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1 **Effects of high-level fishmeal replacement by plant proteins supplemented with**  
2 **different levels of lysine on growth performance and incidence of systemic non-**  
3 **infectious granulomatosis in meagre (*Argyrosomus regius*)**

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21

## 22 **Abstract**

23 The potential use of plant protein (PP) blends (soybean, wheat, rapeseed, corn gluten and  
24 wheat gluten) in the diet of juvenile meagre (*Argyrosomus regius*) was investigated at  
25 increasing levels to replace fishmeal (FM) (33 and 56%) in six isonitrogenous (480 g/kg)  
26 and isoenergetic (22 MJ/kg) diets, which were supplemented with crystalline lysine.  
27 Meagre juveniles ( $36 \pm 0.6$  g initial weight) were reared in triplicate for 60 days at  $19.4 \pm$   
28  $2.4$  °C in order to evaluate their growth performance, feed utilization parameters, body  
29 proximate composition and the prevalence of systemic non-infectious granulomatosis.  
30 Results indicated that there was no significant difference (GLM ANOVA,  $P > 0.05$ ) in  
31 growth performance and feed utilization parameters in meagre fed the diet containing 300  
32 g/kg FM (33% FM replacement) compared to the control group (450 g/kg FM inclusion),  
33 although a trend showing inferior body gain and feed conversion ratio was observed.  
34 However, higher levels of FM replacement (56%) by PP blends (200 g/kg FM inclusion)  
35 significantly impaired growth performance, feed conversion and protein efficiency rates  
36 (GLM ANOVA,  $P < 0.05$ ), which may be linked to a decrease in feed intake and/or  
37 reduced levels of bioactive compounds or other micronutrients present in FM. On the  
38 other hand, increasing dietary lysine levels from 25 g/kg to 29 g/kg in the diets containing  
39 the same PP content and 200 g/kg inclusion of FM, significantly improved growth  
40 performance in juvenile meagre. The replacement of FM did not affect lipidosomatic and  
41 hepatosomatic indexes in any of the experimental groups evaluated (GLM ANOVA,  $P >$   
42  $0.05$ ). The etiology of granulomatosis found in different tissues was not due to the  
43 presence of bacteria, since no bacterial structures were detected in histological slides  
44 when samples were stained with the Gram, Ziehl-Neelsen and Fite-Faraco staining.  
45 Presence of chronic systemic non-infectious granulomatosis was observed in meagre from  
46 all the experimental groups regardless the level of FM replacement by PP blends,

47 indicating that the onset and progression of granulomatosis occurred insidiously at earlier  
48 life stages of meagre and persisted at variable levels thereafter. The liver and kidney were  
49 found to be the most severely affected tissues.

50

51 *Keywords:* meagre, plant protein-based ingredients, fishmeal substitution, lysine, non-  
52 infectious systemic granulomas.

53

## 54 **1 INTRODUCTION**

55 Meagre (*Argyrosomus regius*) is the most recent species whose intensive culture has been  
56 developed in the Mediterranean basin. In 2014, the European production of this species  
57 was estimated at 2,055 t, Spain (53% of total production, 1,090 t), France (377 t) and  
58 Greece (300 t) being the main producers (APROMAR, 2015). This species is  
59 characterized by its large size and higher growth rates than those of most of the common  
60 Mediterranean-cultured species, such as gilthead sea bream (*Sparus aurata*) and European  
61 sea bass (*Dicentrarchus labrax*) (Quémener et al., 2002). In addition, the body and fillet  
62 traits of this large species have shown a very high dressing content with a negligible  
63 amount of mesenteric and muscular fat in comparison to other cultured fish that makes  
64 this species even more interesting for industrial processing and human consumption (Poli  
65 et al., 2003; Hernández et al., 2009). As an emerging species for aquaculture, little  
66 information is available on the dietary requirements of meagre, including their amino acid  
67 requirements, as well as in the development and optimization of sustainable feeds for  
68 maximum growth (Chatzifotis et al., 2010, 2012; Estévez et al., 2011; Velazco-Vargas et  
69 al., 2014).

70 Feeding costs represent 50 to 70 percent of total production costs of an intensive finfish  
71 aquaculture farm (Rana et al., 2009). In this context, fishmeal (FM) has become one of the

72 most expensive raw materials in fish feeds and is the main protein source in the feed for  
73 marine fish cultured species during the last decades (Tacon and Metian, 2008). However,  
74 the increasing demand, price, restricted availability, fluctuations of supply of this  
75 commodity and the unpredictability of the market, have directed the most recent research  
76 into looking for abundant and available alternative protein and oil sources to meet the  
77 future needs of the emerging finfish aquaculture (Naylor et al., 2009). In this sense,  
78 proteins derived from plants (PP) have received considerable attention by fish nutritionists  
79 during the last two decades and consequently, the level of FM inclusion within compound  
80 diets for marine finfish has steadily declined during the last years (Tacon and Metian,  
81 2008). Many studies have shown considerable success in partially or totally replacing FM  
82 with PP in diets for various marine fish species (Robaina et al., 1995; Kaushik et al.,  
83 2004; Hernández et al., 2007; Salze et al., 2010; Moxley et al., 2014). In addition to  
84 soybean meal, which is the most used PP source in fish feeds, other PP sources (*e.g.* corn,  
85 wheat, rapeseed and lupin) have also been examined as alternative protein sources for FM  
86 because of their availability and low market price in comparison to FM (Fournier et al.,  
87 2004; Kaushik et al., 2004; Tacon and Metian, 2008). When substituting FM by vegetal  
88 ingredients, diets need to be supplemented with essential amino acids, especially lysine  
89 since most PP sources are lower in this essential amino acid compared to FM, whereas  
90 this is one of the most limiting amino acids in diets for warm-water fish (NRC, 2011).  
91 However, the impact of a blend of plant origin in practical diets for different warm water  
92 marine fish species has not been thoroughly evaluated, especially in new aquaculture  
93 species like the meagre (Estévez et al., 2011; de Rodrigáñez et al., 2013; Ribeiro et al.,  
94 2015) or in other sciaenid species (Minjarez-Osorio et al., 2016). Furthermore, to date, no  
95 available information is available on essential amino acid requirements of meagre,  
96 especially fed on commercial feed formulations. Non-infectious systemic granulomatosis

97 is a common disease with high morbidity rates in cultured meagre (Ghittino et al., 2004).  
98 Although the etiology of this disorder is still unknown, several authors have suggested  
99 that it may be nutritionally induced (Paperna et al., 1980; Tixerant et al., 1984; Ghittino et  
100 al., 2004); thus, it is of interest to evaluate the potential effect of FM substitution in  
101 experimental diets on the prevalence of this disease in this species.

102 Consequently, the objectives of this study were i) to evaluate the effects of FM  
103 substitution by different vegetable protein blends (mainly by soybean, corn gluten and  
104 wheat gluten) on growth performance, voluntary feed intake, feed utilization and health  
105 condition, especially on the presence of granulomatosis, in meagre juveniles, and ii) to  
106 investigate the synergistic effect of lysine supplementation in the different PP blend  
107 inclusions.

108

109

## 110 **2 MATERIAL AND METHODS**

111

### 112 *2.1 Fish and rearing conditions*

113 Juvenile meagre were obtained from a commercial fish farm (Argosaronikos SA,  
114 Salamina Island, Greece) that imports juveniles from France for rearing purposes and  
115 were transferred to the Hellenic Center for Marine Research (HCMR) facility in Agios  
116 Kosmas, Athens. Once acclimated, all fish with an initial average body weight (BW) of  
117  $36 \pm 0.6$  g (mean  $\pm$  standard deviation;  $n = 360$ ) were assigned to 18 experimental small  
118 cages (1.0 m  $\times$  1.5 m  $\times$  1.5 m) with 20 fish per cage (3 replicates-cages per diet). All  
119 cages were placed in two large rectangular concrete tanks of 36 m<sup>3</sup> water capacity; they  
120 suspended about 20 cm from the tank bottom and were continuously supplied with filtered  
121 sea water (salinity 35 ppt). A piece of tarp was placed inside and perimetrically in each

122 cage, ten centimeters above and below the water level to avoid pellets escape from one  
123 cage to the other. Sea water was distributed in each 36 m<sup>3</sup> tank from 10 different pipes at  
124 400 L/h and aerated using stone diffusers to maintain oxygen saturation over 80%. Water  
125 temperature followed the ambient seasonal temperature throughout the experiment with  
126 an average value of 19.4 ± 2.6 °C. The photoperiod followed the natural cycle of the  
127 season (LD 11:13 h). Water quality was regularly checked, and total ammonia levels were  
128 always below 0.3 mg/L. Fish were hand-fed twice a day (09:00 and 15:00 h) carefully to  
129 apparent satiation (judged by the drop in feeding response, and indicated especially as the  
130 point at which the first one or two pellets were not approached by fish, sank and remained  
131 on the bottom of the tank), six days a week with the experimental diets for a period of 60  
132 days.

### 133 *2.2 Experimental diets*

134 Six isonitrogenous (480 g/kg) and isoenergetic (22 MJ/kg) commercial extruded diets  
135 (pellet size = 2.0-2.5 mm) with different FM levels and graded levels of PP blends were  
136 formulated to feed grow-out meagre as shown in Table 1. A basal diet (Diet FM45)  
137 containing 450 g/kg FM inclusion and 37.7 g/kg lysine was used as the control diet. Five  
138 of the experimental diets, FM30, FM20a, FM20b, FM20c and FM20d, were formulated in  
139 a way to contain low levels of FM inclusion (300 g/kg, and 200 g/kg, respectively) and  
140 decreasing levels of lysine (37.4, 37.0, 28.5, 24.8 and 20.9 g/kg feed, respectively). Since  
141 lysine requirements have not yet been established for meagre, a wide range of lysine  
142 intakes was used in order to formulate diets theoretically deficient (20.9 g/kg), adequate  
143 (24.8-28.5 g/kg) or excess (37.0-37.4 g/kg) in lysine containing different fishmeal  
144 inclusion levels (20,30,45%). In these diets, FM was substituted by a mixture of PP blends  
145 including mainly soybean, corn gluten and wheat gluten, while graded levels of lysine  
146 were obtained by supplementing experimental diets with appropriate levels of crystalline

147 L-lysine HCl. Different PP combinations and lysine supplementation in the formulation of  
148 experimental diets were employed in order to attain the same protein and energy levels in  
149 diets having different fishmeal inclusion and accomplishing at the same time a gradual  
150 reduction of lysine. Thus, diet FM20d was not supplemented with lysine, while it was  
151 formulated to contain the lowest soybean (75.5 g/kg diet) and the highest corn gluten and  
152 wheat gluten (300 and 127.9 g/kg diet, respectively), compared to other diets, achieving  
153 by this way the lowest lysine concentration (20.9 g/kg feed) among the experimental  
154 diets. In order to increase feed palatability of diets with lower fishmeal inclusion than  
155 control diet, krill and squid meal (20 g/kg each) were incorporated in diets FM30 to  
156 FM20d. The diets were produced at IRIDA S.A., a commercial fish feed mill located in  
157 Agrinio (Greece), and stored at 4 °C until used at HCMR facilities.

158

### 159 *2.3 Fish sampling, growth performance and feed utilization indexes*

160 All animal experimental procedures and handling in the present study were conducted in  
161 accordance with the Guidelines of the European Union Council (86/609/EU) for the use  
162 of laboratory animals. At the end of the trial, fish were anaesthetized with 2-  
163 phenoxyethanol (250 mg/L) and individually weighed (BW, g) and counted to calculate  
164 growth performance and survival rate. In addition, 10 fish per tank were randomly  
165 selected ( $n = 30$  fish per diet) and euthanized with an overdose of anesthetic (500 mg/L)  
166 for analytical purposes. Five fish from each tank were pooled and analyzed for carcass  
167 composition, whereas the remaining five fish were dissected to calculate the following  
168 parameters: lipidosomatic index (LSI) (%) = perivisceral fat weight (g) x 100 / BW (g);  
169 hepatosomatic index (HSI) = liver weight (g) x 100 / BW (g).

170 In addition, the fillet of the latter specimens was flash-frozen and kept at -80 °C for  
171 further amino acid analyses, and their internal organs (hepatopancreas, intestine, muscle,

172 kidney, spleen, skin and heart) were fixed in buffered formaldehyde (pH = 7.2) for  
 173 histological purposes. Samples for histology were dehydrated in a graded series of  
 174 ethanol, cleared with xylene, embedded in paraffin, cut in serial sections (3–5  $\mu\text{m}$  thick)  
 175 and stained with hematoxylin-eosin for their histomorphological examination and to  
 176 evaluate the potential effect of experimental diets on granulomatosis. Samples were also  
 177 stained with Gram, Ziehl-Neelsen and Fite-Faraco staining in order to detect the presence  
 178 of bacterial structures, especially *Nocardia* sp., in granulomas as described in Elkesh et al.  
 179 (2013). In order to evaluate the incidence of granulomatosis, a qualitative scale with  
 180 values ranging from 0 (absent) to 4 (severe) was used to measure the severity of this  
 181 disorder. This scale considered the incidence of granulomas in different tissues (skin,  
 182 muscle, gut, liver, kidney, spleen and heart) per specimen, the number and size of  
 183 granulomas in each tissue and whether they were calcified or not.

184 Fish growth performance and feed consumption indexes were calculated according to the  
 185 following equations:

186 Specific growth rate (SGR; %/day) =  $100 \times [(\ln BW_f - \ln BW_i) / \text{days}]$

187 Daily growth index (DGI; %) =  $(BW_f^{1/3} - BW_i^{1/3}) / \text{days} \times 100$

188 Thermal growth coefficient (TGC) =  $(BW_f^{1/3} - BW_i^{1/3}) / (\Sigma D_0)$

189 Total feed intake per fish (TFI) = dry feed consumed / fish

190 Feed conversion ratio (FCR) = dry feed consumed / BW gain

191 Protein efficiency ratio (PER, %) = BW gain / protein intake

192 Protein productive value (PPV) =  $(P_f - P_i) / (P_d \times \text{TFI})$ ,

193 where  $BW_i$  and  $BW_f$  are the initial and final body weights,  $\Sigma D_0$  is the thermal sum  
 194 (feeding days  $\times$  average temperature,  $^{\circ}\text{C}$ ),  $P_f$  and  $P_i$  are the initial and final protein levels  
 195 of fish, and  $P_d$  is the protein concentration of the feed on a dry basis, respectively.

196

197 *2.4 Proximate composition and amino acid analyses*

198 The proximate composition of experimental diets, fish carcass and fillets from each  
199 dietary group were analyzed as follows: crude protein was determined by the Kjeldahl  
200 method ( $N \times 6.25$ ), crude fat using a Soxtec<sup>TM</sup> 2050 extraction unit (FOSS, Hillerød,  
201 Denmark) using petroleum ether as solvent, and dry matter and ash content according to  
202 standard procedures (AOAC, 1995). Gross energy content of diets was determined by an  
203 adiabatic bomb calorimeter (IKA, Werke GmbH & Co).

204 The amino acid composition of experimental diets and fish muscle was determined after  
205 acid hydrolysis (6N, 110 °C, 24 h), and calculation by AccQ-Tag<sup>TM</sup> Ultra according to the  
206 amino acid analysis application solution (Waters Corporation, Milford, MA, U.S.A.). DL-  
207 Norvaline (Sigma) 2.5 mM was used as an internal standard. UPLC was performed on an  
208 ACQUITY system (Waters Corporation, Milford, MA, U.S.A.) equipped with PDA  
209 detector and the detection wavelength was set at 260 nm. The column used was BEH C18  
210 column (100mm  $\times$  2.1 mm i.d., 1.7 $\mu$ m) from Waters. The flow rate was 0.7 ml min<sup>-1</sup> and  
211 column temperature was kept at 55 °C. Peak identification and integration was performed  
212 by the software Empower v.2.0 (Waters Corporation) using an Amino Acid Standard H  
213 (Pierce) as an external standard. All analyses were performed in duplicate. In case the  
214 values between replicates did not meet the standardized acceptance criteria based on the  
215 mean and standard deviation (<5%), new duplicate analyses were performed according to  
216 the above-mentioned procedures.

217

218 *2.5 Statistical analyses*

219 Cages were considered as experimental units and fish represented sample units. The  
220 average value calculated from the three cages (replicates) exposed to the same diet were  
221 used for comparisons among experimental groups and data were presented as mean  $\pm$

222 standard deviation. All data were tested for normality and homogeneity of variance prior  
223 to being subjected to ANOVA using Kolmogorov- Smirnov and Levene tests,  
224 respectively. Data expressed as percentages were arcsine transformed prior to their  
225 analysis. General Linear Model (GLM) ANOVA with 'Fishmeal' and 'Lysine' as fixed  
226 factors was applied separately for each dependent variable. In particular, 'Fishmeal' was  
227 incorporated as a fixed factor with three dietary levels (200, 300 and 450 g/kg), while  
228 'Lysine' was arranged as a fixed factor with six levels (20.9, 24.8, 28.5, 37.0, 37.4 and  
229 37.7 g/kg feed). Since not all lysine levels could be present in every 'Fishmeal' level, due  
230 to feed composition constraints, a nested design involving two fixed factors (Neter et al.,  
231 1996) was applied using 'Lysine' as a nested fixed factor within 'Fishmeal' and the Type  
232 IV sum of squares in the between-subjects effects. Significant differences between means  
233 were determined by Tukey's post-hoc tests. The level of significance was set at  $P < 0.05$ .  
234 All statistical tests were performed using SPSS<sup>®</sup> for Windows, Release 13, 2004 (SPSS  
235 Inc<sup>®</sup>).

236

237

### 238 **3 RESULTS**

#### 239 *3.1 Growth performance and feed utilization*

240 Results from the GLM ANOVA revealed high R-squared values (0.81-0.96), which  
241 indicated that the applied statistical model fit very well the data, explaining most of the  
242 variability in each of the analyzed growth parameters (Table 3). Moreover, the partial  $\eta^2$   
243 indicated that FM inclusion levels had a higher relative impact on growth parameters than  
244 lysine levels, except for the PER. Scatter plots of 12 growth parameters against lysine  
245 content in the three fish meals levels (FM20, FM30 and FM45) are shown in Fig. 1,

246 revealing the trend of each growth parameter in relation to lysine concentration within  
247 each fishmeal level.

248 Post-hoc test results for growth performance and feed utilization parameters are shown in  
249 Table 4. At the end of the two-month trial, no differences in survival were found among  
250 different diets ( $P > 0.05$ ). Meagre fed diets FM45 and FM30 showed the highest  $BW_f$   
251 values. On the contrary, fish fed diets FM20c and FM20d, which contained the high  
252 inclusion of PP and the lowest lysine concentrations (24.8 and 20.9 g/kg, respectively,  
253 Table 2), showed the poorest growth performance, 25% lower than diets FM45 and FM30  
254 ( $P < 0.05$ ). Of the rest of the tested diets, FM20a and FM20b that contained the high  
255 inclusion of PP and in-between lysine concentrations (37.0 and 28.5 g/kg, respectively,  
256 Table 2) showed intermediate  $BW_f$  values, which were significantly lower compared to  
257 FM45 and FM30 groups but significantly higher compared to FM20c and FM20d groups  
258 ( $P < 0.05$ ). Similar results regarding WG, SGR, TGC and DGI parameters were found  
259 among different diets ( $P < 0.05$ ). A gradual decline in the TFI was observed among  
260 experimental groups correlated with the increase in the level of FM substitution by PP  
261 sources, although it was not found to be statistically significant ( $P > 0.05$ ). Fish fed diets  
262 FM45 and FM30 showed the best results in terms of FCR and PER, whereas meagre fed  
263 diets FM20c and FM20d showed the poorest FCR and PER results, which were 20 and  
264 27.4%, respectively, lower than in fish from the above-mentioned treatments ( $P < 0.05$ ).  
265 Fish fed diets FM20a and FM20b showed intermediate values with regard to those  
266 displaying the best and worst results in FCR and PER values. Different levels of FM  
267 substitution by different PP blends and levels did not affect HSI and LSI values among  
268 groups ( $P > 0.05$ ).

269

270 *3.2 Protein productive value, body proximate composition and fillet AA profile*

271 The levels of FM substitution by graded levels of PP blends affected the protein  
272 productive values between experimental diets (Table 4,  $P < 0.05$ ). The highest PPVs were  
273 found in meagre fed diets FM45 and FM30, while the lowest in FM20c. The rest of the  
274 experimental diets exhibited intermediate PPVs values. Results of the body proximate  
275 composition and AA profile of fillet of meagre fed diets containing different levels of FM  
276 substitution are shown in Tables 5 and 6. The level of FM substitution in diets had little  
277 influence on body composition, significantly affecting only the protein content of meagre  
278 body ( $P < 0.05$ ). Thus, fish fed FM30, FM20b, and FM20c diets exhibited the highest  
279 levels of body protein content, whereas the lowest values were found in meagre fed diets  
280 FM45 and FM20d (Table 5). Fish fed FM20a showed intermediate values in body protein  
281 content among the above-mentioned groups. No differences in moisture, lipid and ash  
282 contents were found between groups ( $P > 0.05$ ). Regardless of the different AA of  
283 experimental diets (Table 2), the AA profile of meagre fillet fed different feeds was quite  
284 constant with just differences in proline content between groups (Table 6,  $P < 0.05$ ), as a  
285 result of the higher content of this AA in diets with high levels of FM substitution by PP  
286 blends.

287

### 288 *3.3 Histological analyses*

289 The qualitative assessment of the incidence of granulomatosis in meagre fed different  
290 experimental diets is shown in Figure 2.

291 Diet FM45: The histological organization of the skin and muscular tissue of most of the  
292 examined animals (5/6) was normal, whereas one specimen (1/6) was severely affected by  
293 numerous small granulomas affecting the hypodermis and adjacent surface muscle. The  
294 digestive tract and pancreatic tissue was normal in all analyzed fish from this  
295 experimental group, whereas one specimen showed three small granulomas present in the

296 perivisceral adipose tissue close to the hypodermis (Fig. 3a). The tissue most severely  
297 affected by granulomatosis was the liver. In particular, five of the six fish analyzed had  
298 multiple small chronic granulomas throughout the hepatic parenchyma (Fig. 3b). The  
299 granulomas revealed concentric layers of macrophages and epithelioid cells and  
300 frequently had necrotic centers, whereas in three fish, adjacent granulomas appeared to  
301 have coalesced. Size of individual granulomas was *ca.* 40 to 80  $\mu\text{m}$ , while coalesced  
302 granulomas measured *ca.* 800  $\mu\text{m}$  in diameter. One fish had no granulomas, but an  
303 occasional small focus of inflammatory cells consisting principally of macrophages. Very  
304 large individual granulomas (100-400  $\mu\text{m}$ ) were present in the kidneys of all fish, whereas  
305 some of them had calcified centers. The histological organization of the spleen was  
306 normal in all examined specimens.

307 Diet FM30: No histological alterations in the skin and muscle samples were observed in  
308 all examined fish. The digestive tract and pancreas was normal in most of the analyzed  
309 specimens (5/6), whereas one animal had multiple granulomas in the *lamina propria* of  
310 the intestinal mucosa, whereas a single small granuloma was found in the peritoneum of  
311 another fish. In three of the six examined fish, multiple granuloma formations were  
312 present together with focal diffuse areas of chronic inflammation in the liver. Multiple  
313 large chronic granulomas, often with calcified centers, were present in the kidneys of all  
314 fish (Fig. 3c). Large granulomas were also present in the spleens of two fish with a  
315 marked eosinophilic granule cell response surrounding them.

316 Diet FM20a: The histological organization of the skin and muscular tissue of all the  
317 examined animals was normal. One fish had two granulomas in the *lamina propria* of the  
318 gut, whereas another had a single granuloma in the same position (Fig. 3d). In one fish,  
319 there were extensive areas of fatty liver degeneration and diffuse areas of chronic  
320 inflammatory response in the absence of developed granuloma formation. In another

321 examined specimen, there were major areas of chronic diffuse inflammatory change with  
322 the presence of one granuloma within this area of chronic inflammation. In a third animal,  
323 there were occasional very small granulomas and areas of chronic inflammatory response.  
324 Two fish showed no inflammatory response or granuloma formation. Granulomas were  
325 present in the kidneys of all fish and in one examined fish, eight granulomas were  
326 detected in just one kidney section.

327 Diet FM20b: No histological alterations in the skin and muscle samples were observed in  
328 all examined fish fed this diet. There was a single very small granuloma in the peritoneum  
329 of one fish, but the other samples were all normal. The histological organization of the  
330 digestive tract and pancreas was normal in all examined specimens. Similar to the other  
331 diets, the liver was the tissue most severely affected by granulomatosis. Three fish  
332 showed small numbers of granulomas, whereas two of them showed more extensive areas  
333 of chronic inflammatory infiltration. Numerous chronic granulomas were present in the  
334 rest of the sampled animals. Several granulomas were found in the kidneys of all samples.  
335 Spleens were normal with the exception of one fish, which contained a single small  
336 granuloma. Focal myocarditis was present in the ventricle of a single fish, but other hearts  
337 were normal.

338 Diet FM20c: No histological alterations in the skin, muscle, gut, pancreas and spleen  
339 samples were observed in all examined fish. The liver of one fish had some very small  
340 inflammatory foci, but the majority were in good condition. One kidney was normal  
341 histologically, but the remainder of the analyzed kidneys each contained four or five  
342 granulomas. Hearts were normal with the exception of one fish, which had focal  
343 myocarditis in the ventricle and a minor pericarditis, but lesions were not extensive.

344 Diet FM20d: The histological organization of the skin, muscular tissue, pancreas and  
345 spleen of all the examined animals was normal. A single granuloma was detected in the  
346 peritoneum of two fish, whereas one of the six examined specimens had a single  
347 granuloma in the *lamina propria* of the gut. The liver showed limited focal chronic  
348 inflammatory response in one fish and a much more extensive and advanced response in a  
349 second fish. The other three fish had very minor and limited chronic inflammatory  
350 response. The kidney had a massive granuloma present in one fish and two to three large  
351 granulomas in each of the remaining fish. There was limited focal chronic inflammatory  
352 change present in the heart of three fish, whereas two granulomas were present in the  
353 ventricle of a fourth.

354 Regardless of the diet analyzed, the results from the Gram, Ziehl-Neelsen and Fite-Faraco  
355 staining used for detecting the presence of bacteria within granulomas revealed that no  
356 distinct bacterial structures were seen in forming granulomas (areas of increased  
357 cellularity and inflammation) or in the necrotic centers of granulomas in any of the tissues  
358 examined.

359

#### 360 **4 DISCUSSION**

361 In recent years, a significant amount of research has been conducted on the replacement  
362 of FM by different PP blends. As Sitjà-Bobadilla et al. (2005) indicated, the suitability of  
363 this replacement in terms of growth performance has resulted in considerable variability  
364 among different fish species and experimental conditions; thus, specific trials have to be  
365 performed for each species. In this study, we evaluated two different levels of FM  
366 replacement (33 and 56%) with different levels of PP blends in diets for juvenile meagre  
367 supplemented with different levels of crystalline lysine. In general, our results analysed

368 using a GLM ANOVA showed that growth performance and feed utilization parameters  
369 in meagre were not significantly affected in fish fed diets in which FM was substituted by  
370 PP mixtures in a diet containing 300 g/kg FM (diet FM30; 33% of FM replacement) in  
371 comparison to fish fed diet FM45 with 450 g/kg FM. These results indicated that when  
372 properly supplemented with essential amino acids, corn and wheat gluten can partially  
373 substitute Super Prime FM in the diet of meagre. In contrast, the reduction in the inclusion  
374 levels of FM up to 20% substantially depressed growth in meagre. In particular, fish fed  
375 diets FM20a, FM20b, FM20c containing 200 g/kg FM (56% FM replacement) and  
376 increasing levels of soybean meal (131-162 g/kg), corn gluten (237-280 g/kg) and wheat  
377 gluten (100 g/kg) showed a reduction in growth of between 18 to 26% in comparison to  
378 the control diet (FM45). In addition, a diet containing 200 g/kg FM (diet FM20d, 56%  
379 FM replacement), and low levels of soybean meal (76 g/kg) and rapeseed meal (32 g/kg)  
380 and increasing levels of corn gluten (300 g/kg) and wheat gluten (130 g/kg) in relation to  
381 the other diets, depressed growth performance of meagre up to 29%, although it contained  
382 theoretically reduced anti-nutritional substances (less dietary soybean meal) and higher  
383 quality protein (higher inclusion of wheat gluten and corn gluten), compared to the rest of  
384 FM20 series of diets.

385 These results are in line with those already reported by Estévez et al. (2011) and Velazco-  
386 Vargas et al. (2013) in which 20 to 25% FM replacement in meagre diets with a plant  
387 protein mixture (soy cake, corn gluten, soy protein concentrate and sunflower cake) and  
388 soybean meal, respectively could be used without a significant decrease in growth  
389 performance.

390 On the contrary, our results were different to those reported by Ribeiro et al. (2015) in  
391 meagre and Minjarez-Osorio et al. (2016) in two other sciaenid carnivorous species. In  
392 meagre, Ribeiro et al. (2015) have successfully substituted up to 50% FM (Peruvian

393 fishmeal 70 LT and fair average quality fishmeal) with a mixture of soybean and pea  
394 protein concentrates, and corn and wheat gluten. In addition, Minjarez-Osorio et al.  
395 (2016) were able to replace up to 75% of menhaden fishmeal protein in the diet with non-  
396 genetically modified soybean meal (SBM-3010) having low levels of anti-nutritional  
397 substances and soybean protein concentrate without affecting growth performance in red  
398 drum (*Sciaenops ocellatus*), whereas they could successfully replace up to 50% when they  
399 used corn protein concentrate. Moreover, in the case of juvenile shortfin corvina  
400 (*Cynoscion parvipinnis*), it was observed that soybean and corn protein concentrates  
401 could replace up to 75% of menhaden fishmeal protein in the diet, while non-genetically  
402 modified soybean meal successfully replaced up to 50% of menhaden fishmeal protein  
403 without compromising fish performance. However, in both of the aforementioned studies  
404 the amino acid profile of the formulated diets was not shown.

405 Such differences between the results of the current study and those reported by Ribeiro et  
406 al. (2015) and Minjarez-Osorio et al. (2016) might be due to different, but not mutually  
407 exclusive, reasons. In our study, voluntary feed intake was affected in fish fed the  
408 vegetable-protein based diets and TFI values tended to decrease with increasing levels of  
409 FM substitution, even though this trend was not statistically significant. This trend could  
410 be partially responsible for the lower performance in terms of growth and feed utilization  
411 parameters of meagre fed diets FM20a to FM20d. However, the TFI was similar in diets  
412 FM20a to FM20c, which contained the same FM level (20%). In order to increase feed  
413 palatability due to the inclusion of increasing levels of PP sources, krill and squid meal  
414 were incorporated in diets FM30 to FM20d as these ingredients have been reported to  
415 increase feed palatability of diets containing high levels of PP blends (Aksnes et al., 2006;  
416 Kader et al., 2010). However, it seems that the level of krill and squid meal (20 g/kg each)  
417 used in the present study as palatability enhancers were not enough to compensate for the

418 loss of feed palatability with increasing levels of FM substitution by PP ingredients. In  
419 addition, and as a second hypothesis, it should also be considered that feed ingredients  
420 from marine resources and plants are different in compounds other than the  
421 macronutrients and amino acid and mineral profiles (Aksnes, 2005), and some of these  
422 may be important in explaining the difficulties in totally replacing fishmeal with plant  
423 protein blends. Thus, differences in taurine, as well as some other peptides like anserine  
424 and carnosine, in addition to nucleotides and other bioactive compounds may partially  
425 explain differences in growth performance between experimental diets (Aksnes et al.,  
426 2006). Finally, a third hypothesis that would explain the above-mentioned differences  
427 might be linked to the occurrence of anti-nutritional compounds or to differences in  
428 apparent digestibility of alternative PP sources (Espe et al., 2007). For instance, Minjarez-  
429 Osorio et al. (2016) incorporated novel non-genetically modified soybean with reduced  
430 levels of anti-nutritional factors, in contrast to the conventional soybean meal that was  
431 used in our study. Previous studies have shown a good dietary tolerance towards PP  
432 sources in meagre (Rodrigues-Olim, 2012; Velazco-Vargas et al., 2014; Ribeiro et al.,  
433 2015). In particular, protein digestibility of wheat gluten, soybean protein concentrate, pea  
434 protein concentrate and corn gluten meal has been reported to be high (>90%) and  
435 moderate (78-84%) for soybean meal, rapeseed meal and sunflower meal. Similarly, the  
436 apparent digestibility of protein in soybean meal products varied between 80 and 93% in  
437 mulloway (*A. japonicus*) and red drum (Gaylord and Gatlin, 1996; McGoogan and Reigh,  
438 1996; Booth et al., 2013). Although in the present study FM was mainly substituted by  
439 corn gluten and wheat gluten, two PP sources with high protein digestibility values in  
440 meagre (Ribeiro et al., 2015), differences in fish performance may be due to differences in  
441 blend and proportions of vegetable ingredients used in the two studies and probably in the  
442 processing conditions of PP ingredients between both studies.

443 Nevertheless, little knowledge on nutrient requirements and scarce information on the  
444 formulation of commercial feeds are the main obstacles for the sustainable farming of  
445 meagre. Chatzifotis et al. (2012) tested dietary formulations with different protein and  
446 lipid levels and high inclusion of FM (> 51%) and concluded that protein requirements of  
447 juvenile meagre were *ca.* 50%. These authors reported lower growth rates of meagre  
448 compared to those observed in our study (SGR = 0.7 – 1.3 vs 1.4 – 2.1%/day,  
449 respectively), although the rearing water temperature was identical (19 °C) and fish initial  
450 mean body weight was similar between the two studies; these differences might be  
451 attributed to different feed formulations. On the contrary, Couto et al. (2016) evaluating  
452 the carob seed germ meal in the diets of meagre found higher growth rates than in present  
453 study (DGI = 3.4 – 3.7 vs 1.76 – 2.76, respectively), probably due the higher rearing  
454 water temperature (23 °C vs 19 °C, respectively).

455 The amino acid (AA) requirements in meagre larvae and juveniles are still unknown.  
456 Saavedra et al. (2016) determined the AA composition of the whole-body tissue of  
457 meagre at different days after hatching in order to estimate the AA requirements of  
458 meagre larvae following a common research practice in case where no available  
459 information on the AA requirements of fish species exist (Kaushik, 1998). Lysine is found  
460 in low concentrations in some plant ingredients, mainly in cereal grain by-products, such  
461 as corn gluten and wheat gluten meal, which are commonly used in fish feeds (Wilson,  
462 2003); it is sensitive to severe processing conditions and for these reasons is commonly  
463 considered as the first limiting EAA in feeds (NRC, 2011) and has attracted a lot of  
464 attention in fish nutrition (Hauler and Carter, 2001). The addition of crystalline Lys (37.0  
465 g/kg) to the FM20a diet at similar levels to diets FM45 and FM30 could not compensate  
466 growth performance of meagre for the decrease of dietary FM from 450 g/kg to 200 g/kg.  
467 Interestingly, in diets with low FM inclusion (200 g/kg) and similar inclusion levels of PP

468 blends, an increase in dietary Lys from 24.8 to 28.5 g/kg diet improved growth  
469 performance of juvenile meagre (diets FM20c and FM20b), while no effect was found  
470 with further increase of Lys from 28.5 to 37.0 g/kg (diets FM20b and FM20a). Diet  
471 FM20d, showed the lowest growth performance among the experimental diets, even  
472 though formulated -as mentioned above- to have a better PP combination in terms of  
473 protein quality, digestibility and reduced anti-nutritional substances compared to FM20  
474 series diets, and this was obviously due to the lowest Lys concentration which this diet  
475 contained.

476 In the present study, 33 to 56% FM substitution by alternative PP sources did not affect  
477 total body lipid content nor the HSI and LSI ratios. Similarly, no changes in total lipid  
478 levels, HSI and mesenteric fat were found in meagre fed diets containing 25 to 50% FM  
479 replacement (Velazco-Vargas et al., 2013; Ribeiro et al., 2015) and red drum and shortfin  
480 corvina fed diets with 50 to 75% FM replacement by PP sources (Minjarez-Osorio et al.,  
481 2016). In contrast, Estévez et al. (2011) reported that replacing variable amounts of FM  
482 protein with different levels of PP affected meagre composition with an overall increase  
483 in the adiposity level in both muscle and liver, as it has also been reported in other  
484 carnivorous fish species (Kaushik et al., 1995; Zhang et al., 2016). As Minjarez-Osorio et  
485 al. (2016) suggested, contradictory results found in different studies may indicate that HSI  
486 and other body condition indexes may not be clear indicators of metabolic effects caused  
487 by FM replacement with plant feedstuffs in fish diets.

488 Different studies in meagre and other scianid species have shown that diets with different  
489 levels of FM replacement by different PP ingredients did not have any effect on the body  
490 proximate composition of fish (Estévez et al., 2011; Velazco-Vargas et al., 2013; Ribeiro  
491 et al., 2015; Minjarez-Osorio et al., 2016). In this study, although no differences were  
492 found in the levels of total lipids in meagre fed diets with different levels of FM

493 substitution, we found slight differences in protein levels. Meagre fed diets FM30, FM20b  
494 and FM20c had on average 4.2 and 4.8% higher protein levels than fish fed diets FM45  
495 (control) and FM20d, respectively; an increase that was linked to a non-statistically  
496 significant decrease in their total lipid content; however, these levels were within the  
497 normal range of values for meagre reported in similar studies (Estévez et al., 2011;  
498 Velazco-Vargas et al., 2013; Ribeiro et al., 2015; Minjarez-Osorio et al., 2016).

499 In this study, the effects of FM substitution by different PP blends was also evaluated by  
500 means of the examination of the histological organization of different organs and tissues,  
501 although no histological changes associated with experimental dietary formulations were  
502 found. However, systemic granulomatosis was evident in most of examined animals with  
503 different levels of severity depending on the organ considered. Systemic granulomatosis  
504 is a common disease in meagre characterized by multiple systemic visceral granulomas  
505 that manifest progressively as calcified and necrotic organs (Ghittino et al., 2004). While  
506 the clear etiology of this disease is not fully understood, there is evidence that it may be  
507 linked to nocardiosis (Elkesh et al., 2013) and to metabolic or nutritional disorders, since  
508 similar systemic granulomas were observed in other cultured fish species such as gilthead  
509 sea bream (*Sparus aurata*) (Paperna, 1987; Ghittino et al., 2004), turbot (*Scophthalmus*  
510 *maximus*) (Tixerant et al., 1984) and in different salmonids species (Herman, 1996; Good  
511 et al., 2016). Specific staining procedures for detecting the presence of acid-fast bacteria  
512 like *Nocardia* sp. were conducted in order to provide evidence of the etiology for the  
513 systemic granulomatosis found in all dietary treatments; however, negative results from  
514 the Gram, Ziehl-Neelsen and Fite-Faraco staining indicated that granulomatosis in this  
515 study could be considered of a non-infectious origin. In this study, the organs mostly  
516 affected by non-infectious granulomatosis were the liver and kidney where numerous  
517 individual and coalescent granulomas were detected in all experimental groups, whereas

518 the digestive tract and pancreas were the organs with the lowest incidence of  
519 granulomatosis. Although different authors have associated this disease with feed  
520 formulation and storage conditions (Herman, 1996; Good et al., 2016), we did not find  
521 any relationship between the prevalence and severity of systemic non-infectious  
522 granulomatosis with different levels of FM substitution with PP sources in meagre. Thus,  
523 the true etiology of the observed pathology remains unknown, and further research needs  
524 to be conducted to enhance our understanding on this disease affecting meagre. However,  
525 the finding that granulomas were assigned a chronic inflammation stage indicates the  
526 onset and progression of granulomatosis occurs insidiously at earlier life stages of  
527 meagre. In addition to non-infectious granulomatosis, the histological observations  
528 revealed a large accumulation of lipid deposits in the hepatic parenchyma, which is in  
529 agreement with recent data from Ribeiro et al. (2015) feeding meagre with diets  
530 containing 51% (DM) protein and 17% (DM) lipids.

531

## 532 **5 CONCLUSIONS**

533 Fishmeal was successfully partially substituted (33% replacement) by corn gluten and  
534 wheat gluten in meagre diets when feeds were supplemented with lysine in order to  
535 balance the AA profile of the diet. Higher level of FM replacement (56%) resulted in a  
536 decrease in growth performance and feed utilization parameters, which may be linked to  
537 different reasons such as a decrease in diet palatability and/or reduced levels of bioactive  
538 compounds and micronutrients present in FM. The increase of dietary Lys levels from 25  
539 to 28.5 g/100g diet in the diets contained similar PP blends and 200 g/kg inclusion of FM  
540 significantly improved the growth performance of juvenile meagre. Furthermore, this  
541 study clearly showed evidences that the appropriate dietary fishmeal level and its

542 adequate replacement level should be taken into account when determining optimal  
543 dietary lysine for meagre in future studies.

544 Chronic systemic non-infectious granulomatosis was observed in meagre from all the  
545 experimental groups regardless of the dietary treatment and fishmeal replacement level  
546 considered, with the liver and kidney found to be the most severely affected tissues. These  
547 findings indicated that further research in earlier life stages of fish is needed to assess the  
548 etiology of this common disease in meagre. The high level of lipid accumulation in the  
549 hepatic parenchyma suggested that dietary lipid levels in this species need to be optimized  
550 in order to avoid potential physiological and metabolic mid or long-term disorders  
551 associated with a fatty liver syndrome.

552

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#### 562 **References**

563

564 Official Methods of Analysis of AOAC International, (2005). *AOAC International*, 18th  
565 ed., Arlington, VA: AOAC.

- 566 Asociación Empresarial de Acuicultura de España (APROMAR), (2015). El Estado de la  
567 Acuicultura en España. [http:// www.apromar.es/](http://www.apromar.es/) (accessed 10.05.2016).
- 568 Aksnes, A. (2005). Feed ingredients. The impacts of nitrogen extractives in aqua feed  
569 ingredients. *International Aqua Feed*, 8, 28–30.
- 570 Aksnes, A., Hope, B., Jönsson, E., Björnsson, B.T., Albrektsen, S. (2006). Size-  
571 fractionated fish hydrolysate as feed ingredient for rainbow trout (*Oncorhynchus*  
572 *mykiss*) fed high plant protein diets. I: Growth, growth regulation and feed  
573 utilization. *Aquaculture*, 261, 305-317.
- 574 Booth, M.A., Allan, G.L., Smullen, R.P. (2013). Digestibility of common feed ingredients  
575 by juvenile mullock *Argyrosomus japonicus*. *Aquaculture*, 414–415, 140–148.
- 576 Couto, A., Barroso, C., Guerreiro, I., Pousão-Ferreira, P., Matos, E., Peres, H., Oliva-  
577 Teles, A., Enes, P. (2016). Carob seed germmeal in diets for meagre (*Argyrosomus*  
578 *regius*) juveniles: Growth, digestive enzymes, intermediary metabolism, liver and  
579 gut histology. *Aquaculture*, 451, 396-404.
- 580 Chatzifotis, S., Panagiotidou, M., Papaioannou, N., Pavlidis, M., Nengas, I., Mylonas, C.  
581 C. (2010). Effect of dietary lipid levels on growth, feed utilization, body  
582 composition and serum metabolites of meagre (*Argyrosomus regius*) juveniles.  
583 *Aquaculture*, 307, 65-70.
- 584 Chatzifotis, S., Panagiotidou, M., Divanach, P. (2012). Effect of protein and lipid dietary  
585 levels on the growth of juvenile meagre (*Argyrosomus regius*). *Aquaculture*  
586 *International*, 20, 91-98.
- 587 de Rodrigáñez, M.S., Fuentes, J., Moyano, F. J., Ribeiro, L. (2013). In vitro evaluation of  
588 the effect of a high plant protein diet and nucleotide supplementation on intestinal  
589 integrity in meagre (*Argyrosomus regius*). *Fish Physiology and Biochemistry*, 39,  
590 1365-1370.

- 591 Elkesh, A., Kantham, K. P. L., Shinn, A. P., Crumlish, M., Richards, R.H.( 2013).  
592 Systemic nocardiosis in a Mediterranean population of cultured meagre,  
593 *Argyrosomus regius* Asso (Perciformes: Sciaenidae). Journal of Fish Disease, 36,  
594 141-149.
- 595 Espe, M., Lemme, A., Petri, A., El-Mowafi, A.( 2007). Assessment of lysine requirement  
596 for maximal protein accretion in Atlantic salmon using plant protein diets.  
597 *Aquaculture*, 263, 168-178.
- 598 Estévez, A., Treviño, L., Kotzamanis, Y., Karacostas, I., Tort, L., Gisbert, E.( 2011).  
599 Effects of different levels of plant proteins on the ongrowing of meagre  
600 (*Argyrosomus regius*) juveniles at low temperatures. *Aquaculture Nutrition*, 17,  
601 e572-e582.
- 602 Fournier, V., Huelvan, C., Desbruyeres, E. (2004). Incorporation of a mixture of plant  
603 feed stuffs as substitute for fishmeal in diets of juvenile turbot (*Psetta maxima*).  
604 *Aquaculture*, 235, 451-465.
- 605 Gaylord, T.G., Gatlin, D.M. (1996). Determination of digestibility coefficients of various  
606 feed stuffs for red drum (*Sciaenops ocellatus*). *Aquaculture*, 139, 303–314.
- 607 Ghittino, C., Manuali, E., Latini, M., Agnetti, F., Rogato, F., Agonigi, R., Colussi, S.,  
608 Prearo, M. (2004). Case of systemic granulomatosis in meagre (*Argyrosomus*  
609 *regius*) and comparison with the histological features present in gilthead seabream.  
610 *Ittiopatologia*, 1, 59-67.
- 611 Good, C., Marancik, D.P., Welch, T.J., May, T., Davidson, J., Summerfelt, S. (2016).  
612 Systemic granuloma observed in Atlantic salmon *Salmo salar* raised to market size  
613 in a freshwater recirculation aquaculture system. *Aquaculture Research*, 47, 3679-  
614 3683.

- 615 Hauler, R.C., Carter, C.G. (2001). Reevaluation of the quantitative dietary lysine  
616 requirements of fish. *Reviews in Fisheries Science*, 9, 133–163.
- 617 Herman, R.L. (1996). Systemic non-infectious granulomatoses in fishes. *US Fish &*  
618 *Wildlife Service. Fish Disease Leaflet* 79, 9.
- 619 Hernández, M.D., Martínez, F.J., Jover, M., García, B. G. (2007). Effects of partial  
620 replacement of fishmeal by soybean meal in sharpsnout seabream (*Diplodus*  
621 *puntazzo*) diet. *Aquaculture* 263, 159-167.
- 622 Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M. (2010). Supplemental  
623 effects of some crude ingredients in improving nutritive values of low fishmeal diets  
624 for red sea bream, *Pagrus major*. *Aquaculture*, 308, 136-144.
- 625 Kaushik, S.J. (1998). Whole body amino acid composition of European seabass  
626 (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta*  
627 *maxima*) with an estimation of their IAA requirement profiles. *Aquatic Living*  
628 *Resources*, 11: 355-358.
- 629 Kaushik, S., Coves, D., Dutto, G., Blanc, D., (2004). Almost total replacement of  
630 fishmeal by plant protein sources in the diet of a marine teleost, the European  
631 seabass, *Dicentrarchus labrax*. *Aquaculture*, 230, 391–404.
- 632 Hernández, M.D., López, M.B., Álvarez, A., Ferrandini, E., García-García, E., Garrido,  
633 M.D. (2009). Sensory, physical, chemical and microbiological changes in  
634 aquacultured meagre (*Argyrosomus regius*) fillets during ice storage. *Food*  
635 *Chemistry*, 114, 237–245.
- 636 Kaushik, S.J., Cravedi, J.P., Lalles, J.P., Sumpter, J., Fauconneau, B., Laroche, M. (1995).  
637 Partial or total replacement of fishmeal by soybean protein on growth, protein  
638 utilization, potential estrogenic or antigenic effects, cholesterolemia and flesh  
639 quality in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, 133, 257–274.

- 640 Kaushik, S.J., Covès, D., Dutto, G., Blanc, D. (2004). Almost total replacement of  
641 fishmeal by plant protein sources in the diet of a marine teleost, the European  
642 seabass, *Dicentrarchus labrax*. *Aquaculture*, 230, 391–404.
- 643 McGoogan, B.B., Reigh, R.C. (1996). Apparent digestibility of selected ingredients in red  
644 drum (*Sciaenops ocellatus*) diets. *Aquaculture*, 141, 233–244.
- 645 Minjarez-Osorio, C., Castillo-Alvarado, S., Gatlin III, D.M., González-Félix, M.L., Perez-  
646 Velazquez, M., Rossi Jr, W. (2016). Plant protein sources in the diets of the  
647 sciaenids red drum (*Sciaenops ocellatus*) and shortfin corvina (*Cynoscion*  
648 *parvipinnis*): A comparative study. *Aquaculture*, 453, 122-129.
- 649 Moxley, J.D., Rossi, W., Buentello, A., Pohlenz, C., Gatlin, D. M., Tomasso, J.R. (2014).  
650 Replacement of fishmeal with plant feedstuffs in the diet of red drum, *Sciaenops*  
651 *ocellatus*: effects on production characteristics and tolerance to aquaculture-related  
652 stressors. *Journal of the World Aquaculture Society*, 45, 192-198.
- 653 Neter, J., Kutner, M., Nachtsheim, C., and Wasserman, W. (1996). *Applied Linear*  
654 *Statistical Models*, McGraw-Hill Companies, Inc., NY.
- 655 Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I.,  
656 Gatlin, D.M., Goldberg, R., Hua, K., Nichols, P.D. (2009). Feeding aquaculture in  
657 an era of finite resources. *Proceedings of the National Academy of Sciences U S A*,  
658 8, 15103–15110.
- 659 National Resaerch Council (NRC) (2011). *Nutrient Requirements of Fish and Shrimp*.  
660 The National Academic Press, Washington, D.C.
- 661 Paperna, I. (1987). Systemic granuloma of sparriid fish in culture. *Aquaculture*, 67, 53-58.
- 662 Poli, B.M., Parisi, G., Zampacavallo, G., Iurzan, F., Mecatti, M., Lupi, P., Bonelli, A.  
663 (2003). Preliminary results on quality and quality changes in reared meagre

- 664 (*Argyrosomus regius*): body and fillet traits and freshness changes in refrigerated  
665 commercial-size fish. *Aquaculture International*, 11, 301–311.
- 666 Quéméner, L., Suquet, M., Mero, D., Gaignon, J.-L. (2002). Selection method of new  
667 candidates for finfish aquaculture: the case of the French Atlantic, the Channel and  
668 the North Sea coasts. *Aquatic Living Resources* 15, 293–302.
- 669 Rana, K.J., Siriwardena, S., Hasan, M. (2009). Impact of rising feed ingredient prices on  
670 aquafeeds and aquaculture production. *FAO Fisheries and Aquaculture Technical  
671 Paper* 541. FAO, Rome.
- 672 Ribeiro, L., Moura, J., Santos, M., Colen, R., Rodrigues, V., Bandarra, N., Soares, F.,  
673 Ramalho, P., Barata, M., Moura, P., Pousão-Ferreira, P., Dias, J. (2015). Effect of  
674 vegetable based diets on growth, intestinal morphology, activity of intestinal  
675 enzymes and haematological stress indicators in meagre (*Argyrosomus regius*).  
676 *Aquaculture*, 447, 116-128.
- 677 Robaina, L., Izquierdo, M. S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D.,  
678 Fernandez-Palacios, H. (1995). Soybean and lupin seed meals as protein sources in  
679 diets for gilthead seabream (*Sparus aurata*): nutritional and histological  
680 implications. *Aquaculture*, 130, 219-233.
- 681 Rodrigues-Olim, C.P. (2012). Apparent digestibility coefficients of feed ingredients for  
682 juvenile meagre (*Argyrosomus regius*, Asso 1801). (MSc Thesis), Faculdade de  
683 Ciências, Universidade do Porto, 79 pp. <http://hdl.handle.net/10216/64953> (accessed  
684 12.05.2016).
- 685 Saavedra, M., Candeias-Mendes, A., Castanho, S., Teixeira, B., Mendes, R., Pousão-  
686 Ferreira, P. (2015). Amino acid profiles of meagre (*Argyrosomus regius*) larvae:  
687 Towards the formulation of an amino acid balanced diet. *Aquaculture*, 448, 315-  
688 320.

- 689 Salze, G., McLean, E., Battle, P.R., Schwarz, M.H., Craig, S.R. (2010). Use of soy  
690 protein concentrate and novel ingredients in the total elimination of fishmeal and  
691 fish oil in diets for juvenile cobia, *Rachycentron canadum*. *Aquaculture*, 298, 294-  
692 299.
- 693 Sitjà-Bobadilla, A., Peña-Llopis, S., Gómez-Requeni, P., Médale, F., Kaushik, S., Pérez-  
694 Sánchez, J. (2005). Effect of fishmeal replacement by plant protein sources on non-  
695 specific defence mechanisms and oxidative stress in gilthead sea bream (*Sparus*  
696 *aurata*). *Aquaculture*, 249, 387-400.
- 697 Tacon, A.G.J., Metian, M. (2008). Global overview on the use of fishmeal and fish oil in  
698 industrially compounded aquafeeds: Trends and future prospects. *Aquaculture*, 285,  
699 146–158.
- 700 Tixerant G., Aldrin, J., Baudin-Laurencin, F. Messenger J.L. (1984). Granulomatous  
701 syndrome and tyrosine metabolism defect in turbot (*Scophthalmus maximus*).  
702 *Bulletin de l'Academie Veterinaire de France*, 57, 75-90.
- 703 Velazco-Vargas, J., Martínez-Llorens, S., Cerda, M.J., Tomás-Vidal, A. (2013).  
704 Evaluation of soybean meal as protein source for *Argyrosomus regius* (Asso, 1801)  
705 (*Sciaenidae*). *International Journal of Fisheries and Aquaculture*, 5, 35–44.
- 706 Velazco-Vargas J., Tomás-Vidal A., Hamdan M., Moyano López F.J., Jover Cerda M.,  
707 Martínez-Llorens S. (2014). Influence of digestible protein levels on growth and  
708 feed utilization of juvenile meagre *Argyrosomus regius*. *Aquaculture Nutrition*, 20,  
709 520-531.
- 710 Wilson, R.P. (2003). Amino acid requirements of finfish and crustaceans. In: D`Mello,  
711 J.P.F. (Ed.), *Amino Acids in Animal Nutrition*. CAB International, Wallingford,  
712 Oxon, United Kingdom, pp. 427–447.

713 Zhang, Y., Ji, W., Wu, Y., Han, H., Qin, J., Wang, Y. (2016). Replacement of dietary  
714 fishmeal by soybean meal supplemented with crystalline methionine for Japanese  
715 seabass (*Lateolabrax japonicus*). *Aquaculture Research*, 47, 243-252.  
716

717 Table 1 Formulation and proximate composition (g per kg of diet in dry matter basis) of  
 718 diets containing different levels of fish meal (FM) substitution by different plant protein  
 719 sources. Data are presented as mean  $\pm$  standard deviation.  
 720

	<b>Diet 1</b>	<b>Diet 2</b>	<b>Diet 3</b>	<b>Diet 4</b>	<b>Diet 5</b>	<b>Diet 6</b>
<b>Ingredient (g/kg of diet)</b>	<b>(FM<sub>45</sub>)</b>	<b>(FM<sub>30</sub>)</b>	<b>(FM<sub>20</sub>)</b>	<b>(FM<sub>20</sub>)</b>	<b>(FM<sub>20</sub>)</b>	<b>(FM<sub>18</sub>)</b>
FM super Prime	450.0	300.0	200.0	200.0	200.0	180.0
Fish oil	124.7	127.7	136.0	135.6	135.3	134.5
SBM 48%	100.0	99.0	162.3	145.9	130.9	75.5
Wheat 90%	95.0	90.0	80.0	80.0	80.0	80.0
Rapeseed meal	80.0	80.0	-	-	-	32.4
Corn gluten	71.3	130.0	236.6	259.6	280.0	300.0
Wheat gluten	50.0	100.0	100.0	100.0	101.0	127.9
Middlings	14.5	3.6	-	-	-	-
Krill meal	0	20.0	20.0	20.0	20.0	20.0
Squid meal	0	20.0	20.0	20.0	20.0	20.0
Premix 0.25%	2.5	2.5	2.5	2.5	2.5	2.5
Choline 60%	2.5	2.5	2.5	2.5	2.5	2.5
Monocalcium phoshate	2.3	12.7	22.8	22.9	23.1	24.5
Lysine HCl	6.7	11.8	16.7	10.6	4.5	-
DL Methionine	0.35	-	0.42	-	-	-
Antioxidant	0.2	0.2	0.2	0.2	0.2	0.2
<b>Total</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>
<b>Determined proximate composition (%)</b>						
Dry matter	93.4 $\pm$ 0.1	92.9 $\pm$ 0.1	92.5 $\pm$ 0.2	92.1 $\pm$ 0.1	91.2 $\pm$ 0.0	91.6 $\pm$ 0.10
Crude protein	47.9 $\pm$ 0.1	47.8 $\pm$ 0.2	47.4 $\pm$ 0.3	47.5 $\pm$ 0.6	47.6 $\pm$ 0.1	47.4 $\pm$ 0.4
Crude fat	17.3 $\pm$ 0.1	16.9 $\pm$ 0.0	16.8 $\pm$ 0.1	16.7 $\pm$ 0.1	17.0 $\pm$ 0.2	16.7 $\pm$ 0.1
Ash	8.8 $\pm$ 0.1	7.7 $\pm$ 0.1	6.6 $\pm$ 0.0	6.5 $\pm$ 0.1	6.4 $\pm$ 0.1	6.1 $\pm$ 0.0
Gross energy (MJ/kg)	21.6 $\pm$ 0.1	21.7 $\pm$ 0.1	21.8 $\pm$ 0.2	21.9 $\pm$ 0.1	21.9 $\pm$ 0.2	21.9 $\pm$ 0.1
Phosphorus*	1.2	1.2	1.2	1.2	1.2	1.2
Cellulose*	2.0	2.0	1.4	1.4	1.4	1.6
Starch*	9.0	9.7	10.3	10.5	10.8	11.2

721 \*Theoretical values

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723

724 Table 2 Amino acid composition (mean  $\pm$  standard deviation) of the experimental diets (g  
725 per 100 g feed).

726

727

	<b>Diets</b>					
	<b>Diet 1 (FM<sub>45</sub>)</b>	<b>Diet 2 (FM<sub>30</sub>)</b>	<b>Diet 3 (FM<sub>20</sub>)</b>	<b>Diet 4 (FM<sub>20</sub>)</b>	<b>Diet 5 (FM<sub>20</sub>)</b>	<b>Diet 6 (FM<sub>18</sub>)</b>
HyPro	0.26 $\pm$ 0.00 <sup>a</sup>	0.22 $\pm$ 0.00 <sup>b</sup>	0.14 $\pm$ 0.00 <sup>c</sup>	0.18 $\pm$ 0.00 <sup>d</sup>	0.14 $\pm$ 0.01 <sup>c</sup>	0.13 $\pm$ 0.00 <sup>c</sup>
His	1.23 $\pm$ 0.02 <sup>a</sup>	1.14 $\pm$ 0.01 <sup>a</sup>	1.03 $\pm$ 0.00 <sup>b</sup>	1.16 $\pm$ 0.03 <sup>ac</sup>	1.10 $\pm$ 0.04 <sup>bc</sup>	1.03 $\pm$ 0.02 <sup>b</sup>
Tau	0.33 $\pm$ 0.00 <sup>a</sup>	0.26 $\pm$ 0.00 <sup>b</sup>	0.17 $\pm$ 0.00 <sup>ce</sup>	0.19 $\pm$ 0.00 <sup>d</sup>	0.18 $\pm$ 0.01 <sup>cd</sup>	0.15 $\pm$ 0.00 <sup>e</sup>
Ser	1.95 $\pm$ 0.01 <sup>a</sup>	1.95 $\pm$ 0.01 <sup>a</sup>	2.04 $\pm$ 0.02 <sup>ac</sup>	2.18 $\pm$ 0.02 <sup>b</sup>	2.11 $\pm$ 0.03 <sup>bc</sup>	2.12 $\pm$ 0.03 <sup>bc</sup>
Arg	2.42 $\pm$ 0.01 <sup>a</sup>	2.22 $\pm$ 0.02 <sup>b</sup>	2.03 $\pm$ 0.01 <sup>c</sup>	2.23 $\pm$ 0.05 <sup>b</sup>	2.08 $\pm$ 0.08 <sup>bc</sup>	1.96 $\pm$ 0.03 <sup>c</sup>
Gly	2.29 $\pm$ 0.01 <sup>a</sup>	2.06 $\pm$ 0.01 <sup>b</sup>	1.77 $\pm$ 0.00 <sup>c</sup>	1.97 $\pm$ 0.06 <sup>bd</sup>	1.86 $\pm$ 0.06 <sup>cd</sup>	1.82 $\pm$ 0.06 <sup>cd</sup>
Asp	3.92 $\pm$ 0.05 <sup>a</sup>	3.91 $\pm$ 0.06 <sup>a</sup>	3.57 $\pm$ 0.01 <sup>b</sup>	3.19 $\pm$ 0.10 <sup>c</sup>	3.33 $\pm$ 0.06 <sup>bc</sup>	3.43 $\pm$ 0.01 <sup>bc</sup>
Glu	8.18 $\pm$ 0.05 <sup>a</sup>	9.21 $\pm$ 0.24 <sup>b</sup>	9.87 $\pm$ 0.16 <sup>bc</sup>	9.37 $\pm$ 0.23 <sup>b</sup>	9.90 $\pm$ 0.23 <sup>bc</sup>	10.64 $\pm$ 0.03 <sup>c</sup>
Thr	1.83 $\pm$ 0.00 <sup>a</sup>	1.72 $\pm$ 0.00 <sup>b</sup>	1.60 $\pm$ 0.00 <sup>c</sup>	1.67 $\pm$ 0.02 <sup>bc</sup>	1.63 $\pm$ 0.03 <sup>c</sup>	1.63 $\pm$ 0.03 <sup>c</sup>
Ala	2.81 $\pm$ 0.00 <sup>a</sup>	2.77 $\pm$ 0.02 <sup>a</sup>	2.79 $\pm$ 0.01 <sup>a</sup>	2.70 $\pm$ 0.05 <sup>a</sup>	2.84 $\pm$ 0.06 <sup>ab</sup>	3.02 $\pm$ 0.05 <sup>b</sup>
Pro	2.52 $\pm$ 0.03 <sup>a</sup>	2.80 $\pm$ 0.01 <sup>b</sup>	3.14 $\pm$ 0.01 <sup>c</sup>	3.28 $\pm$ 0.02 <sup>d</sup>	3.30 $\pm$ 0.01 <sup>d</sup>	3.54 $\pm$ 0.06 <sup>e</sup>
Cys	0.23 $\pm$ 0.00 <sup>a</sup>	0.24 $\pm$ 0.00 <sup>a</sup>	0.27 $\pm$ 0.00 <sup>b</sup>	0.33 $\pm$ 0.00 <sup>c</sup>	0.31 $\pm$ 0.00 <sup>cd</sup>	0.29 $\pm$ 0.01 <sup>bd</sup>
Lys	3.77 $\pm$ 0.05 <sup>a</sup>	3.74 $\pm$ 0.03 <sup>a</sup>	3.70 $\pm$ 0.01 <sup>a</sup>	2.85 $\pm$ 0.10 <sup>b</sup>	2.48 $\pm$ 0.05 <sup>c</sup>	2.09 $\pm$ 0.01 <sup>d</sup>
Tyr	1.30 $\pm$ 0.02 <sup>a</sup>	1.29 $\pm$ 0.01 <sup>a</sup>	1.40 $\pm$ 0.01 <sup>ac</sup>	1.57 $\pm$ 0.03 <sup>b</sup>	1.49 $\pm$ 0.03 <sup>bc</sup>	1.44 $\pm$ 0.02 <sup>c</sup>
Met	1.05 $\pm$ 0.01 <sup>a</sup>	0.91 $\pm$ 0.00 <sup>b</sup>	0.91 $\pm$ 0.01 <sup>b</sup>	0.96 $\pm$ 0.02 <sup>b</sup>	0.95 $\pm$ 0.03 <sup>b</sup>	0.95 $\pm$ 0.0 <sup>b</sup>
Val	2.11 $\pm$ 0.01 <sup>a</sup>	2.01 $\pm$ 0.02 <sup>b</sup>	1.92 $\pm$ 0.00 <sup>c</sup>	1.95 $\pm$ 0.00 <sup>bc</sup>	1.97 $\pm$ 0.02 <sup>bc</sup>	2.00 $\pm$ 0.03 <sup>b</sup>
Ile	1.82 $\pm$ 0.02 <sup>a</sup>	1.76 $\pm$ 0.01 <sup>ab</sup>	1.71 $\pm$ 0.01 <sup>b</sup>	1.76 $\pm$ 0.01 <sup>ab</sup>	1.76 $\pm$ 0.02 <sup>ab</sup>	1.76 $\pm$ 0.03 <sup>ab</sup>
Leu	3.69 $\pm$ 0.06 <sup>a</sup>	3.80 $\pm$ 0.01 <sup>a</sup>	4.30 $\pm$ 0.02 <sup>b</sup>	4.52 $\pm$ 0.01 <sup>bc</sup>	4.59 $\pm$ 0.03 <sup>c</sup>	4.74 $\pm$ 0.12 <sup>c</sup>
Phe	1.90 $\pm$ 0.02 <sup>a</sup>	1.89 $\pm$ 0.01 <sup>a</sup>	2.04 $\pm$ 0.01 <sup>ac</sup>	2.32 $\pm$ 0.07 <sup>b</sup>	2.22 $\pm$ 0.07 <sup>bc</sup>	2.13 $\pm$ 0.04 <sup>bc</sup>

728

729 Differences in amino acid composition between experimental diets are indicated by  
730 different letters (ANOVA,  $P < 0.05$ ,  $n = 2$ ).

731



**Table 3.** Growth performance indices of meagre (*A. regius*) fed diets containing different levels of fish meal (FM) substitution by different plant protein sources. Data are presented as mean  $\pm$  standard deviation.

	Diets					
	Diet 1 (FM <sub>45</sub> )	Diet 2 (FM <sub>30</sub> )	Diet 3 (FM <sub>20</sub> )	Diet 4 (FM <sub>20</sub> )	Diet 5 (FM <sub>20</sub> )	Diet 6 (FM <sub>18</sub> )
Survival (%)	98.0	98.0	100	98.0	100	100
Initial mean body weight (g)	36.0 $\pm$ 0.6	36.0 $\pm$ 0.6	36.0 $\pm$ 0.6	36.0 $\pm$ 0.0	36.0 $\pm$ 0.6	36.0 $\pm$ 0.0
Final mean body weight (g)	105.8 $\pm$ 3.1 <sup>a</sup>	98.6 $\pm$ 3.2 <sup>a</sup>	84.4 $\pm$ 2.5 <sup>bc</sup>	86.8 $\pm$ 0.9 <sup>b</sup>	78.6 $\pm$ 2.7 <sup>cd</sup>	75.2 $\pm$ 2.8 <sup>d</sup>
WG (g fish <sup>-1</sup> )	70 $\pm$ 3.7 <sup>a</sup>	63 $\pm$ 3.2 <sup>a</sup>	49 $\pm$ 2.7 <sup>bc</sup>	51 $\pm$ 0.5 <sup>b</sup>	43 $\pm$ 2.7 <sup>cd</sup>	39 $\pm$ 2.4 <sup>d</sup>
TFI (g)	85 $\pm$ 6.7	80 $\pm$ 5.0	73 $\pm$ 5.1	75 $\pm$ 6.5	78 $\pm$ 9.5	69 $\pm$ 3.4
DGI (%)	2.76 $\pm$ 0.13 <sup>a</sup>	2.55 $\pm$ 0.10 <sup>a</sup>	2.11 $\pm$ 0.09 <sup>bc</sup>	2.20 $\pm$ 0.01 <sup>c</sup>	1.92 $\pm$ 0.10 <sup>bd</sup>	1.76 $\pm$ 0.08 <sup>d</sup>
FCR	1.22 $\pm$ 0.13 <sup>a</sup>	1.27 $\pm$ 0.02 <sup>a</sup>	1.50 $\pm$ 0.05 <sup>ab</sup>	1.47 $\pm$ 0.14 <sup>ab</sup>	1.81 $\pm$ 0.19 <sup>b</sup>	1.76 $\pm$ 0.18 <sup>b</sup>
PER	1.74 $\pm$ 0.20 <sup>ab</sup>	1.80 $\pm$ 0.03 <sup>b</sup>	1.71 $\pm$ 0.05 <sup>ab</sup>	1.68 $\pm$ 0.16 <sup>ab</sup>	1.39 $\pm$ 0.15 <sup>ac</sup>	1.18 $\pm$ 0.12 <sup>c</sup>
SGR (% day <sup>-1</sup> )	2.09 $\pm$ 0.09 <sup>a</sup>	1.95 $\pm$ 0.06 <sup>a</sup>	1.66 $\pm$ 0.07 <sup>bc</sup>	1.72 $\pm$ 0.01 <sup>c</sup>	1.53 $\pm$ 0.07 <sup>bd</sup>	1.41 $\pm$ 0.05 <sup>d</sup>
TGC x 1000	1.42 $\pm$ 0.06 <sup>a</sup>	1.31 $\pm$ 0.05 <sup>a</sup>	1.09 $\pm$ 0.05 <sup>bc</sup>	1.13 $\pm$ 0.01 <sup>c</sup>	0.99 $\pm$ 0.05 <sup>bd</sup>	0.91 $\pm$ 0.04 <sup>d</sup>
LSI (%)	0.25 $\pm$ 0.06	0.23 $\pm$ 0.06	0.46 $\pm$ 0.15	0.32 $\pm$ 0.14	0.45 $\pm$ 0.12	0.42 $\pm$ 0.21
HSI (%)	3.39 $\pm$ 0.44	3.83 $\pm$ 0.68	3.66 $\pm$ 0.48	3.15 $\pm$ 0.52	3.47 $\pm$ 0.24	3.74 $\pm$ 0.48
PPV	2.11 $\pm$ 0.20 <sup>a</sup>	2.07 $\pm$ 0.15 <sup>a</sup>	1.58 $\pm$ 0.06 <sup>bc</sup>	1.72 $\pm$ 0.17 <sup>ab</sup>	1.28 $\pm$ 0.13 <sup>c</sup>	1.43 $\pm$ 0.19 <sup>bc</sup>

Differences in proximate composition between experimental diets are indicated by different letters (ANOVA,  $P < 0.05$ ,  $n = 3$ ).

*Abbreviations*, WG: weight gain ( $\text{g fish}^{-1}$ ); DGI: Daily growth index; TFI: Total feed intake (g) per fish; DFC: Daily growth index (%); FCR: Feed conversion ratio; PER: Protein efficiency ratio; SGR ( $\% \text{ day}^{-1}$ ): Specific growth rate; TGC: Thermal growth coefficient. LSI: Lipidosomatic index (%); HSI: hepatosomatic index (%); PPV: Protein productive value.

1 **Table 4.** Whole body proximate composition (% in fresh weight) of meagre (*A. regius*) fed  
 2 diets containing different levels of fish meal (FM) substitution by different plant protein  
 3 sources. Data are presented as mean  $\pm$  standard deviation.

	<b>Diets</b>					
	<b>Diet 1 (FM<sub>45</sub>)</b>	<b>Diet 2 (FM<sub>30</sub>)</b>	<b>Diet 3 (FM<sub>20</sub>)</b>	<b>Diet 4 (FM<sub>20</sub>)</b>	<b>Diet 5 (FM<sub>20</sub>)</b>	<b>Diet 6 (FM<sub>18</sub>)</b>
Water	72.0 $\pm$ 0.3	72.8 $\pm$ 0.4	72.0 $\pm$ 1.0	72.4 $\pm$ 0.4	72.5 $\pm$ 0.6	72.9 $\pm$ 1.1
Protein	16.1 $\pm$ 0.4 <sup>a</sup>	16.7 $\pm$ 0.2 <sup>b</sup>	16.3 $\pm$ 0.1 <sup>ab</sup>	16.9 $\pm$ 0.1 <sup>b</sup>	16.8 $\pm$ 0.2 <sup>b</sup>	16.0 $\pm$ 0.2 <sup>a</sup>
Lipid	7.3 $\pm$ 0.4	7.1 $\pm$ 0.2	7.0 $\pm$ 0.2	6.8 $\pm$ 0.2	6.8 $\pm$ 0.3	7.3 $\pm$ 0.3
Ash	3.6 $\pm$ 0.2	3.7 $\pm$ 0.1	3.8 $\pm$ 0.1	3.9 $\pm$ 0.2	3.8 $\pm$ 0.1	3.8 $\pm$ 0.2

5  
 6 Differences in proximate composition between experimental diets are indicated by  
 7 different letters (ANOVA,  $P < 0.05$ ,  $n = 3$ ).

8

9

10 **Table 5.** Amino acid composition of the fillet of meagre (*A. regius*) fed diets containing  
 11 different levels of fish meal (FM) substitution by different plant protein sources. Data are  
 12 presented as mean  $\pm$  standard deviation.

13

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	Diets					
	Diet 1 (FM <sub>45</sub> )	Diet 2 (FM <sub>30</sub> )	Diet 3 (FM <sub>20</sub> )	Diet 4 (FM <sub>20</sub> )	Diet 5 (FM <sub>20</sub> )	Diet 6 (FM <sub>18</sub> )
HyPro	0.10 $\pm$ 0.00 <sup>a</sup>	0.07 $\pm$ 0.01 <sup>b</sup>	0.07 $\pm$ 0.00 <sup>b</sup>	0.07 $\pm$ 0.00 <sup>ab</sup>	0.07 $\pm$ 0.01 <sup>b</sup>	0.07 $\pm$ 0.01 <sup>b</sup>
His	0.42 $\pm$ 0.01	0.41 $\pm$ 0.00	0.43 $\pm$ 0.05	0.42 $\pm$ 0.01	0.46 $\pm$ 0.05	0.38 $\pm$ 0.02
Tau	0.08 $\pm$ 0.01	0.06 $\pm$ 0.01	ND	ND	ND	ND
Ser	0.84 $\pm$ 0.02	0.87 $\pm$ 0.02	0.86 $\pm$ 0.04	0.88 $\pm$ 0.05	0.90 $\pm$ 0.03	0.82 $\pm$ 0.01
Arg	1.24 $\pm$ 0.01	1.25 $\pm$ 0.02	1.26 $\pm$ 0.07	1.26 $\pm$ 0.01	1.34 $\pm$ 0.05	1.20 $\pm$ 0.02
Gly	1.19 $\pm$ 0.03	1.09 $\pm$ 0.08	1.10 $\pm$ 0.06	1.10 $\pm$ 0.03	1.10 $\pm$ 0.04	1.00 $\pm$ 0.02
Asp	2.14 $\pm$ 0.07	2.32 $\pm$ 0.06	2.21 $\pm$ 0.07	2.30 $\pm$ 0.13	2.36 $\pm$ 0.07	2.16 $\pm$ 0.08
Glu	3.33 $\pm$ 0.10	3.57 $\pm$ 0.07	3.42 $\pm$ 0.11	3.55 $\pm$ 0.16	3.65 $\pm$ 0.08	3.34 $\pm$ 0.11
Thr	0.92 $\pm$ 0.02	0.95 $\pm$ 0.01	0.94 $\pm$ 0.05	0.95 $\pm$ 0.02	1.01 $\pm$ 0.02	0.92 $\pm$ 0.01
Ala	1.22 $\pm$ 0.03	1.31 $\pm$ 0.03	1.28 $\pm$ 0.05	1.33 $\pm$ 0.05	1.35 $\pm$ 0.03	1.27 $\pm$ 0.04
Pro	0.73 $\pm$ 0.02 <sup>a</sup>	0.81 $\pm$ 0.03 <sup>ab</sup>	0.86 $\pm$ 0.05 <sup>ab</sup>	0.87 $\pm$ 0.01 <sup>ab</sup>	0.93 $\pm$ 0.03 <sup>b</sup>	0.89 $\pm$ 0.01 <sup>b</sup>
Cys	0.08 $\pm$ 0.00	0.09 $\pm$ 0.00	0.10 $\pm$ 0.01	0.09 $\pm$ 0.01	0.10 $\pm$ 0.01	0.09 $\pm$ 0.00
Lys	1.93 $\pm$ 0.06	2.06 $\pm$ 0.05	1.98 $\pm$ 0.08	2.04 $\pm$ 0.09	2.09 $\pm$ 0.05	1.90 $\pm$ 0.07
Tyr	0.68 $\pm$ 0.00	0.72 $\pm$ 0.01	0.72 $\pm$ 0.04	0.72 $\pm$ 0.01	0.76 $\pm$ 0.03	0.68 $\pm$ 0.01
Met	0.60 $\pm$ 0.01	0.63 $\pm$ 0.01	0.63 $\pm$ 0.03	0.63 $\pm$ 0.01	0.67 $\pm$ 0.02	0.60 $\pm$ 0.00
Val	0.92 $\pm$ 0.01	0.96 $\pm$ 0.01	0.96 $\pm$ 0.05	0.98 $\pm$ 0.02	1.01 $\pm$ 0.02	0.92 $\pm$ 0.01
Ile	0.84 $\pm$ 0.01	0.88 $\pm$ 0.02	0.89 $\pm$ 0.05	0.91 $\pm$ 0.01	0.93 $\pm$ 0.02	0.84 $\pm$ 0.02
Leu	1.55 $\pm$ 0.03	1.62 $\pm$ 0.03	1.62 $\pm$ 0.08	1.63 $\pm$ 0.02	1.72 $\pm$ 0.03	1.56 $\pm$ 0.02
Phe	0.76 $\pm$ 0.01	0.79 $\pm$ 0.03	0.83 $\pm$ 0.05	0.81 $\pm$ 0.01	0.84 $\pm$ 0.03	0.77 $\pm$ 0.00

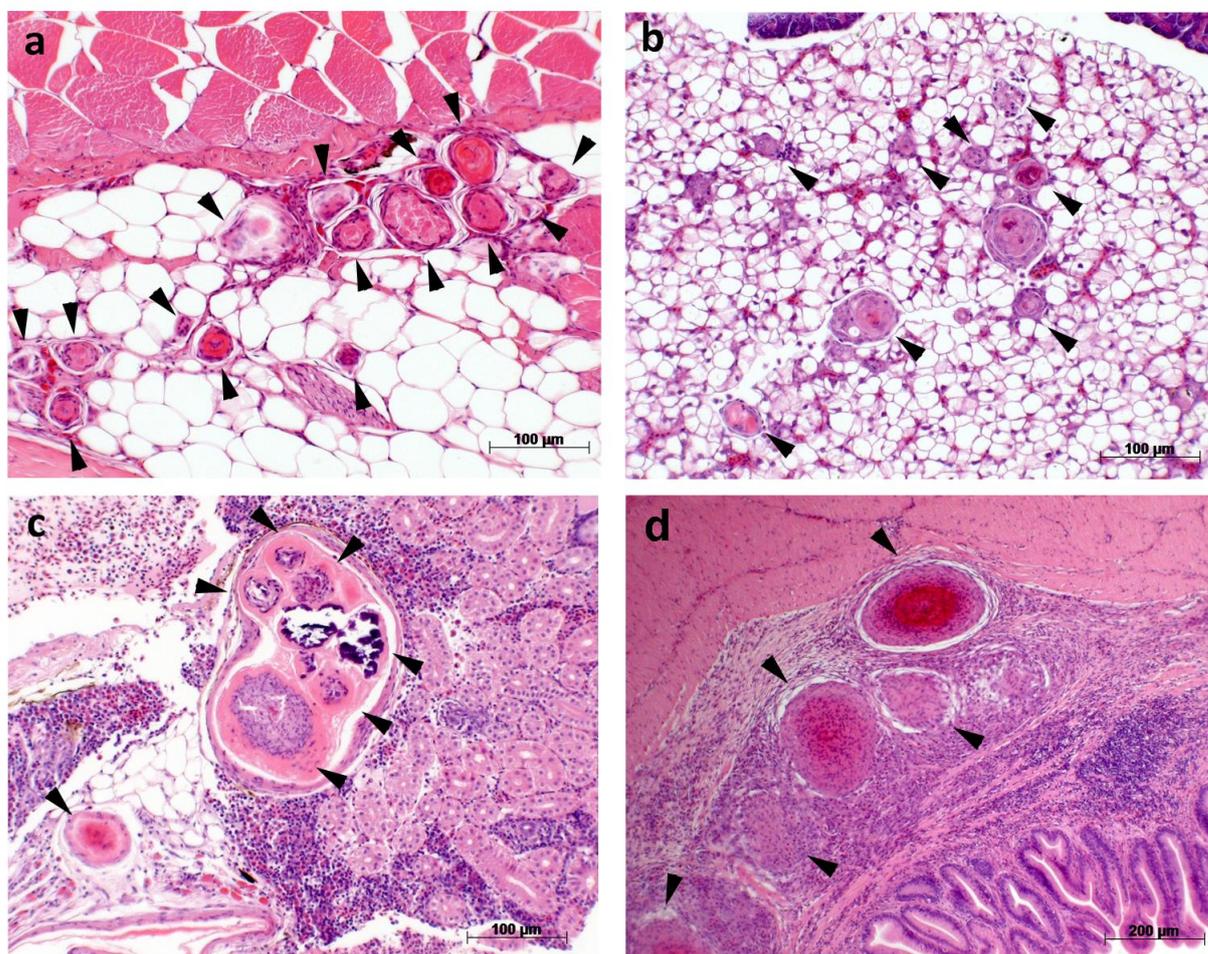
15

16 Differences in amino acid composition between experimental diets are  
 17 indicated by different letters (ANOVA,  $P < 0.05$ ,  $n = 2$ ).

18

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20 Figure 1. Histological images of different granulomas in several organs in meagre (*A.*  
21 *regius*). a, scattered small granulomas affecting the hypodermis and adjacent muscular  
22 tissue; b, small granulomas scattered along the hepatic parenchyma; c, large granuloma  
23 with calcified center in the kidney, as well as a small granuloma in the left side of the view  
24 field; d, granulomas at different stages of development in the *lamina propria* of the gut.  
25 Arrow heads indicate granulomas at different stages of development. Staining:  
26 hematoxylin-eosin (histological slides from Gram, Zhiel-Neelsen and Fite-Faraco stainings  
27 not shown).



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