Deliverable Report

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Deliverable Title	The effect of high plant protein diets in the development of Systemic Granulomatosis in meagre			
WP No:	24		**WP Lead beneficiary:**	P1. HCMR
WP Title:	Fish health - meagre			
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Task Title:	Subtask 24.1.1 Feeding trials. Trial 3			
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Objective: The objective of this task is to investigate the possible causes of systemic granulomatosis (SG). This report refers to the effect of high plant protein diets in the development of Systemic Granulomatosis in meagre (trial 3).

Introduction

One of the most important bottlenecks of meagre (*Argyrosomus regius*) production is Systemic Granulomatosis (SG), a pathological condition affecting the majority of farmed populations. SG is characterized by multiple granulomas in all soft tissues, which progressively become calcified and necrotic. The aetiology of the disease is unknown, however it is suspected that it may be a metabolic disorder (Katharios et al. 2011) similar to systemic granulomas observed in other cultured fish species such as the gilthead sea bream (*Sparus aurata*), the rainbow trout (*Oncorhynchus mykiss*) and the turbot (*Scophthalmus maximus*). In all cases, the development of the disease has been associated with nutritional imbalance in minerals and vitamins or inadequacy due to the use of plant protein (PP) sources or long-term stored formulated feeds, fish meal (FM), or frozen fish (Herman, 1996). One of the objectives of Work Package 24 – Fish health –meagre (WP24) is to identify the causes of SG via several feeding trials. On the first trial we concluded that vitamin D_3 has no effect on the development of SG (D24.1), while the second trial showed that the high phosphorus (P) content in the diets (15 g kg^{-1}) ameliorated the severity of granulomatosis (D24.2). The purpose of this third trial was to examine whether FM replacement by PP sources affects the development of SG. Furthermore, due to the results obtained in D24.2 we also investigated whether P supplementation in PP diets has any effect on SG.

Fishmeal from wild harvested fish species such as anchovies, sardines and mackerel has been used as the main protein source in aquafeeds because of its wide availability and digestibility, high protein content and high levels of most essential amino acids and minerals (Gatlin et al. 2007; NRC 2011; Olsen and Hasan 2012; Tacon, Hasan, and Metian 2011). However, as aquaculture production increases rapidly and because of the limited amount of available FM in the market, its lack of sustainability and its increasing price, many efforts are being made to reduce its inclusion in the diets used (Apper-Bossard et al. 2013; NRC 2011; Tacon and Metian 2008). Alternative replacements of FM protein could be products from land animals, single cell proteins, and PP sources (NRC 2011). According to Gatlin et al. (2007) FM alternatives must have certain characteristics including wide availability, competitive price, easy handling, shipping and storage, low levels of non-soluble carbohydrates and anti-nutrients, high protein content, good amino acid profile, high digestibility and palatability. Plant proteins represent an interesting candidate to replace FM in the diets. Among plant ingredients, vital wheat gluten and soy protein concentrate are the primary alternatives to FM because of their availability and their low prices. However, PP based diets, especially when intended for carnivorous species, have several nutritional disadvantages compared to FM, such as the relatively low protein content and the presence of anti-nutritional components that may reduce the digestion or the absorption of the nutrients, the function of vitamins or may even be toxic for the fish (Francis, Makkar, and Becker 2001; Gatlin et al. 2007; Olsen and Hasan 2012). Therefore, it seems that for the successful production of aquafeeds without FM, a combination of plant-derived feed ingredients will be required with supplementation of amino acids, flavourings and possibly exogenous enzymes (Gatlin et al. 2007). During the last decades, several studies have been made in order to investigate the effect of partial or total replacement of FM by PP sources among various marine fish species such as red drum (*Sciaenops ocellatus*) (Minjarez-Osorio et al. 2016; Rossi, Tomasso, and Gatlin 2015), turbot (Imsland et al. 2016; Sevgili et al. 2015), Senegalese sole (*Solea senegalensis*) (Cabral et al. 2013), Atlantic cod (*Gadus morhua*) (Olsen et al. 2007), Atlantic salmon (*Salmo salar*) (Espe, El-Mowafi, and Ruohonen 2012), red sea bream (*Pagrus major*) (Kader et al. 2012), sharpsnout seabream (*Diplodus putazzo*) (Hernández et al. 2007), European seabass (*Dicentratrchus labrax*) (Kaushik et al. 2004; Messina et al. 2013; Tibaldi et al. 2003) and gilthead sea bream (Dias et al. 2009; Francesco et al. 2007; Gomez-Requeni et al. 2004; Robaina et al. 1995; Sitjà-Bobadilla et al. 2005). Concerning meagre, few data exist on the effect of FM replacement by PP in the diets but it seems that meagre showed a good ability to accept plant based diets. Estévez et al. (2011) found that a reduction of FM to 20% of the formulation did not affect growth or feed utilization of meagre juveniles. Also, Velazco-Vargas et al. (2013) showed that 30% soybean inclusion could be an excellent plant meal to substitute 25% of FM as no effects on growth and feed efficiency parameters were detected, while Ribeiro et al. (2014) concluded that fish fed diets with high incorporation levels of PP sources showed a similar growth performance in comparison to FM based diets.

Materials and methods

Experimental diets

Four experimental diets were formulated at the SKRETTING Aquaculture Research Centre (SARC). The first diet was based on 60% FM and the other three on 14% FM and increasing levels of P in the diets with 14% FM. Fishmeal replacement in the diet with 14% FM was done with PP derived from corn gluten, wheat gluten and soya concentrate. PP+High P diet has equal concentration of P with the FM diet, PP diet was not supplemented with P, while PP+Medium was supplemented to be between the PP+High P and PP diets. All the diets were formulated to contain about 53% crude protein and 16% crude lipid (**Table 1**).

Table 1. Formulation and chemical analysis of the experimental diets (% dry weight).

Formulation

	FM	PP	PP+Medium P	PP+High P
Wheat	16.98	12.08	10.19	8.39
Corn gluten	5.00	10.00	10.00	10.00
Wheat gluten	5.00	26.28	26.61	26.94
Soya concentrate	5.00	24.70	24.70	24.70
Fish meal	60.18	14.00	14.00	14.00
Fish oil	7.48	10.94	10.99	11.03
DL-Methionine	0.00	0.34	0.33	0.32
L-Lysine	0.00	1.51	1.48	1.46

Phosphate	0.35	0.13	1.69	3.24
Premix min vit	0.01	0.01	0.01	0.01
Chemical analysis				
Dry matter	93.00	93.00	93.00	93.00
Moisture	7.00	7.00	7.00	7.00
Protein	53.50	53.50	53.50	53.50
Lipid	16.00	16.00	16.00	16.00
Ash	9.04	4.09	5.57	7.04
Starch	11.23	9.68	8.50	7.33
Phosphorus (gkg^{-1})	14.32	5.46	9.75	14.00

Experimental fish and feeding trial

Meagre juveniles of 2 g average weight, produced in June 2016 at the facilities of the Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Crete, Greece were used for the feeding trial. In total, 600 fish were weighed and placed into 12 500-l cylindrical tanks at a density of 50 fish per tank. Three replicates were allocated to each diet. The feeding trial lasted 3 months (August-November 2016).

Tanks were supplied with borehole water (salinity 35‰, pH 7.5) and all had similar light conditions and temperature (20°C). Air stones in each tank provided aeration and nets were placed over the tanks in order to prevent fish escape. The fish were hand-fed to visual satiety twice a day for 7 days a week, while feed intake was recoded daily.

Sampling and analytical methods

Before sampling, the fish were starved for 24 hours to reduce handling stress and allow digestion and tract evacuation. Fish were then anaesthetized with 2-phenoxyethanol and weighed.

Every month, 3-4 fish from each tank (10/treatment) were sampled for granulomas evaluation and histology.

The following indices were calculated:

Specific Growth Rate (SGR)	$SGR\% = 100\ x\ \frac{\ln\,(Final\ BW - Initial\ BW)}{\Delta T}$
Feed conversion ratio (FCR)	$FCR = \frac{feed\ intake\ (g)}{wet\ weight\ gained\ (g)}$

BW: body weight, ΔT: (Time, days)

Visual evaluation of granulomatosis

To assess fish status regarding the presence of granulomas, a semi-quantitative ordinal-scale scoring system was developed based on stepwise evaluation of the severity of the lesions in the internal organs of the examined individuals. Each fish was dissected and internal organs were examined macroscopically. Fresh squash preparation of heart, liver, intestine, spleen, swim bladder, peritoneum and kidney were assessed under a stereoscope. For the general state of each individual, the sum of the scores from the various tissues was calculated.

The assessment scale used was according to the following scoring system:

Score 0	No granulomas

Score 1	Granulomas visible only with microscopy
Score 2	Granulomas visible macroscopically
Score 5	Tissue calcification

Histology

Samples of heart, liver, intestine, spleen and kidney were fixed in 4% formaldehyde: 1% glutaraldehyde. Subsequently they were dehydrated in gradually increased ethanol solutions (70-96%) and then embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer). Sections of 4 μm were obtained with a microtome (RM 2035, Leica, Germany). After drying, slides were stained with methylene blue/azure II/basic fuchsin according to Bennett et al. (1976) and examined under a light microscope.

Plasma analysis

Blood samples were collected from the caudal vasculature into heparinized syringes. After centrifugation for 15 min at 6000 r.p.m., the plasma was removed and stored at −80°C for further analysis. Specifically, alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), calcium (Ca), P, magnesium (Mg), glucose (Glu) and total proteins (TP) were assessed using commercially available kits (Biosis, Greece). Analyses were performed at the Department of Biology, University of Crete.

Statistical analysis

One-Way Analysis of Variance (ANOVA) was performed to test any differences in growth and blood biochemical parameters among the four experimental diets followed by Tukey's post-hoc test in cases where significant differences were found with ANOVA ($p<0.05$). Kruskal-Wallis test was performed to compare the severity of granulomatosis in the various organs among groups as well as the overall condition of the fish. Statistical analyses were made using IBM SPSS Statistics 20 software.

Results

Growth performance

Juvenile meagre grew from ~2 to ~40 g over the course of the feeding trial. FM replacement by PP sources affected negatively fish growth, while P supplementation in the PP diets had a positive effect on growth. Specifically, fish fed the FM and PP+High P diets exhibit significant higher body weight than fish fed the PP+Medium P and PP diets. Furthermore, the length of the fish fed the FM was significant higher than that of the fish fed the PP diet (**Fig. 1, 2**). SGR was significantly higher in fish fed FM and PP+High P diets compared with those fed PP diet, while FCR was lower in FM group but no significant differences were observed among the diets (**Fig. 3**).

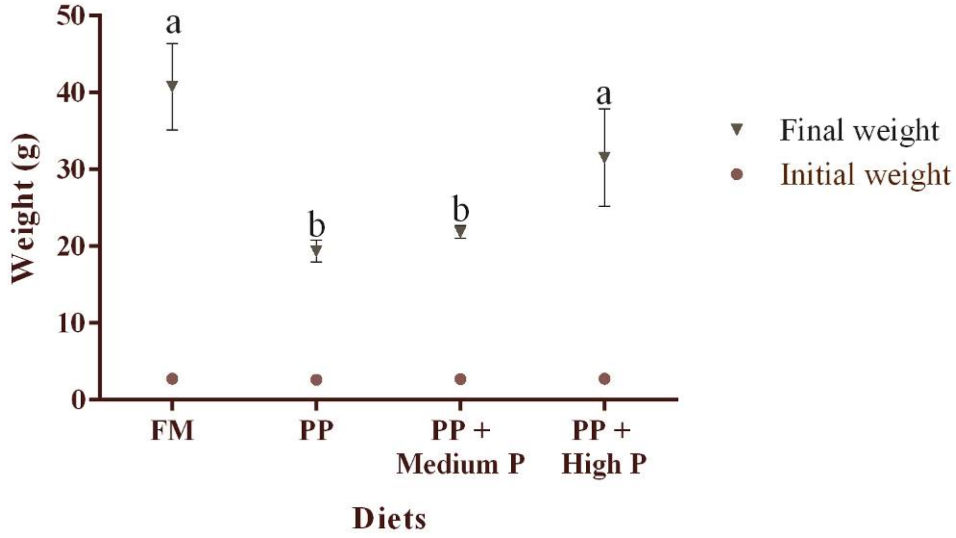

Figure 1. Average weights (g) of meagre fed diets with plant proteins and increasing levels of P in the beginning of the experiment and after 3 months. Values are means ± SD. Different letters indicate the statistically significant differences between the diets in each time point ($p<0.05$).

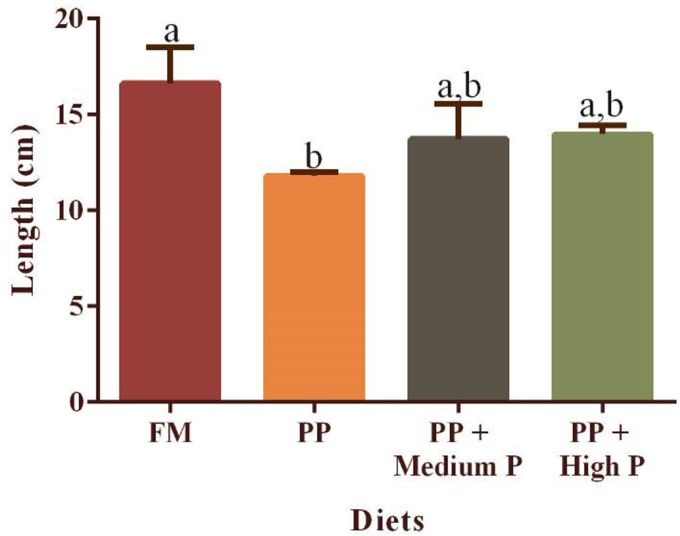

Figure 2. Average standard length (cm) of meagre fed diets with plant proteins and increasing levels of P at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets ($p<0.05$).

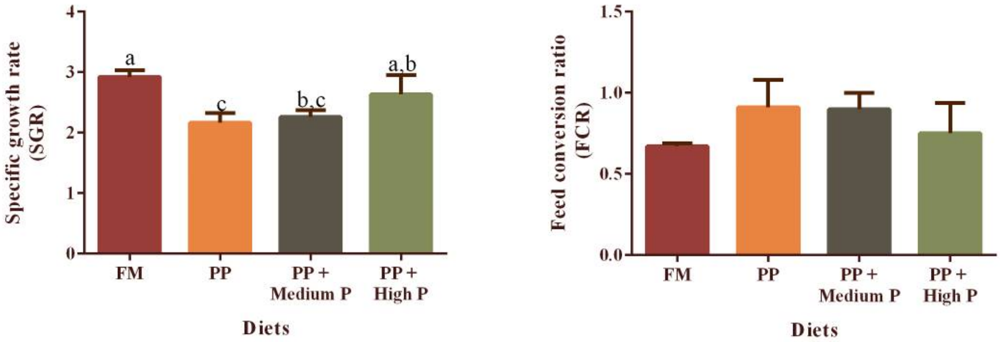

Figure 3. Specific growth rate (SGR) and feed conversion ratio (FCR) of meagre fed diets with plant proteins and increasing levels of P at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets ($p<0.05$).

Evaluation of granulomas

Fish did not exhibit granulomas in the tissues studied at the beginning of the feeding trial. After 3 months of feeding, granulomas were observed in all groups of fish. **Fig. 4** shows the boxplots of total scores of granulomas for the different diets at the end of the experiment. Plant proteins were found to negatively affect SG. Specifically, the median of the FM group was significant lower compared to all PP diets, which was confirmed by Kruskal–Wallis test ($H(3)=41.455$, $p=0.000$).

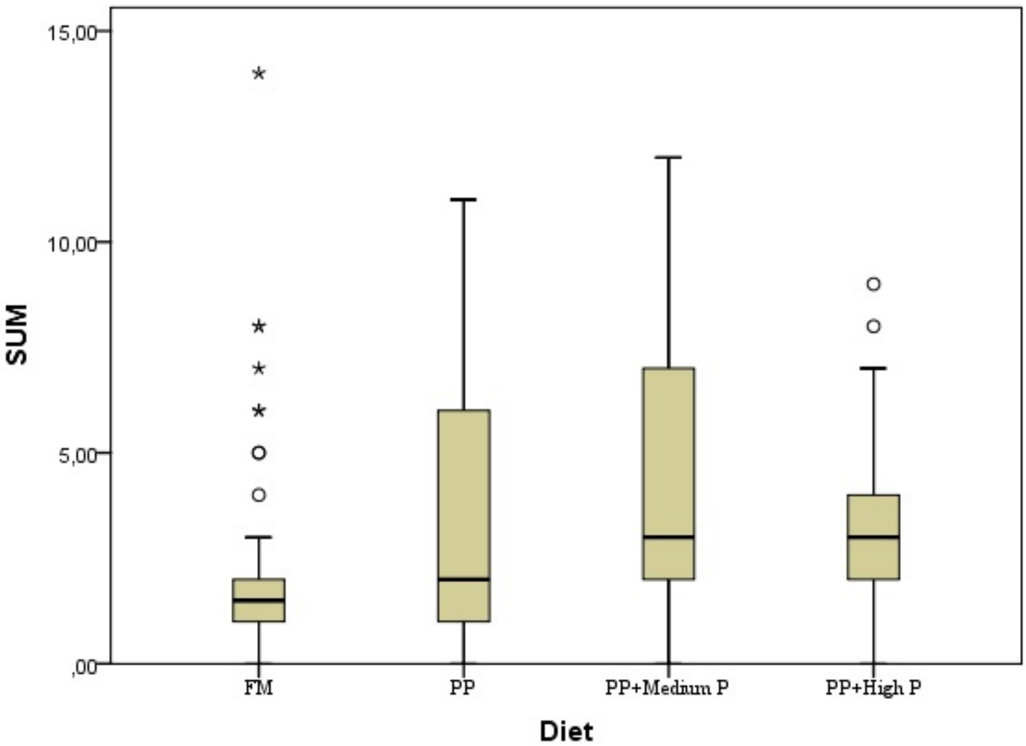

Figure 4. Boxplots of meagre's total score of granulomas at the end of the experiment fed diets with plant proteins and increasing levels of P at the end of the feeding trial. Outliers are presented as circles and extreme scores as asterisks. The medians of the groups are significantly different ($H(3)=41.455$, $p=0.000$).

The organs that appeared to be mostly affected by granulomatosis were the kidney and the liver followed by the spleen. Kidneys and livers from the fish of all diets presented all levels of granulomatosis severity: no granulomas, granulomas visible only with microscopy, granulomas visible macroscopically and tissue calcification. **Fig. 5** shows the percentage of fish in each of the 4 categories of the granulomas scoring system at the end of the experiment for every tissue examined. Statistically significant differences exist between the diets and the development of granulomatosis for the liver (p=0.000), the intestine (p=0.000) and the spleen (p=0.000). For the liver and the spleen, after the pairwise comparison, we found that the fish fed the FM diet had more organs with no granulomas and less with calcification compared to fish fed the PP, PP+Medium P and PP+High P diets. For the intestine, statistically significant differences were found between the fish fed the FM, PP and PP+High P diets compared to those fed the PP+Medium P diet. No granulomas were observed in the peritoneum and the swim bladder in any of the fish examined irrespective of the diet.

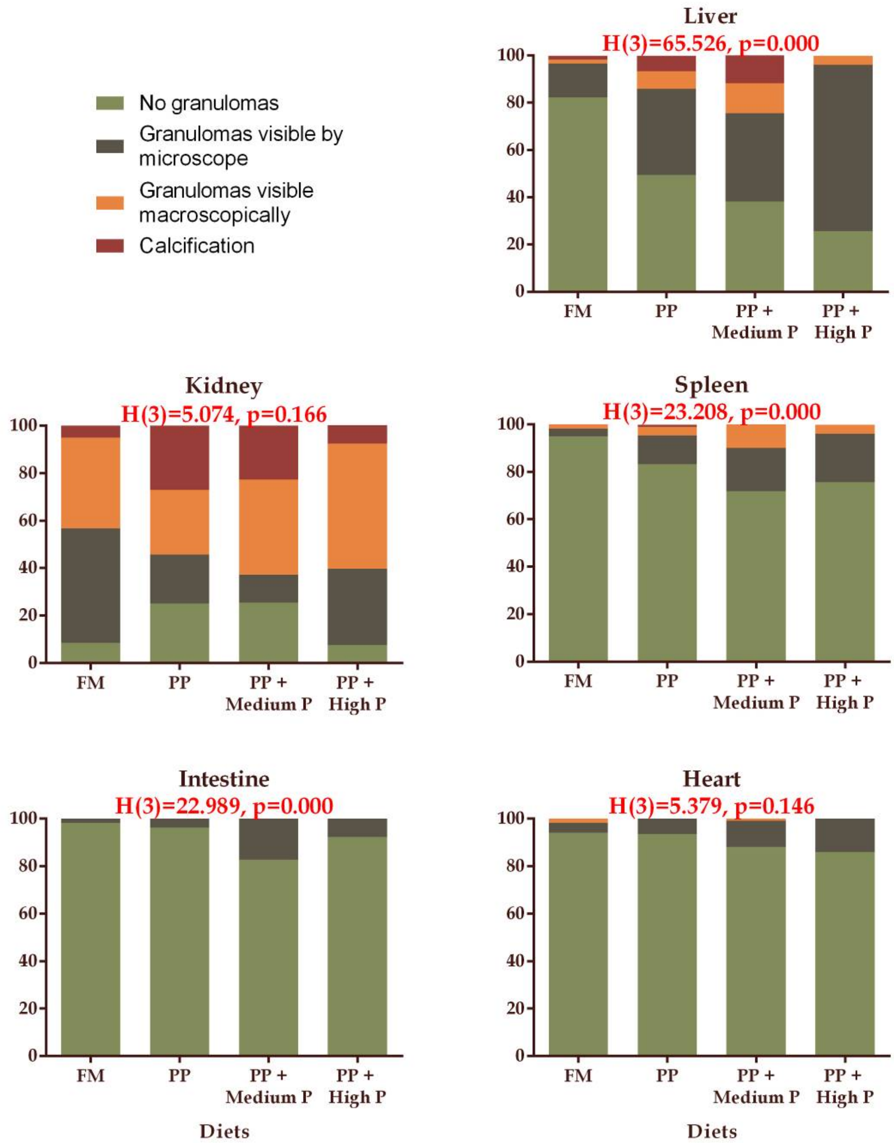

Figure 5. Percentage of the fish fed diets with plant proteins and increasing levels of P in each of the 4 categories of the granulomas scoring system (no granuloma, granulomas visible only with microscopy, granulomas visible macroscopically and tissue calcification) for every tissue examined. Kruskal-Wallis test results are indicated with red letters.

Histology

The fish of all different experimental groups had similar lesions concerning SG and the histological assessment was in accordance to the visual examination of the fresh preparations. The overall pathology was not different to that described by Katharios et al. (2011). Several stages of the granuloma formation with the characteristic epithelioid cells could be identified in the examined tissues ranging from immature granulomas, multilayer mature granulomas to big nodules, possibly a result of the merging of several adjacent granulomas that had big areas of central necrosis with dystrophic calcification **(Fig. 6).** The kidney and liver were the organs mainly affected although granulomas were also found in heart and spleen. The livers of the fish of all the different experimental groups are characterized by lipoid degeneration. Rodlet cells were present in large numbers in all tissues aligned like epithelial cells in the peritoneal membranes and surrounding blood vessel walls **(Fig. 7).** Furthermore, the intestines of fish fed the diets with 14% FM exhibited higher incidence of vacuoles in the intestinal villi compared to those fed the diet with 60% FM **(Fig. 8).**

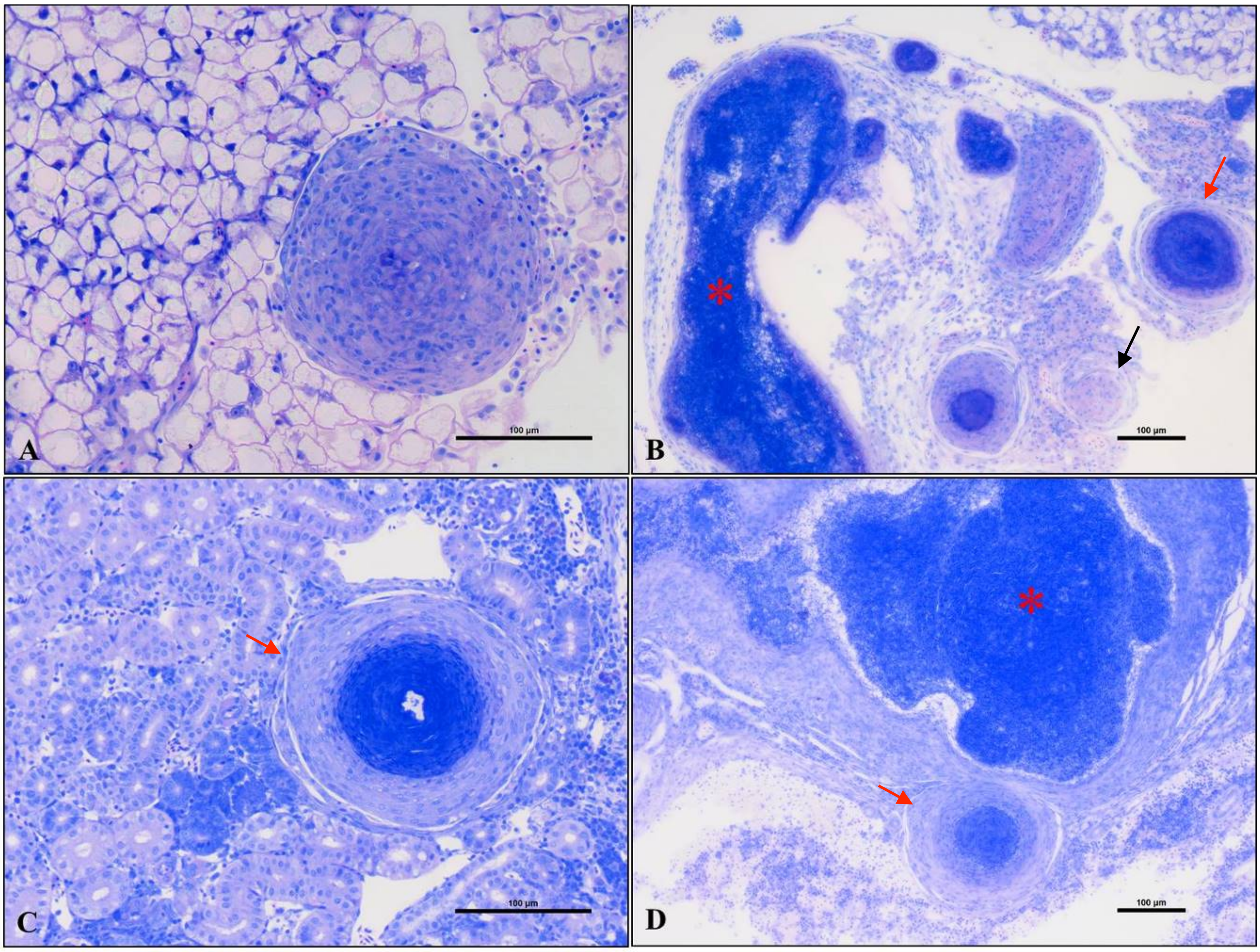

Figure 6. Immature granuloma in the liver of meagre. Lipoid degeneration is evident (A). Immature granuloma (black arrow), mature granulomas (red arrows) and areas with dystrophic calcification (*) in the liver (B) and the kidney of meagre (C, D).

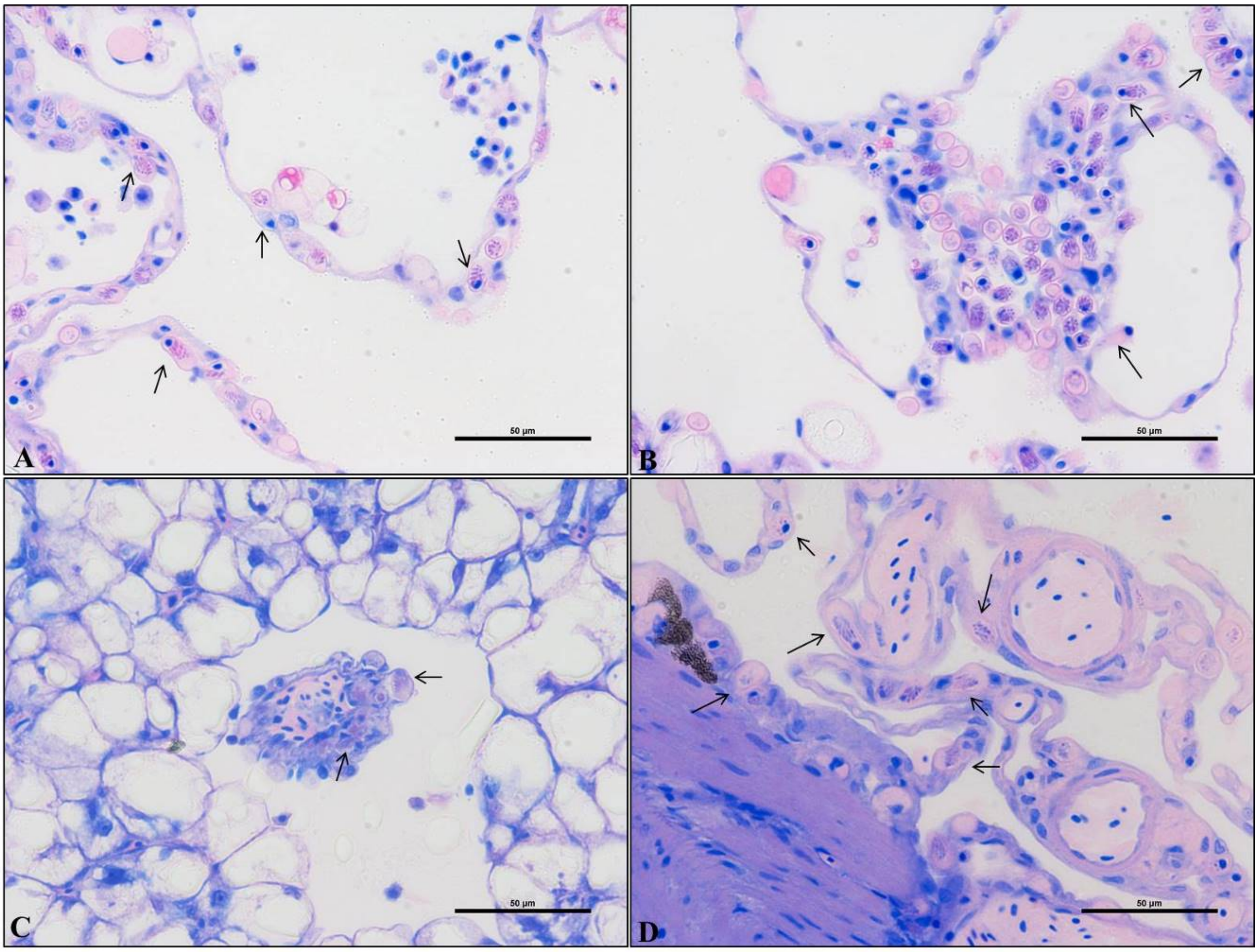

Figure 7. Rodlet cells (black arrows) aligned in the peritoneal membranes (A, B) and surrounding blood vessel walls (C,D)

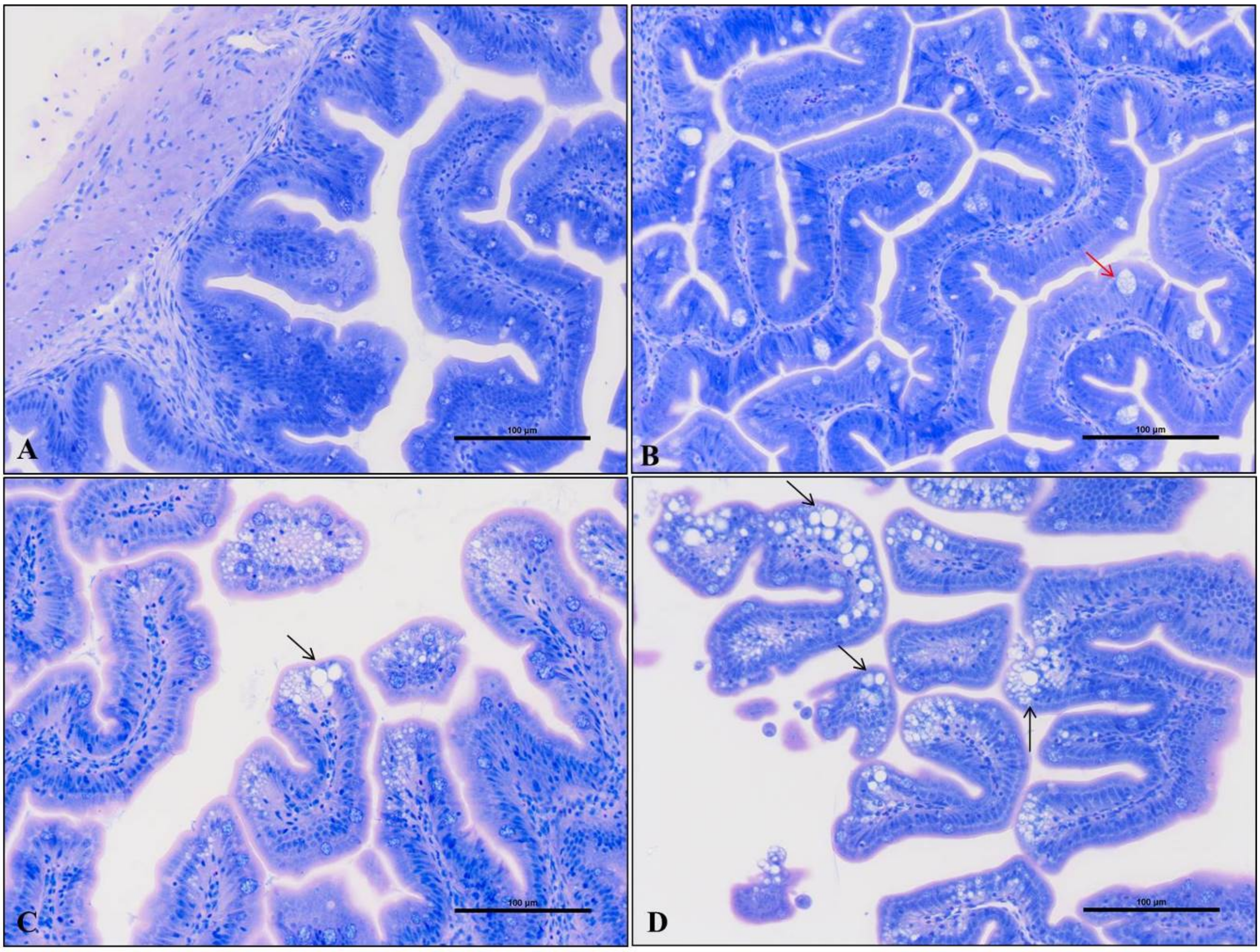

Figure 8. Histological sections of intestines of meagre. Normal appearance of intestines of meagre fed the diet with 60% fishmeal (A, B). Lipidic vacuolization in intestinal villi of meagre fed the diets with 14% fishmeal (black arrows C, D).

Plasma analysis

Fishmeal replacement by PP sources affected only the enzyme activity of ALP. Fish fed the PP diet exhibit the highest levels of ALP followed by fish fed the PP+Medium P, FM and PP+High P diets. No significant differences were observed for ALT and AST enzyme activities among the different diets **(Fig. 9)**. Concerning the other plasma parameters, fish of the FM group had significantly higher levels of P followed by the group of PP+High P, PP+Medium P and PP group. Furthermore, fish fed the FM diet had significantly higher levels of plasma Ca compared to those fed the PP diet. Plasma glucose and total protein concentrations did not differ among the different diet groups **(Fig. 10).**
On the other hand, when the values were expressed by ranks of the sum of the granulomas score, no statistical significant differences were observed neither on the enzymatic activities nor on the other plasma metabolites **(Fig. 11, 12)**.

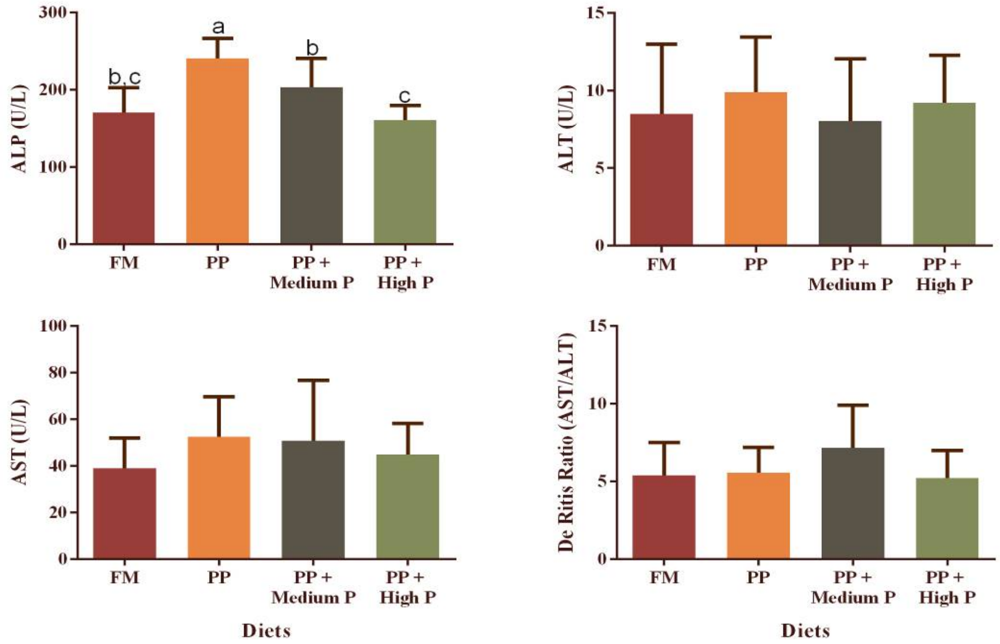

Figure 9. Plasma enzyme activities of meagre fed diets with plant proteins and increasing levels of P at the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets ($p<0.05$).

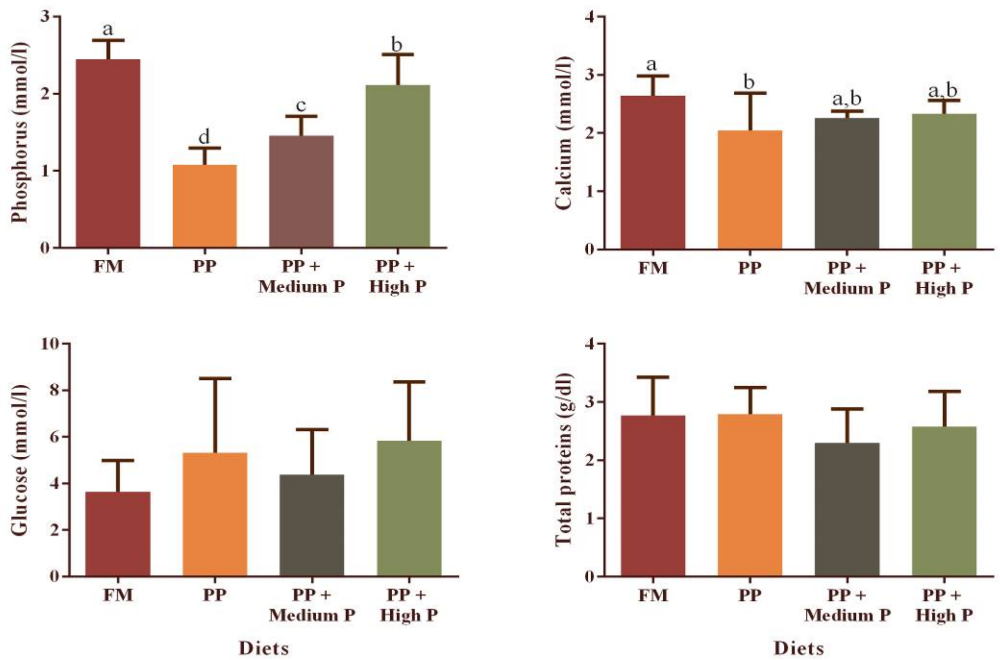

Figure 10. Plasma metabolites of meagre fed diets with plant proteins and increasing levels of P in the end of the feeding trial. Values are means ± SD. Different letters indicate the statistically significant differences between the diets ($p<0.05$).

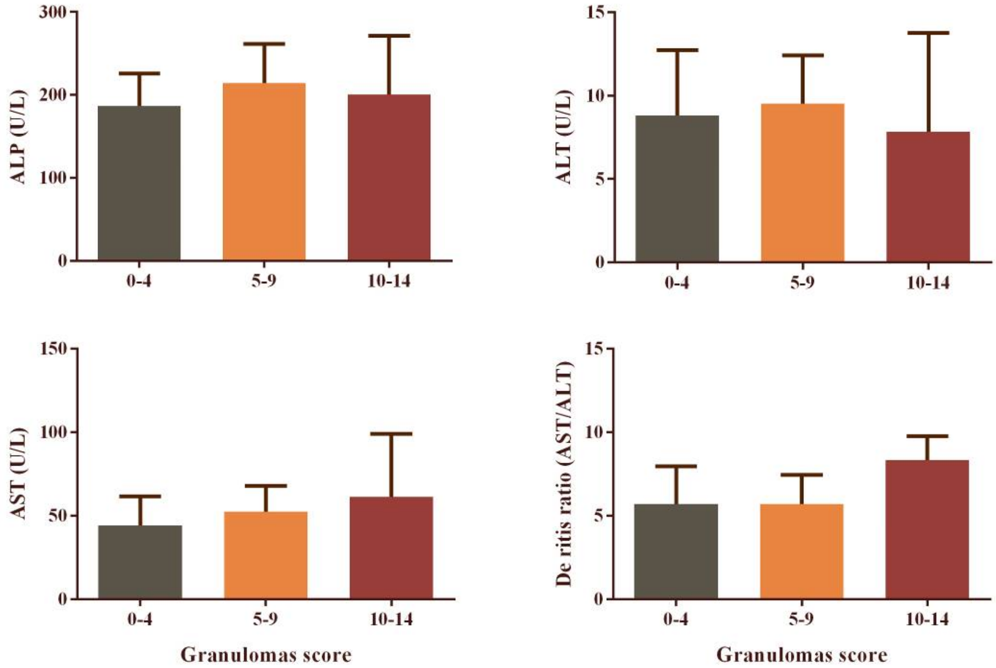

Figure 11. Plasma enzyme activities of meagre by rank of granulomas sum score (0-4: with no tissue calcification, 5-9: with calcification in one tissue, 10-14: with calcification in two tissues) at the end of the feeding trial. Values are means ± SD.

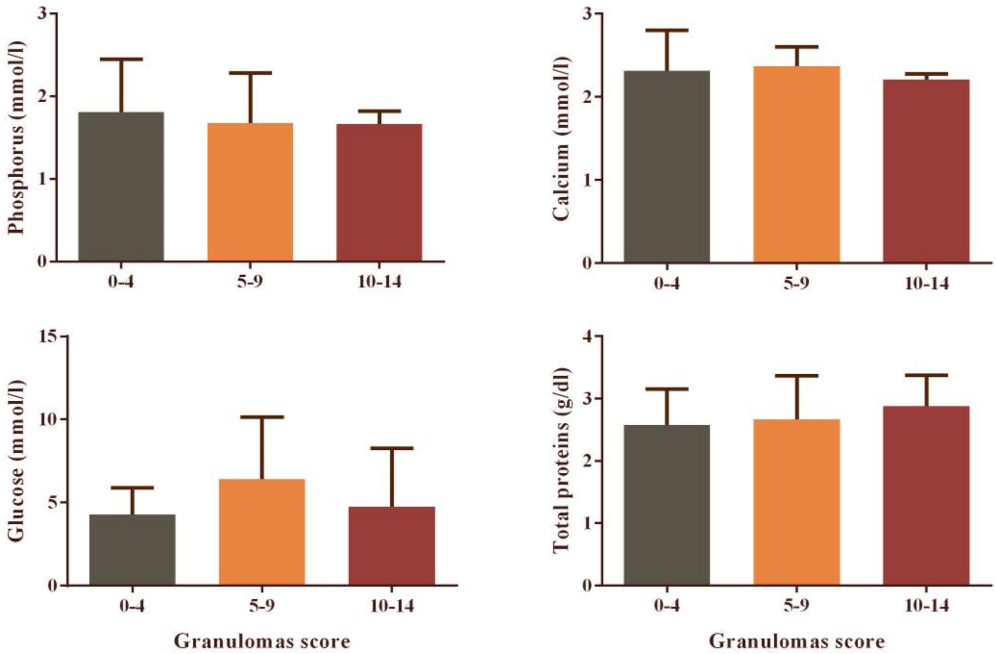

Figure 12. Plasma metabolites of meagre by rank of granulomas sum score (0-4: with no tissue calcification, 5-9: with calcification in one tissue, 10-14: with calcification in two tissues) at the end of the feeding trial. Values are means ± SD.

Discussion

The present trial showed that PP in the diets of meagre negatively affects SG. Fish fed 60% FM were in a significantly better state regarding the total score of granulomas in all tissues. Furthermore, fish of this diet group exhibited a significantly lower percentage of liver and spleen calcification and there was a significantly higher percentage of fish with no granulomas in these organs compared to those fed the PP, PP+Medium P and PP+High P diets. Phosphorus supplementation in the PP diets did not affect the overall condition of the fish (as assessed by the granulomas scoring system) but had a positive effect in the liver of the fish. Specifically, fish fed the PP+High P diet exhibited lower percentage of liver calcification and liver with macroscopically visible granulomas compared to those fed the PP and PP+Medium P diets. Despite the fact that the fish of the FM group were in a better state, they also exhibited granulomas in all examined tissues. Some of plant derived ingredients such as wheat and wheat gluten are used as pellet binders in extruded feeds (Apper-Bossard et al. 2013). Thus, FM based diets also contained small amounts of those, which may be responsible for the appearance of granulomas in meagre.

Fishmeal reduction from 60% to 14% affected the weight of fish while P supplementation positively influenced the development of fish. Fish fed FM diet and PP+High P showed similar growth performance. Plant protein meals contain less P than FM. Phytic acid is the storage form of P in plant ingredients and is poorly available to fish, thus supplementation with inorganic P may be necessary to maintain body P requirements (Hardy 2010; Kaushik et al. 2004). Phosphorus is an essential mineral for fish as a constituent of bones and scales but it also serves as a buffer to maintain optimal pH in body fluids and has an integral role in cellular fraction, as it is a key component of nucleic acids, phospholipids, phosphoproteins, ATP and several key enzymes (Coote et al. 1996; Lall 2002). Phosphorus deficiency results in reduced growth, decreased feed efficiency, reduced bone mineralization and skeletal abnormalities (Lall 2002; Lall and Lewis-McCrea 2007). In summary, the results obtained in this trial showed that a reduction of FM from 60% to 14% is possible for juvenile meagre in terms of growth performance, only in combination with high levels of P supplementation (14 g kg^{-1}). Other studies on meagre showed that a reduction of FM to 20% of the formulation did not affect growth or feed utilization of juveniles. However, further reduction of FM to 10% affects the growth performance, but the inclusion of fish protein hydrolysate in those diets may reverse this effect (Estévez et al. 2011). Furthermore, Velazco-Vargas et al. (2013) suggested that 30% inclusion of soybean meal could be an excellent plant meal in the diets of meagre while Ribeiro et al. (2014) found that a 50% replacement of marine proteins by PP sources has no effect on the growth performance of meagre. Concerning other fish species, replacing up to 70% FM protein by PP sources in diets did not affect the growth performance of European seabass (Dias et al. 2009; Francesco et al. 2007; Messina et al. 2013) but total replacement of FM does not seem feasible (Gomez-Requeni et al. 2004; Sitjà-Bobadilla et al. 2005). Seabass can also accept diets with partial or almost total replacement of FM by PP without adverse effects on growth performance (Kaushik et al. 2004; Tibaldi et al. 2003). In general, FM replacement by PP can be successfully used in diets for marine fish species, provided that essential amino acid requirements are met, the palatability of the feed is guaranteed and the levels of anti-nutritional factors are low (Dias et al. 2009; Gatlin et al. 2007).

Histological assessment confirmed the results of the visual inspection performed in fresh preparations. There was a clear chronic inflammation manifested with the granulomatous lesions. In this trial, as it also happened in the previous two, the failure of demonstrating infectious agents in histological sections enforces the hypothesis that SG is a metabolic disorder and not of an infectious agent. As we described also in D24.1 and D24.2 another interesting finding is the involvement of the enigmatic rodlet cells in SG. The distinctive characteristics of these pear-shaped cells are the collection of the rodlets (linear crystal structures) within their cytoplasm and the thick surrounding membrane. Under specific conditions rodlet cells expel their rodlets into the extracellular environment (Depasquale 2014). It has been shown that rodlets contain the antimicrobial peptide piscidin (Silphaduang et al., 2006). Thus, their secretory nature might be connected to the defense mechanism of meagre against infection. In this trial, we also observed that following rodlet expulsion, empty cells with intensely eosinophilic stained membranes, remain in the area and fuse together. However, the role of rodlet cells in the disease cannot be elucidated since there is no data on the presence of these cells in normal or wild specimens. An interesting histological finding, which is not directly linked to SG is that high PP diets were found to induce excessive vacuolization in the intestinal villi of meagre. However, such accumulation did not cause damage to the intestine epithelium, with no signs of necrosis or degeneration. Lipid vacuoles in the intestine are considered as temporary storage site of lipids when the rate of lipid absorption exceeds the rate of lipoprotein synthesis (Escaffre et al., 2007; Rodrigues et al., 2010). In addition, lipid accumulation in enterocyte regions is considered a result of reduced re-acylation and lowered rate of lipid synthesis from lipids of plant origin (Ostaszewska et al., 2005). In accordance to our results, lipid accumulation in the enterocytes was found also in other fish species fed diets with PP, such as rainbow trout (Burrells et al., 1999; Escaffre et al., 2007; Ostaszewska et al., 2005), gilthead seabream (Sitjà-Bobadilla et al. 2005) and orange spotted grouper (Wang et al. 2017).

Measurement of plasma constituent levels can be an important indicator of the nutritional, physiological and clinical status of the fish. The advantage of this analysis is that it is easy to measure several samples in a short period using commercial kits, and also it can be carried out without killing the animal (Maita 2007). However, the diagnostic value of this examination is questionable, especially in fishes, due to the lack of reliable references of the normal values. Various factors such as age, sex, water quality, temperature, handling and analytic methods may contribute to variability in hematological data, thus it is difficult to compare results from different studies or set normal ranges (Maita 2007; Tavares-Dias and Moraes 2007). The analysis of serum liver enzymes such as ALT, AST and ALP was carried out since these have been proposed to be the main biomarkers for liver diseases (Hall and Cash 2012). In general, the elevation of ALT and AST concentrations may indicate hepato-cellular diseases while the elevation in ALP may indicate cholestatic diseases of the liver (Sahoo, Rukmini, and Ray 2015). Our results showed that FM replacement by PP affects the plasma P and Ca content as well as the ALP activity. Elevated plasma ALP activity corresponds to an inflammatory reaction of the bile ducts (Maita 2007) maybe from the use of PP sources. Many studies in various fish species observed that supplementation of dietary P led to an increase of plasma P concentration (Dougall et al. 1996; Roy and Lall 2003; Vielma et al., 2002; Vielma and Lall 1998; Yang et al. 2006). Our results are in agreement with these, since the high P content diets (FM and PP+High P) led to a significantly higher concentration of plasma P in meagre. Concerning other fish species, Gomez-Requeni et al. (2004) found in gilthead seabream decreased levels of plasma glucose with the increase of FM replacement by PP sources, while Sitjà-Bobadilla et al. (2005) found in the same fish species that plasma glucose concentrations did not differ among fish fed the four diets with PP replacement, but plasma total proteins were significantly reduced in fish fed the diets with 75% and 100% replacement. In great sturgeon (*Huso huso*), replacing FM with PP caused a significant higher levels of plasma glucose and proteins and significant lower levels of P (Jahanbakhshi et al. 2013).

The evolution of the severity of SG did not affect the enzymatic activities or any other plasma metabolites measured in this trial. On the contrary, in D24.1 we found that, regardless of the diets, ALP, ALT and AST activity increased in meagre with granulomas or tissue calcification compared with fish without. Increases in AST and ALT activities indicate injury of liver cells caused by various chemicals or lipid peroxidation (Maita 2007). In damaged tissues, cell membranes become more permeable, releasing some enzymes into the blood and thus modifying normal plasma values. In fish, elevated plasma ALP and AST have been associated with liver or bone disorders (Peres et al., 2013, 2014). Furthermore, the ratio of the serum activities of AST and ALT is a useful indicator of the aetiology of hepatitis and it can be more clinically valid than assessing individual concentration (Botros and Sikaris 2013; Sahoo et al. 2015). All the ratios of AST/ALT revealed both from the results of this trial but also from the first trial (D24.1) were >5, which suggests severe hepatic dysfunction, probably related to both SG and lipoid liver degeneration, a condition common in farmed fish (Botros and Sikaris 2013).

In summary, PP in the diets of meagre were found to negatively affect SG while P supplementation in the PP diets did not affect the overall condition but had a positive effect in the liver of the fish. Moreover, the results obtained in this trial showed that a reduction of FM from 60% to 14% is possible for juvenile meagre in terms of growth performance, only in combination with high levels of P supplementation (14 g kg^{-1}).

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