European sea bass (*Dicentrarchus labrax*) fed mannanoligosaccharides. *Fish*
836 *Shellfish Immunol.* Vol. 23 (5). 2007 Nov. 969-981. DOI:
837 10.1016/j.fsi.2007.03.007
- 838 Torrecillas S., Makol A., Caballero M. J., Montero D., Dhanasiri A.K.S., Sweetman J.
839 and Izquierdo M. S. Effects on mortality and stress response in European seabass,
840 *Dicentrarchus labrax* (L.), fed mannanoligosaccharides (MOS) after *Vibrio*
841 *anguillarum* exposure. *J. of Fish Dis.* Vol. 35 (8). 2012 Aug. 591-602. DOI:
842 10.1111/j.1365-2761.2012.01384.x
- 843 Torrecillas S., Makol A., Betancor M. B., Montero D., Caballero M. J., Sweetman J.,
844 Izquierdo M. S. Enhanced intestinal epithelial barrier health status on European
845 sea bass (*Dicentrarchus labrax*) fed mannan oligosaccharides. *Fish Shellfish*
846 *Immunol.* Vol. 34 (6). 2013 Jun. 1485-1495. DOI: 10.1016/j.fsi.2013.03.351
- 847 Torrecillas S., Montero D., Izquierdo M. S. Improved health and growth of fish fed
848 mannan oligosaccharides: Potential mode of action. *Fish Shellfish Immunol.* Vol.
849 36 (2). 2014 Feb. 525-544. DOI: 10.1016/j.fsi.2013.12.029
- 850 Torrecillas S., Montero D., Caballero M. J., Robaina L., Zamorano M. J., Sweetman J.,
851 Izquierdo M. S. Effects of dietary concentrated mannan oligosaccharides
852 supplementation on growth, gut mucosal immune system and liver lipid
853 metabolism of European sea bass (*Dicentrarchus labrax*) juveniles. *Fish Shellfish*
854 *Immunol.* Vol. 42 (2). 2015 Feb. 508-516. DOI: 10.1016/j.fsi.2014.11.033
- 855 Torrecillas S., Román L., Rivero-Ramírez F., Caballero M. J., Pascual C., Robaina L.,
856 Izquierdo M. S., Acosta F., Montero D. Supplementation of arachidonic acid rich
857 oil in European sea bass juveniles (*Dicentrarchus labrax*) diets: Effects on
858 leucocytes and plasma fatty acid profiles, selected immune parameters and
859 circulating prostaglandins levels. *Fish Shellfish Immunol.* Vol. 64. 2017 May.
860 437-445. DOI: 10.1016/j.fsi.2017.03.041
- 861 Torrecillas S., Caballero M. J., Mompel D., Montero D., Zamorano M. J., Robaina L.,
862 Rivero-Ramírez F., Karalazos V., Kaushik S., Izquierdo M. S. Disease resistance
863 and response against *Vibrio anguillarum* intestinal infection in European seabass
864 (*Dicentrarchus labrax*) fed low fish meal and fish oil diets. *Fish Shellfish*
865 *Immunol.* Vol. 67. 2017 Aug. 302-311. DOI: 10.1016/j.fsi.2017.06.022
- 866 Torrecillas S., Betancor M. B., Caballero M. J., Rivero F., Robaina L., Izquierdo M. S.,
867 Montero D. Supplementation of arachidonic acid rich oil in European seabass
868 juveniles (*Dicentrarchus labrax*) diets: effects on growth performance, tissue fatty
869 acid profile and lipid metabolism. *Fish physiol. and Biochem.* Vol. 44 (1). 2018
870 Feb. 283–300. DOI: 10.1007/s10695-017-0433-5
- 871 Villalta M., Estévez A., Bransden M. P. Arachidonic acid enriched live prey induces
872 albinism in Senegal sole (*Solea senegalensis*) larvae. *Aquac.* Vol. 245 (1–4). 2005
873 Mar. 193-209. DOI: 10.1016/j.aquaculture.2004.11.035
- 874 Wallace J. L. and Devchand P. R. Emerging roles for cyclooxygenase-2 in
875 gastrointestinal mucosal defense. *Br. J. Pharmacol.* Vol. 145 (3). 2005 Jun. 275–
876 282. DOI: 10.1038/sj.bjp.0706201

- 877 Willey S., Bengtson D. A. and Harel M. Arachidonic acid requirements in larval
878 summer flounder, *Paralichthys dentatus*. *Aquac. Int.* Vol. 11 (1–2). 2003 Jan.
879 131–149. DOI: 10.1023/A:1024148625202
- 880 Xu H., Ai Q., Mai K., Xu W., Wang J., Ma H., Zhang W., Wang X., Liufu Z. Effects of
881 dietary arachidonic acid on growth performance, survival, immune response and
882 tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus*.
883 *Aquac.* Vol. 307 (1–2). 2010 Sep. 75-82. DOI: 10.1016/j.aquaculture.2010.07.001
- 884 Xu H., Wang C., Zhang Y., Wei Y. & Liang M. Moderate levels of dietary arachidonic
885 acid reduced lipid accumulation and tended to inhibit cell cycle progression in the
886 liver of Japanese seabass *Lateolabrax japonicus*. *Scientific Reports* Vol. 8
887 (10682). 2018 Dic. DOI: 10.1038/s41598-018-28867-z.
- 888 Yang X., Wei H., Qin L., Zhang S., Wang X., Zhang A., Du L., Zhou H. Reciprocal
889 interaction between fish TGF- β 1 and IL-1 β is responsible for restraining IL-1 β
890 signaling activity in grass carp head kidney leukocytes. *Dev. Comp. Immunol.*
891 Vol. 47 (2). 2014 Dec. 197-204. DOI: 10.1016/j.dci.2014.07.023.
- 892 Yaqoob P. and Calder P. C. Fatty acids and immune function: new insights into
893 mechanisms. *British J. of Nutr.* Vol. 98 (1). 2007 Oct. S41–S45. DOI:
894 10.1017/S0007114507832995
- 895 Zapata, A.G. and Cooper, E.L. *The Immune System: Comparative Histophysiology*,
896 John Wiley & Sons, New York,
897 1990. ISBN 0-471-92361-3. 335 pp., illustrated edition.
- 898 Zou J. and Secombes C. J. The Function of Fish Cytokines. *Biol. (Basel)* Vol. 5 (2).
899 2016 May. 23-57. DOI:10.3390/biology5020023

900

901 **Captions to figures**

902

903 **Figure 1.** RT-qPCR of immune-related genes in distal intestine of *D. labrax* juveniles,
904 at basal time and at 2 days post infection: (a) *tnfa*; (b) *il-1 β* ; (c) *il-10*; (d) *cox-2*. N= 9
905 fish/diet. All values of relative expression are represented as mean \pm SD. Differences
906 were significant when $P < 0.05$, after One-way ANOVA. Significant ($p < 0.05$) differences
907 among diets within same sampling point indicate with letters: lowercase for Basal and
908 uppercase for 2DPI.

909

910 Table 1. Ingredients and biochemical composition analyzed for the different experimental diets containing
 911 graded levels of ARA (% of dry matter).

912

| | DIETS | | | | |
|---|--------------|-------|-------|-------|-------|
| | ARA0.6 | ARA1 | ARA2 | ARA4 | ARA6 |
| Fish Meal¹ | -- | 52.50 | 52.50 | 52.50 | 52.50 |
| Fish oil¹ | -- | 14.50 | 12.60 | 11.40 | 10.10 |
| Defatted Fish Meal² | 46.50 | -- | -- | -- | -- |
| Corn Meal³ | 7.00 | 6.00 | 6.00 | 6.00 | 6.00 |
| Soy 44 Meal³ | 10.00 | 10.00 | 10.00 | 10.00 | 10.00 |
| Wheat Meal³ | 5.50 | 5.50 | 5.50 | 5.50 | 5.50 |
| Wheat Gluten³ | 7.00 | 7.00 | 7.00 | 7.00 | 7.00 |
| Vegetable fats and oils | 14.50 | -- | -- | -- | -- |
| Vitamins Mix⁴ | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Mineral Mix⁵ | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| CMC⁶ | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| ARA⁷ | -- | -- | 0.50 | 1.50 | 2.50 |
| DHA & EPA⁸ | 5.00 | -- | 1.40 | 1.60 | 1.90 |
| <i>Analyzed Proximate composition (g·kg⁻¹; d.w.)</i> | | | | | |
| Crude Lipids | 20.77 | 21.33 | 20.87 | 21.12 | 22.02 |
| Crude Protein | 43.71 | 43.32 | 44.93 | 44.61 | 45.14 |
| Ash | 9.75 | 10.51 | 10.47 | 10.39 | 10.49 |
| Moisture | 8.94 | 6.57 | 7.63 | 7.25 | 7.39 |

913 1.

914 2. Fish meal and oil, South American origin, (65% protein, 12% lipid).

915 3. Defatted soymeal (GIA-ECOQUA laboratory, produced by 3 x chloroform extraction; 73% protein, 2%
 916 lipid).

917 4. Vegetable ingredients locally found (SBM:46% protein, 3% lipid).

918 5. Vitamin premix contains (mg kg⁻¹ or IU/kg of dry diet): thiamine 40 mg, riboflavin 50 mg, pyridoxine
 919 40 mg, calcium pantothenate 117 mg, nicotinic acid 200 mg, biotin 1 mg, folic acid 10 mg, cyanocobalamin,
 920 0.5 mg, choline chloride 2700 mg, Myo-inositol 2000 mg, ascorbic acid 5000 mg, menadione 20 mg,
 921 cholecalciferol 2000 IU, ethoxyquin 100 mg, retinol acetate 5000 IU.

922 6. Mineral premix contains (g/kg of dry diet): calcium orthophosphate 1.60 g, calcium carbonate 4 g, ferrous
 923 sulphate 1.5 g, magnesium sulphate 1.6 g, potassium phosphate 2.8 g, sodium phosphate 1 g, aluminum
 924 sulphate 0.02 g, zinc sulphate 0.24 g, copper sulphate 0.20 g, manganese sulphate 0.08 g, potassium iodate
 925 0.02 g.

926 7. Carboxymethyl cellulose (sodium salt, Sigma-Aldrich, Munich, Germany).

927 8. Vevodar®, DSM Food Specialties, Netherlands.

928 9. DHA50 and EPA50, CRODA, East Yorkshire, UK.

929

930 Table 2. Fatty acid composition (% of total identified FA) of total lipids in experimental diets.

931

| | Diets | | | | |
|--------------------|--------------|--------------|--------------|--------------|--------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| 14:0 | 0.19 | 4.53 | 4.99 | 4.76 | 4.29 |
| 15:0 | 0.04 | 0.53 | 0.56 | 0.53 | 0.48 |
| 16:0 | 6.15 | 16.55 | 18.10 | 17.92 | 16.66 |
| 17:0 | 0.01 | 0.55 | 0.50 | 0.48 | 0.48 |
| 18:0 | 3.19 | 3.92 | 4.57 | 4.89 | 5.04 |
| 20:0 | 0.25 | 0.29 | 0.35 | 0.38 | 0.40 |
| Σ Saturates | 9.83 | 26.36 | 29.07 | 28.97 | 27.35 |
| 16:1 n-7 | 0.28 | 5.78 | 6.04 | 5.73 | 5.19 |
| 18:1 n-9 | 17.31 | 17.36 | 18.31 | 18.02 | 17.09 |
| 18:1 n-7 | 0.89 | 3.00 | 3.15 | 3.01 | 2.75 |
| 20:1 n-9 | 0.11 | 0.33 | 0.34 | 0.31 | 0.28 |
| 20: 1n-7 | 0.73 | 2.54 | 2.58 | 2.43 | 2.16 |
| 22:1 n-11 | 0.19 | 2.27 | 2.12 | 1.98 | 1.71 |
| 22:1 n-9 | 0.28 | 0.44 | 0.43 | 0.41 | 0.36 |
| Σ Monoenes | 19.92 | 32.61 | 33.91 | 32.80 | 30.35 |
| 18:2 n-6 | 13.94 | 5.86 | 5.98 | 6.06 | 6.12 |
| 18: 3n-6 | 0.06 | 0.33 | 0.42 | 0.55 | 0.73 |
| 20:2 n-6 | 0.15 | 0.37 | 0.40 | 0.41 | 0.42 |
| 20:3 n-6 | 0.05 | 0.15 | 0.23 | 0.42 | 0.61 |
| 20:4 n-6 | 0.59 | 1.03 | 2.03 | 4.03 | 6.35 |
| 22:4 n-6 | 0.14 | 0.17 | 0.18 | 0.19 | 0.20 |
| 22:5 n-6 | 0.52 | 0.46 | 0.50 | 0.49 | 0.53 |
| Σ n-6 | 15.45 | 8.39 | 9.75 | 12.16 | 14.96 |
| 16:4n-3 | 0.03 | 0.80 | 0.67 | 0.64 | 0.66 |
| 18:3 n-3 | 42.28 | 1.61 | 1.59 | 1.36 | 1.27 |
| 18:4 n-3 | 0.08 | 1.39 | 1.11 | 1.02 | 1.02 |
| 20: 3n-3 | 0.24 | 0.18 | 0.17 | 0.16 | 0.15 |
| 20:4 n-3 | 0.14 | 0.66 | 0.56 | 0.53 | 0.52 |
| 20:5 n-3 | 2.06 | 9.65 | 7.68 | 7.32 | 7.61 |
| 22:5 n-3 | 0.57 | 1.82 | 1.40 | 1.33 | 1.40 |
| 22:6 n-3 | 9.14 | 14.65 | 12.19 | 11.89 | 13.01 |
| Σ n-3 | 54.57 | 30.93 | 25.54 | 24.42 | 25.79 |
| Σn-3LC-PUFA | 12.15 | 26.97 | 22.00 | 21.22 | 22.69 |
| ARA/EPA | 0.29 | 0.11 | 0.26 | 0.55 | 0.84 |
| DHA/EPA | 4.43 | 1.52 | 1.59 | 1.62 | 1.71 |
| DHA/ARA | 15.51 | 14.23 | 6.02 | 2.95 | 2.05 |
| n-3/n-6 | 3.53 | 3.69 | 2.62 | 2.01 | 1.72 |

932

933

934

935

936
937
938
939

Table 3. References, annealing temperatures, sequences and sources of primers for RT-qPCR.

| Genes | Genbank reference | Annealing temperature | Primers sequence 5'-3' | From |
|-----------|-------------------|-----------------------|---|---------------------------------------|
| IL-10 | AM268529 | 52°C | F'ACCCCGTTCGCTTGCCA R'CATCTGGTGACATCACTC | Buonocore <i>et al.</i> , 2007. |
| IL1-β | AJ311925 | 58°C | F'GGTGGACAAAGCCAGTC R'CCGAGCCTTCAACATCG | Picchietti <i>et al.</i> , 2009 |
| TNF- α | DQ070246.1 | 58°C | F'ACAGCGGATATGGACGGTG R'GCCAAGCAAACAGCAGGAC | Román <i>et al.</i> , 2013 |
| COX- 2 | AJ630649 | 52°C | F'CATTCTTTGCCCAGCACTTCACC R'AGCTTGCCATCCTTGAAGAGTC | Picchietti <i>et al.</i> , 2009 |
| EF-1 | AJ866727 | 60°C | F'GCTTCGAGGAAATCACCAAG R'CAACCTTCCATCCCTTGAAC | Geay <i>et al.</i> , 2011 |

940
941
942

943 Table 4. Lipid class composition (% of lipid classes detected) in distal intestine of *D. labrax*. All results
 944 are expressed as mean±SD. Letters denote significant differences ($P<0.05$) after ANOVA analysis.

| | Diets | | | | |
|-----------------------|------------|-------------|-------------|-------------|------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| TAG (1) | 55.20±9.36 | 55.77±11.30 | 56.40±9.06 | 51.27±10.47 | 53.87±2.81 |
| FFA (2) | 10.20±4.79 | 7.50±5.20 | 7.97±4.10 | 9.07±4.04 | 6.57±0.74 |
| Cholesterol/sterols | 8.97±1.03 | 9.83±0.65 | 9.13±0.31 | 9.47±0.32 | 10.37±0.83 |
| Unknown neutral lipid | 4.40±2.43 | 3.87±2.11 | 4.03±1.97 | 4.03±1.83 | 3.07±0.23 |
| Σ neutral lipids | 78.77±1.56 | 76.97±5.31 | 77.53±3.54 | 73.83±4.42 | 73.87±2.05 |
| PA/PGI/CL (3) | 1.20±0.82 | 1.13±0.55 | 1.03±0.51 | 1.13±0.67 | 0.97±0.15 |
| PtdCho | 6.03±0.42 | 6.63±1.50 | 5.87±0.23 | 7.07±0.59 | 7.70±0.17 |
| PtdSer | 2.07±0.31 | 3.27±2.80 | 3.63±2.50 | 4.60±2.78 | 2.70±0.95 |
| PtdEtn | 3.90±0.46a | 4.57±0.98ab | 4.47±0.47ab | 5.10±0.40ab | 5.77±0.67b |
| PtdIns | 1.83±1.07 | 1.80±0.75 | 1.83±0.74 | 2.17±0.76 | 1.97±0.38 |
| SPM (4) | 2.23±0.32a | 2.03±0.51a | 1.97±0.64a | 2.60±0.40ab | 3.17±0.32b |
| LSC (5) | 0.43±0.15 | 0.73±0.40 | 0.57±0.21 | 0.40±0.17 | 0.47±0.31 |
| Pigmented material | 3.53±0.64 | 2.87±0.45 | 3.10±0.61 | 3.10±0.85 | 3.40±0.53 |
| Σ polar lipids | 21.23±1.56 | 23.03±5.31 | 22.47±3.54 | 26.17±4.42 | 26.13±2.05 |

945 (1) Triacylglycerols, (2) Free fatty acids, (3) Phosphatidic acid/Phosphatidylglycerol/cardiolipin, (4)
 946 sphingomyelin (5) Lysophosphatidylcholine

947

948

949

950 Table 5. Selected fatty acids composition (% fatty acid identified) of the different
 951 glycerophospholipids (GPs). a: Phosphatidylcholine (PC); b: Phosphatidylserine (PS); c:
 952 Phosphatidyletanolamine (PE); d: Phosphatidylinositol (PI) analyzed in distal intestine
 953 of European sea bass fed graded levels of ARA in diet. GPsARA/ARA diet: Ratio
 954 between ARA in GPs and ARA in diet. Different letters within the same row denote
 955 significant ($p < 0.05$) differences.

956

| a. phosphatidylcholine (PC) | DIETS | | | | |
|-----------------------------------|-------------|--------------|---------------|--------------|--------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| 16:0 | 19.86±1.09a | 26.98±0.54b | 27.37±0.80b | 27.91±1.56b | 25.93±0.59b |
| 18:0 | 9.25±0.40 | 9.26±0.73 | 8.95±0.91 | 8.85±0.55 | 9.27±0.88 |
| Σ saturated | 30.88±1.17a | 39.55±0.41b | 40.32±1.17b | 40.38±1.68b | 38.90±1.49b |
| 18:1 n-9 | 13.36±0.36b | 10.51±1.04a | 11.08±0.37ab | 10.84±1.72ab | 10.60±1.13ab |
| Σ monoenes | 18.95±0.78 | 18.12±1.08 | 19.13±0.52 | 17.95±2.02 | 18.04±1.09 |
| 18:2 n-6 | 6.71±0.42c | 2.51±0.18b | 2.01±0.07a | 1.87±0.04a | 1.67±0.13a |
| 20:4 n-6 | 3.10±0.08a | 3.08±0.19a | 5.26±0.13b | 8.56±0.39c | 11.28±0.12d |
| Σ n-6 PUFA | 13.09±0.57d | 7.10±0.16a | 8.90±0.20b | 12.05±0.38c | 14.62±0.28e |
| 18:3 n-3 | 11.82±1.06c | 0.34±0.05b | 0.29±0.04ab | 0.26±0.02ab | 0.23±0.05a |
| 20:5 n-3 | 5.25±0.37a | 8.20±0.79c | 7.21±0.19bc | 6.09±0.09ab | 5.47±0.74a |
| 22:6 n-3 | 18.89±2.23 | 25.17±1.70 | 22.82±0.73 | 22.13±3.47 | 21.65±1.29 |
| Σ n-3 PUFA | 37.09±1.35c | 35.22±1.33bc | 31.65±1.07abc | 29.60±3.58ab | 28.41±2.23a |
| Σ PUFA | 50.17±0.81b | 42.32±1.45a | 40.55±1.26a | 41.67±3.45a | 43.06±2.56a |
| Σ n-3/n-6 | 2.84±0.22a | 4.96±0.13c | 3.56±0.05a | 2.46±0.34ab | 1.94±0.12b |
| Σ n-3 LC-PUFA | 24.15±2.44a | 33.37±1.21b | 30.02±0.91ab | 28.22±3.56ab | 27.12±2.03ab |
| GPsARA/ARA diet | 5.26±0.14d | 2.99±0.19c | 2.60±0.07b | 2.12±0.10a | 1.78±0.02a |

957
958

| b. phosphatidylserine (PS) | DIETS | | | | |
|----------------------------------|-------------|-------------|--------------|--------------|--------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| 16:0 | 7.72±1.63 | 7.74±1.40 | 6.54±0.37 | 6.08±0.81 | 7.82±2.57 |
| 18:0 | 34.04±1.43 | 32.64±1.14 | 34.29±0.84 | 34.74±2.41 | 32.57±2.95 |
| Σ saturated | 44.90±0.73 | 43.54±2.78 | 43.89±1.12 | 43.89±1.26 | 43.87±2.17 |
| 18:1 n-9 | 7.39±1.61 | 5.38±0.49 | 4.60±0.65 | 4.69±0.44 | 7.22±2.35 |
| Σ monoenes | 12.90±1.58 | 10.19±0.84 | 9.45±1.06 | 9.36±1.06 | 12.33±2.91 |
| 18:2 n-6 | 2.11±0.39b | 1.05±0.24a | 0.71±0.15a | 0.62±0.04a | 0.77±0.11a |
| 20:4 n-6 | 1.31±0.22a | 1.82±0.50a | 2.44±0.25ab | 4.03±1.07bc | 4.27±0.75c |
| Σ n-6 PUFA | 8.10±0.22b | 5.48±0.55a | 6.16±0.33ab | 7.72±1.33b | 8.03±0.87b |
| 18:3 n-3 | 2.69±0.30a | 0.25±0.20b | 0.16±0.03b | 0.13±0.03b | 0.16±0.04b |
| 20:5 n-3 | 1.06±0.13a | 1.92±0.35b | 1.39±0.09ab | 1.06±0.11a | 1.00±0.27a |
| 22:6 n-3 | 29.15±2.33a | 36.39±2.65b | 37.06±0.61b | 35.87±1.99b | 32.93±3.46ab |
| Σ n-3 PUFA | 34.05±1.87a | 40.73±3.01b | 40.47±0.72ab | 38.97±1.64ab | 35.75±3.88ab |
| Σ PUFA | 42.20±1.59 | 46.27±3.47 | 46.66±1.01 | 46.75±2.26 | 43.80±4.80 |

| | | | | | |
|------------------------|-------------|-------------|-------------|-------------|--------------|
| Σ n-3/n-6 | 4.21±0.35a | 7.44±0.25b | 6.58±0.24b | 5.15±0.96ab | 4.45±0.02a |
| Σ n-3 LC-PUFA | 30.21±2.21a | 38.31±3.01b | 38.44±0.53b | 36.94±1.90b | 33.93±3.60ab |
| GPsARA/ARA diet | 2.22±0.38d | 1.77±0.49c | 1.21±0.13bc | 1.00±0.27ab | 0.67±0.12a |

959

| c. phosphatidyletanolamine (PE) | DIETS | | | | |
|------------------------------------|--------------|-------------|--------------|-------------|-------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| 16:0 | 6.97±0.68 | 10.02±1.86 | 9.73±0.99 | 9.26±1.02 | 8.27±1.00 |
| 18:0 | 16.69±0.96 | 17.30±1.66 | 17.47±0.79 | 17.38±2.13 | 16.88±1.38 |
| Σ saturated | 25.78±1.68 | 30.12±1.87 | 29.84±2.01 | 29.15±3.11 | 27.34±0.36 |
| 18:1 n-9 | 6.66±0.13a | 3.92±0.60b | 4.05±0.11b | 4.14±0.84ab | 3.53±0.42b |
| Σ monoenes | 14.87±0.45 | 12.72±0.80 | 11.79±1.52 | 12.22±1.17 | 12.77±1.44 |
| 18:2 n-6 | 2.72±0.33a | 0.98±0.14b | 0.80±0.14b | 0.84±0.19b | 0.96±0.68ab |
| 20:4 n-6 | 6.43±0.78a | 5.96±0.48a | 9.27±0.57b | 13.13±1.29c | 16.13±1.13d |
| Σ n-6 PUFA | 14.17±1.20bc | 8.71±0.60a | 12.24±0.85b | 16.18±1.37c | 19.50±0.54d |
| 18:3 n-3 | 3.70±0.41a | 0.18±0.01b | 0.16±0.01b | 0.19±0.03b | 0.22±0.11b |
| 20:5 n-3 | 5.01±0.27b | 7.46±0.34d | 6.00±0.41c | 4.46±0.47ab | 3.68±0.17a |
| 22:6 n-3 | 35.10±0.86a | 38.98±2.33b | 38.40±2.09b | 36.29±0.26a | 35.07±1.61a |
| Σ n-3 PUFA | 45.14±0.52bc | 48.41±2.10c | 46.02±2.59bc | 42.38±0.70a | 40.39±1.76a |
| Σ PUFA | 59.34±1.51 | 57.16±2.48 | 58.36±3.53 | 58.64±1.95 | 59.89±1.33 |
| Σ n-3/n-6 | 3.20±0.25bc | 5.57±0.32d | 3.76±0.05c | 2.63±0.19ab | 2.07±0.14a |
| Σ n-3 LC-PUFA | 40.11±0.76a | 46.44±2.13b | 44.41±2.50b | 40.75±0.64a | 38.75±1.75a |
| GPsARA/ARA diet | 10.91±1.32d | 5.79±0.47c | 4.58±0.28c | 3.26±0.32ab | 2.54±0.18a |

960

| d. phosphatidylinositol (PI) | DIETS | | | | |
|---------------------------------|-------------|--------------|--------------|-------------|-------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| 16:0 | 10.52±5.29 | 10.11±2.06 | 10.89±2.05 | 9.43±1.15 | 9.29±1.70 |
| 18:0 | 22.42±2.40 | 26.35±1.22 | 24.50±1.51 | 24.39±2.09 | 25.01±1.18 |
| Σ saturated | 37.00±6.13 | 39.52±1.59 | 42.44±3.96 | 41.05±4.24 | 40.22±3.94 |
| 18:1 n-9 | 10.29±1.47b | 6.06±0.65a | 5.69±0.41a | 7.00±2.63a | 6.13±1.00a |
| Σ monoenes | 16.13±3.18 | 11.14±1.29 | 11.42±1.19 | 12.27±4.26 | 11.48±2.20 |
| 18:2 n-6 | 2.20±0.38b | 1.12±0.15a | 0.85±0.14a | 0.93±0.22a | 0.87±0.15a |
| 20:4 n-6 | 14.48±1.86a | 17.90±1.66ab | 17.39±3.05ab | 21.50±0.51b | 21.13±0.62b |
| Σ n-6 PUFA | 19.16±2.22a | 20.51±1.77ab | 19.75±3.07a | 23.69±0.63b | 23.50±0.54b |
| 18:3 n-3 | 3.49±0.90b | 0.22±0.05a | 0.21±0.03a | 0.21±0.14a | 0.32±0.31a |
| 20:5 n-3 | 2.37±0.49a | 3.86±0.82b | 2.87±0.21ab | 2.00±0.22a | 1.92±0.26a |
| 22:6 n-3 | 20.71±4.14 | 22.90±3.23 | 22.04±3.45 | 19.57±1.96 | 21.13±2.70 |
| Σ n-3 PUFA | 27.52±4.88 | 28.73±2.64 | 26.37±3.61 | 22.94±1.68 | 24.60±3.02 |
| Σ PUFA | 46.86±7.10 | 49.34±2.59 | 46.14±4.98 | 46.68±1.40 | 48.30±3.50 |
| Σ n-3/n-6 | 1.43±0.12c | 1.41±0.20c | 1.35±0.27bc | 0.97±0.09a | 1.05±0.11ab |
| Σ n-3 LC-PUFA | 23.08±4.56 | 26.76±2.62 | 24.91±3.58 | 21.57±1.81 | 23.06±2.90 |
| GPsARA/ARA diet | 24.57±3.16e | 17.39±1.61d | 8.59±1.50c | 5.33±0.13b | 3.33±0.10a |

961

962

963 **Table 6.** Morphometric analysis and number of goblet cells in distal intestine of European sea bass
964 fed graded levels of ARA in diet. All measures considering individual fish weight (g) as co-variable. All
965 results are expressed as mean±SD. FA =fold area ($\mu\text{m}^2/\text{g}$), FP=fold perimeter ($\mu\text{m}/\text{g}$), FL= fold length
966 ($\mu\text{m}/\text{g}$), FW= fold width ($\mu\text{m}/\text{g}$), SW= submucosa width ($[\mu\text{m}/\text{g}] * 100$). GC= goblet cells/area (arbitrary
967 units * 10^4)

968

969

| | DIETS | | | | |
|----|--------------|--------------|--------------|--------------|--------------|
| | ARA0.5 | ARA1 | ARA2 | ARA4 | ARA6 |
| FA | 252.99±21.12 | 198.76±22.27 | 215.94±36.01 | 222.36±26.11 | 225.45±32.28 |
| FP | 12.26±2.90 | 14.17±3.36 | 13.67±2.08 | 13.29±1.36 | 12.84±1.40 |
| FL | 3.90±0.85 | 3.74±0.51 | 4.04±0.51 | 4.09±0.20 | 4.08±0.46 |
| FW | 1.38±0.43 | 1.25±0.16 | 1.29±0.11 | 1.38±0.12 | 1.35±0.07 |
| SW | 68.24±7.56 | 50.38±4.55 | 60.04±13.58 | 50.54±6.18 | 50.85±6.04 |
| GC | 34.84±9.22 | 29.69±1.16 | 33.24±2.09 | 32.21±1.54 | 29.53±0.86 |

970

971

972

