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

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Abstract

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

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

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

1 INTRODUCTION

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

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

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

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

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

2 MATERIAL AND METHODS

2.1 Fish and rearing conditions

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

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

2.2 Experimental diets

Six isonitrogenous (480 g/kg) and isoenergetic (22 MJ/kg) commercial extruded diets (pellet size = 2.0-2.5 mm) with different FM levels and graded levels of PP blends were formulated to feed grow-out meagre as shown in Table 1. A basal diet (Diet FM45) containing 450 g/kg FM inclusion and 37.7 g/kg lysine was used as the control diet. Five of the experimental diets, FM30, FM20a, FM20b, FM20c and FM20d, were formulated in a way to contain low levels of FM inclusion (300 g/kg, and 200 g/kg, respectively) and decreasing levels of lysine (37.4, 37.0, 28.5, 24.8 and 20.9 g/kg feed, respectively). Since lysine requirements have not yet been established for meagre, a wide range of lysine intakes was used in order to formulate diets theoretically deficient (20.9 g/kg), adequate (24.8-28.5 g/kg) or excess (37.0-37.4 g/kg) in lysine containing different fishmeal inclusion levels (20,30,45%). In these diets, FM was substituted by a mixture of PP blends including mainly soybean, corn gluten and wheat gluten, while graded levels of lysine 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 ºC 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 ºC for
171 further amino acid analyses, and their internal organs (hepatopancreas, intestine, muscle,

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

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

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

Daily growth index (DGI; %) = $(BW_f\ 1/3 – Bw_i\ 1/3) / days \times 100$

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

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

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

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

Protein productive value (PPV) = $(P_f – P_i) / (P_d \times TFI)$,

where BW_i and BW_f are the initial and final body weights, ΣD_0 is the thermal sum (feeding days × average temperature, ºC), P_f and P_i are the initial and final protein levels of fish, and P_d is the protein concentration of the feed on a dry basis, respectively.

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 × 6.25), crude fat using a Soxtec™ 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 ºC, 24 h), and calculation by AccQ-Tag™ 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 × 2.1 mm i.d., 1.7μm) from Waters. The flow rate was 0.7 ml min^{-1} and
211 column temperature was kept at 55 ºC. 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 ±

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

3 RESULTS

3.1 Growth performance and feed utilization

Results from the GLM ANOVA revealed high R-squared values (0.81-0.96), which indicated that the applied statistical model fit very well the data, explaining most of the variability in each of the analyzed growth parameters (Table 3). Moreover, the partial η^2 indicated that FM inclusion levels had a higher relative impact on growth parameters than lysine levels, except for the PER. Scatter plots of 12 growth parameters against lysine 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*

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

3.3 Histological analyses

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

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

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

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

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

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

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

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

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

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

4 DISCUSSION

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

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

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

On the contrary, our results were different to those reported by Ribeiro et al. (2015) in meagre and Minjarez-Osorio et al. (2016) in two other sciaenid carnivorous species. In 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 ºC) 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 ºC vs 19 ºC, 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

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

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

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716

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

Ingredient (g/kg of diet)	**Diet 1 (FM_{45})**	**Diet 2 (FM_{30})**	**Diet 3 (FM_{20})**	**Diet 4 (FM_{20})**	**Diet 5 (FM_{20})**	**Diet 6 (FM_{18})**
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
Total	1000	1000	1000	1000	1000	1000
Determined proximate composition (%)						
Dry matter	93.4 ± 0.1	92.9 ± 0.1	92.5 ± 0.2	92.1 ± 0.1	91.2 ± 0.0	91.6 ± 0.10
Crude protein	47.9 ± 0.1	47.8 ± 0.2	47.4 ± 0.3	47.5 ± 0.6	47.6 ± 0.1	47.4 ± 0.4
Crude fat	17.3 ± 0.1	16.9 ± 0.0	16.8 ± 0.1	16.7 ± 0.1	17.0 ± 0.2	16.7 ± 0.1
Ash	8.8 ± 0.1	7.7 ± 0.1	6.6 ± 0.0	6.5 ± 0.1	6.4 ± 0.1	6.1 ± 0.0
Gross energy (MJ/kg)	21.6 ± 0.1	21.7 ± 0.1	21.8 ± 0.2	21.9 ± 0.1	21.9 ± 0.2	21.9 ± 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

*Theoretical values

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

726
727

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

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

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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 ± standard deviation.

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

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

Abbreviations, WG: weight gain (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 (% day^{-1}): Specific growth rate; TGC: Thermal growth coefficient. LSI: Lipidosomatic index (%); HSI: hepatosomatic index (%); PPV: Protein productive value.

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

	Diets					
	Diet 1 (FM_{45})	**Diet 2 (FM_{30})**	**Diet 3 (FM_{20})**	**Diet 4 (FM_{20})**	**Diet 5 (FM_{20})**	**Diet 6 (FM_{18})**
Water	72.0 ± 0.3	72.8 ± 0.4	72.0 ± 1.0	72.4 ± 0.4	72.5 ± 0.6	72.9 ± 1.1
Protein	16.1 ± 0.4^{a}	16.7 ± 0.2^{b}	16.3 ± 0.1^{ab}	16.9 ± 0.1^{b}	16.8 ± 0.2^{b}	16.0 ± 0.2^{a}
Lipid	7.3 ± 0.4	7.1 ± 0.2	7.0 ± 0.2	6.8 ± 0.2	6.8 ± 0.3	7.3 ± 0.3
Ash	3.6 ± 0.2	3.7 ± 0.1	3.8 ± 0.1	3.9 ± 0.2	3.8 ± 0.1	3.8 ± 0.2

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

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

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

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

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