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Effects of different levels of plant proteins on the ongrowing of meagre (*Argyrosomus regius*) juveniles at low temperatures

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Abstract

Four experimental diets with different inclusion levels of plant proteins and fish protein hydrolysates were compared with a commercial diet for meagre (*Argyrosomus regius*) ongrowing at optimal and suboptimal water temperature. Results in terms of growth in length and weight, conversion efficiency, dietary feed intake and utilization, body composition (whole fish and liver) as well as enzyme and immunological activities are presented. Fish growth was significantly reduced by the inclusion of plant proteins, although further addition of fish protein hydrolysates improved the results. Daily feed intake was not affected by plant protein inclusion in the diets, although the group fed the highest inclusion level showed lower ingestion than the rest of the groups, probably as a consequence of a reduced dietary palatability. The decrease in water temperature during the second part of the experiment had a negative effect on feed intake and fish growth. Gross visceral morphology of meagre fed the experimental diets was not affected, but muscle weight was significantly reduced. Whole body and liver composition was not affected with plant protein inclusion. However, the inclusion of fish protein hydrolysates resulted in a significant increase in fat content, especially in liver cholesterol and steryl esters, with a parallel reduction in protein. Brush border enzymes were affected by plant protein inclusion as well as serum lysozyme that significantly increased in the fish fed the highest inclusion level. As a conclusion, up to 315 g kg^{-1} plant protein (76.2% of total protein content) can be included in the diet for meagre without affecting growth or feed utilization. Higher inclusion levels can also be used if at least 5% fish protein hydrolysate is also included.

KEY WORDS: enzymes, growth, lysozyme, meagre, plant proteins, temperature

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Introduction

Fish meal (FM) has been the main protein in the feed for marine fish cultured species during the last decades (Tacon & Metian 2008); however, the increasing demand, price, restricted availability, fluctuations of supply of this ingredient and the unpredictability of the market, as well as the restrictions on the use of several animal derived proteins in fish feed formulations, have directed the most recent research in marine fish ongrowing into looking for abundant and available alternative protein sources. In this sense, proteins derived from plants have received most of the attention (FAO 2001).

The use of plant protein (PP) as a dietary substitute of FM protein in aquafeeds has been the focus of several studies that show large variability among various carnivorous marine fish species in terms of growth performance, feed and nutrient conversion efficiencies, being impaired when a high replacement of dietary FM is used (Robaina *et al.* 1995; Krogdahl *et al.* 2003; Kaushik *et al.* 2004). Most of these studies have been carried out using soybean meal (SBM) as the only plant protein source, and, actually, this is the most commonly used plant source in fish feeds (Azaza *et al.* 2009b). However, different dose–response nutritional studies have evidenced a reduced overall performance with diets including high amounts of conventionally processed SBM as a consequence of reduced nutrient and energy availability and/or the presence of indigestible components and anti-nutritional factors (Francis *et al.* 2001; Urán *et al.* 2008). Recent advances in processing technology have provided more suitable products for use as protein sources in aquaculture. In this sense, different approaches have been evaluated to overcome the

nutritional imbalances resulting from the inclusion of SBM as the sole PP ingredient in aquafeeds, which included the use of processed SBM (Tibaldi *et al*. 2006), the addition of crystalline amino acids (Robaina *et al*. 1995; Pereira & Oliva-Teles 2002), phosphorus (Robaina *et al*. 1998) and taurine (Chatzifotis *et al*. 2008), the use of mixtures of different plant ingredients (Burel *et al*. 2000; Gómez-Requeni *et al*. 2004; Hansen *et al*. 2007), as well as the use of technological processes for the deactivation or removal of endogenous anti-nutritional factors contained in the SBM (Francis *et al*. 2001). In addition, alternative PP sources to decrease the dietary level of SBM incorporation into fish feeds have also been investigated (e.g. Pereira & Oliva-Teles 2002; Santigosa *et al*. 2008; Torstensen *et al*. 2008; Yue & Zhou 2008; Azaza *et al*. 2009b).

Meagre, *Argyrosomus regius*, is a medium grower species with growth rates higher than those of most of the common Mediterranean-cultured species, such as gilthead seabream and European sea bass, and, consequently, a high interest for the intensive culture of this species exists among producers. Body and fillet traits of meagre 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). However, because of its recent introduction in the aquaculture industry, little is known about the nutritional requirements and the optimal ingredients and feed formulation for this species, and diets formulated for sea bass or gilthead sea bream are currently used for meagre. In this sense, these diets are mainly formulated with high levels of FM incorporation.

The objectives of this study were to evaluate growth performance, body composition, digestive enzyme activity as well as changes in the non-specific immune system response in meagre (*Argyrosomus regius*) juveniles fed diets including plant proteins and fish protein hydrolysates to replace varying levels of fish meal.

Material and methods

Experimental diets

Four isoproteic (420 g kg^{-1}) and isolipidic (190 g kg^{-1}) extruded diets based on FM or graded levels of PP (soy cake, corn gluten, wheat gluten and beans) were formulated to be compared with a commercial diet with a total 210 g kg^{-1} protein derived from FM (Table 1). Additionally, a further substitution of FM and PP sources by 50 g kg^{-1} fish protein hydrolysate (FPH; BioCP, Profish, Chile) was also tested (diets PP1H and PP2H, Table 1). All diets were formulated to contain similar levels of digestible protein (DP, 378 g kg^{-1}) and digestible energy (DE, 17.46 MJ kg^{-1}) without any addition of crystalline amino acids. The diets were produced at BioMar research pilot plant in Brande, Denmark, and stored at 4 °C until used.

Table 1 Feed ingredients and feed composition (given as g 100 g^{-1} except for gross energy given as MJ Kg^{-1})

Ingredients (g kg^{-1})	Control Commercial diet	PP1	PP1H	PP2	PP2H
Fish meal (CP 67%)[1]	300	200	150	100	50
BioCP[2]			50		50
Soy cake[3]	180	158	158	225	225
Corn gluten meal[4]	70	129	127	207	206
Wheat gluten[4]	12				
Beans	208	230	231	199	200
Soy protein concentrate[3]	71	61	61	61	61
Sunflower cake	85	74	74	74	74
Fish oil[1]	116	153	147	157	151
Vitamins + Minerals[5]	3	3	3	3	3
Proximate composition (%)					
Dry matter	95.5	93.1	93.7	94.7	94.2
Crude protein	46.7	45.3	45.0	41.9	42.0
Crude fat	11.5	13.4	13.8	10.8	12.1
Starch	10.4	10.5	10.5	10.6	10.5
Ash	6.8	5.5	5.2	5.0	4.5
Gross energy (KJ Kg^{-1})	19.3	19.3	19.3	19.3	19.3

[1] Austral group, Chile.
[2] Profish S.A, Chile.
[3] Imcopa, Brasil.
[4] Roquette, France; Tate&Lyle, Great Britain; Cerestar, France.
[5] DSM, Netherlands.

Growth trials

Juvenile meagre reared from a wild broodstock fish at IRTA were used in the study. Fish were individually weighed and measured, distributed into 24 fibreglass tanks of 400-L capacity in groups of 60 fishes and fed a Skretting Excel D2 commercial diet. Acclimation period to experimental conditions lasted for 20 days (3 weeks). Water flow was 8.5 L min^{-1}, oxygen content was daily recorded in the outlet water remaining between 6 and 8 mg L^{-1}, water temperature ranged from 22.9 to 13.1 °C, salinity varied between 33.4 and 34.5 g L^{-1} and photoperiod followed natural changes according to the time of the year (September–November).

Each diet was randomly tested in triplicate groups (tanks) during 56 days (8 weeks). Because of seasonal changes in water temperature, data from the experiment were analysed according to two different feeding periods. During the first period (from October 3rd to November 6th), water

temperature varied between 22.9 and 16.9 °C (average 20.2 ± 1.9 °C) considered as optimal temperature for meagre, while in the second period (from November 7th to 24th), water temperature decreased from 17.2 to 13.7 °C (average 15.6 ± 1.7 °C), which is below the optimum threshold for this species. Feed was offered by means of automatic feeders, and feed intake was estimated based on records of the amount of feed supplied and feed waste collected. Waste feed was collected from each tank using waste collectors similar to those described by Robaina *et al.* (1995), and its dry weight was estimated gravimetrically after drying for 24 h at 103 °C.

Sampling to monitor fish growth took place after 3 and 8 weeks from the onset of the feeding period with the experimental diets. At the end of the experiment, all the fish (fasted overnight) were measured (standard length – SL, cm) and weighed (BW, g), and 10 fish per tank were sacrificed by immersion in iced water for biochemical and blood analyses. Five fish were used for proximate biochemical analysis (whole fish), whereas from the other five, blood samples were taken from the caudal vein with a plastic syringe, and the fish were subsequently dissected to collect the liver, anterior and posterior intestine, stomach and muscle for enzyme quantification and biochemical composition analysis. Dissection of sacrificed specimens was conducted on a prechilled glass plate maintained at 0 °C to not alter digestive enzyme activity from dissected tissues. The total left-hand side of the muscle fillet was also dissected, and samples were immediately frozen on dry ice for the analysis of proximate composition. Blood samples and dissected tissues for biochemical and enzyme analysis were immediately frozen in liquid nitrogen and stored at −80 °C.

The data obtained were analysed for fish growth and feed utilization, and the following indices were used:

Weight gain (WG) = Final BW − Initial BW/Initial BW (g) × 100

Specific growth rate (SGR) = [ln BW_{final} (g) − ln $BW_{initial}$ (g)] × 100/time (days)

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

Protein efficiency ratio (PER) = [BW_{final} (g) − $BW_{initial}$ (g)]/weight of protein consumed (g)

Mean feed intake (MIF) = Feed intake (g)/fish per day

Condition factor (CF) = 100 × [BW (g)/SL^3 (cm)]

Hepatosomatic index (HIS) = liver weight (g) × 100/BW (g).

Feed and body composition analyses

Chemical analysis of diets for protein, fat and ash was performed according to AOAC (1990) methods. Whole body composition was determined in pooled sample of fish at the beginning (n = 13) and the end of the trial (5 fish per tank). Specimens for body analysis were ground, and small aliquots were dried (120 °C, 24 h) to estimate water content and burned (450 °C, 24 h) for measuring ash content. The remaining samples were freeze-dried and chemical analysis for protein, fat and ash was performed as mentioned previously.

Pooled liver samples of each tank were also analysed for total lipids and protein content by Folch *et al.*'s (1957) and Lowry *et al.*'s (1951) methods, respectively. Fat was determined by gravimetry. Lipid class composition of the liver was carried out by HPTLC following Olsen & Henderson (1989). All chemical analyses were run either in duplicates (dry weight, ash, lipids) or in triplicates (proteins).

Enzyme activities

The activity of digestive enzymes from the stomach and intestine was quantified to assess the effect of different experimental diets on the digestive physiology of meagre juveniles. For the determination of pepsin (gastric enzyme), the stomach was homogenized (Ultra-Turrax T25 basic, IKA© – Werke, Germany) in five volumes (v/w) of ice-cold Milli-Q water, centrifuged at 3300 ***g*** for 3 min at 4 °C, and the supernatant was removed for enzyme quantification. For the determination of intestinal (brush border membrane) enzymes (alkaline phosphatase and aminopeptidase N), samples were homogenized in cold Mannitol 50 mM, Tris–HCl 2 mM buffer, pH 7.0, and the intestinal brush border membranes were purified according to the method developed for intestinal scrapping (Crane *et al.* 1979).

Alkaline phosphatase (E.C. 3.1.3.1) was quantified at 37 °C using 4-nitrophenyl phosphate (PNPP) as substrate in Na_2CO_3 30 mM buffer (pH = 9.8). One unit (U) was defined as 1 μg BTEE released per min and ml of brush border homogenate at 407 nm (Bessey *et al.* 1946). Aminopeptidase N (E.C.3.4.11.2) was determined at 25 °C according to Maroux *et al.* (1973), using sodium phosphate buffer 80 mM (pH = 7.0) and L-leucine p-nitroanilide as substrate (in 0.1 mM DMSO). One unit of enzyme activity (U) was defined as 1 μg nitroanilide released per min and ml of brush border homogenate at 410 nm. Pepsin (E.C. 3.4.23.1), an acidic protease, was quantified at 37 °C using 2% haemoglobin in HCl buffer 1 N as substrate. Pepsin's activity (U) was defined as the μmol of haemoglobin liberated per min at 37 °C and ml of tissue homogenate at 280 nm (Worthington 1972). Soluble protein of crude enzyme extracts was quantified by means of the Bradford's method (Bradford

1976) using bovine serum albumin as standard. All the enzyme quantifications were made by triplicate from five fish per tank. All the chemicals used were provided by Sigma (Germany).

Blood and serum analysis

Blood was obtained by caudal puncture with a 1-mL plastic syringe, placed in Eppendorf tubes and allowed to clot for 2 h. Serum was separated by centrifugation and stored at −80 °C for lysozyme activity determination. Lysozyme level in blood serum was determined by turbidimetry according to Anderson & Siwicki (1994) using hen egg white lysozyme (Sigma, Germany) in PBS as standard. The results are given as mUnits L^{-1}.

Statistics

Data were analysed using linear regression and one-way ANOVA followed by a *post hoc* analysis (Holm–Sidak or Tukey's test) set at $P < 0.05$ using Sigma Stat statistical package (Systat Software Inc, Point Richmond, CA, USA). Prior to ANOVA analysis, all data were checked for normality and homogeneity of variances. Variables were correlated by means of the Pearson product moment correlation. When a statistical correlation was detected between two variables, data were analysed by means of linear regression. Data are given as mean ± SD of three replicates.

Results

Growth, feed intake and biochemical composition

At the beginning of the trial, all the experimental groups were homogeneous in body weight (20.5 ± 3.04 g, $P > 0.05$). At the end of the experimental period, final body weight and length of the fish were significantly higher in the control group fed the commercial diet, decreasing with increasing PP substitution in the diets ($P < 0.001$; Fig. 1, Table 2). Addition of FPH in PP2H diet produced a significant positive effect on fish growth in weight in comparison with the PP2 group (*t*-test, $P < 0.05$). In all experimental groups, condition factor was close to 1.0 ($P > 0.05$; Table 2).

Values of the SGR and WG (Table 2) over the total period of 8 weeks decreased significantly with increasing levels of PP inclusion ($P < 0.05$). During the first period of feeding (3 weeks, optimal rearing temperatures), SGR decreased concomitantly with PP substitution in the diet, showing significant differences when control, PP2 and PP2H groups were compared (ANOVA followed by Holm–Sidak *post hoc* test, $P = 0.002$). During the second period (cold temperatures), growth in terms of SGR was negligible in the PP2H group, while in the rest of the groups, fish lost weight and showed negative SGR values ($P > 0.05$). WG decreased also with increasing PP substitution, and, in this case, PP2 and PP2H groups showed significant differences in WG when compared to the control group during the first feeding period (ANOVA followed by Tukey's *post hoc* test, $P = 0.002$), whereas during the second experimental period, a negative growth was recorded for all the groups. Mean feed ingestion (voluntary feed intake) expressed as g of feed per fish and day decreased significantly for PP2 group for all the periods considered and especially during the low-temperature period during which all the groups showed almost half the amount recorded during the first period ($P < 0.05$). PP2H diet with a total inclusion of 385 g kg^{-1} of PP showed a higher although not significantly different FCR at the end of the feeding trial than the rest of dietary groups. FCR for the second period is not shown because of negative growth of the fish. At the end of the trial, the protein efficiency ratios (PER) from different dietary groups ranged between 0.79 and 1.11, but they were not significantly different. However, PER values tended to decrease with increasing levels of PP inclusion ($P = 0.096$). In contrast, when PER values were calculated in terms of the level of FM-derived protein of the diet, fish fed PP2H showed higher values than the rest of the experimental groups (ANOVA, $P < 0.001$).

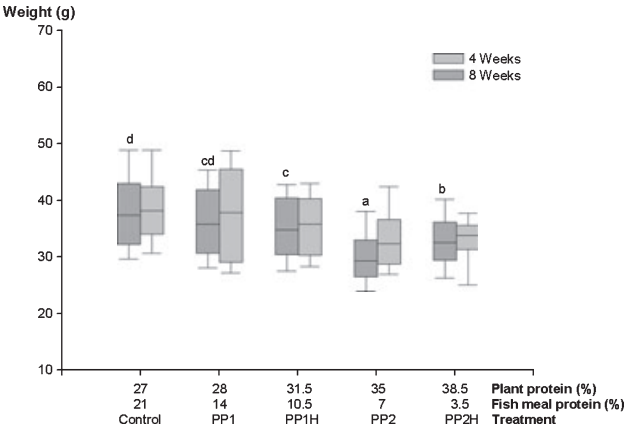

Figure 1 Wet weight of meagre juveniles fed different levels of plant protein (PP) substitution of fish meal (FM) at mid and final sampling. Box plot showing the median (black line inside the box), 25 and 75 percentiles (boundaries of the box) and 5 and 95 percentiles (whiskers above and below the box). Different letters indicate statistical differences among the groups (ANOVA, $P < 0.05$ followed by Tukey's *post hoc* test).

Table 2 Standard growth rate (SGR, % per day), weight gain (WG, g per fish), mean feed ingestion (MFI, g per fish per day) and feed conversion ratio (FCR), and Protein efficiency ratio (PER) for period 1, 2 and for the whole trial

	Control	PP1	PP1H	PP2	PP2H	*P*
Initial BW	20.51 ± 0.31	20.58 ± 0.18	20.38 ± 0.57	20.28 ± 0.36	20.62 ± 0.24	
Period 1(5 weeks, October)						
BW	38.77 ± 6.32^{c}	37.34 ± 8.37^{c}	35.52 ± 5.57^{b}	33.06 ± 5.21^{a}	32.64 ± 4.63^{a}	< 0.001
SGR	1.82 ± 0.12^{b}	1.67 ± 0.29	1.58 ± 0.32	1.40 ± 0.12^{a}	1.31 ± 0.05^{a}	0.002*
WG	0.89 ± 0.08^{b}	0.80 ± 0.18	0.75 ± 0.19	0.63 ± 0.07^{a}	0.58 ± 0.03^{a}	0.002*
MFI	0.68 ± 0.03^{b}	0.64 ± 0.03^{ab}	0.66 ± 0.0.4^{b}	0.56 ± 0.05^{a}	0.62 ± 0.03^{ab}	0.018
FCR	1.33 ± 0.13	1.39 ± 0.27	1.58 ± 0.33	1.54 ± 0.27	1.80 ± 0.11	0.160
Period 2(3 weeks, November)						
BW	38.11 ± 7.55^{d}	36.34 ± 6.62^{cd}	35.15 ± 6.02^{c}	30.23 ± 5.78^{a}	32.81 ± 5.61^{b}	< 0.001
SGR	−0.08 ± 0.16	−0.12 ± 0.35	−0.04 ± 0.46	−0.44 ± 0.32	0.02 ± 0.17	0.457
WG	−0.017 ± 0.03	−0.024 ± 0.07	−0.005 ± 0.09	−0.086 ± 0.06	0.005 ± 0.04	0.530
MFI	0.54 ± 0.03^{b}	0.50 ± 003^{ab}	0.51 ± 0.02^{ab}	0.46 ± 0.02^{a}	0.46 ± 0.02^{a}	0.013
Whole trial (8 weeks)						
Final BW	38.11 ± 7.55^{d}	36.34 ± 6.62^{cd}	35.15 ± 6.02^{c}	30.23 ± 5.78^{a}	32.81 ± 5.61^{b}	< 0.001
SGR	1.11 ± 0.12^{c}	1.00 ± 0.06^{bc}	0.97 ± 0.04^{abc}	0.71 ± 0.17^{a}	0.83 ± 0.05^{ab}	0.006
WG	0.86 ± 0.13^{b}	0.75 ± 0.06^{ab}	0.73 ± 0.04^{ab}	0.49 ± 0.14^{a}	0.59 ± 0.04^{a}	0.005
MFI	0.63 ± 0.03^{b}	0.59 ± 0.03^{ab}	0.60 ± 0.03^{b}	0.52 ± 0.03^{a}	0.56 ± 0.02^{ab}	0.007
FCR	2.02 ± 0.37	2.11 ± 0.15	2.28 ± 0.05	3.13 ± 1.08	2.57 ± 0.16	0.139
PER	1.02 ± 0.17	1.11 ± 0.06	1.01 ± 0.02	0.79 ± 0.22	0.90 ± 0.05	0.096
PER (FM based)	1.16 ± 0.19^{a}	1.50 ± 0.09^{a}	1.57 ± 0.03^{a}	2.06 ± 0.59^{a}	2.91 ± 0.17^{b}	< 0.001

Superscript letters indicate significant differences (ANOVA).

* In the case of SGR and WG in the first period, the ANOVA among Control, PP2 and PP2H diets was significant ($P = 0.002$), and the superscript letters correspond to that analysis.

Inclusion of FPH (Bio CP) to substitute 50 g kg^{-1} of FM in the diet did not have any effect on the results obtained from PP1-fed fish in terms of growth and food utilization; however, FPH inclusion in the diet containing high levels of PP protein resulted in a significant increase in growth in length and weight (Table 3, Fig. 1), SGR and WG (Table 2) in comparison to fish fed the PP2 diet ($P < 0.05$).

The decrease in water temperature observed during the second part of the experiment had a negative effect on feed intake and, consequently, on the overall fish growth (Table 2). Thus, all the groups showed significant negative regressions between temperature and daily feed intake (Fig. 2), with the exception of PP2 diet in which, either at low or at high temperature, daily feed intake did not vary and remained between 20 and 30 g per tank and day (0.33 to 0.5 g per fish and day).

At the end of the trial, the HSI ranged from 1.48 to 1.79% and was not influenced by the increase in dietary PP or by the addition of FPH (Table 3, $P > 0.05$). No dietary effect was observed in stomach relative weight although a slight but significant lower muscle proportion was observed in PP1H group ($P = 0.05$). Thus, feeding diets including different levels of PP did not affect gross visceral morphology of meagre relative to the control fed FM protein.

Results of the proximate biochemical composition of the samples are presented in Tables 3 and 4. Whole fish protein and fat content did not vary significantly with the inclusion of higher levels of PP ($P > 0.05$); however, the addition of FPH in PP1H and PP2H diets resulted in a significant increase in fat content with a concomitant reduction in protein (Table 3; $P < 0.05$). Similar results were also found in terms of liver composition, being the accumulation of fat in the liver higher in the fish fed the diets in which FPH were included ($P < 0.05$) when compared with their counterparts fed the same diet without FPH addition. In this case, fish fed diet PP1H showed the highest degree of liver adiposity. Data on the lipid composition of the liver revealed that the increase in fat content in PP2 group was mainly attributable to higher, significant levels of cholesterol and sterol esters and lower amounts of polar lipids (Table 4, $P < 0.05$). In this case, FPH addition had a fat-reducing effect on liver lipid class composition showing the fish fed PP1H and PP2H diets had lower content of cholesterol and steryl esters than their counterparts feeding PP1 and PP2 diets.

Digestive enzyme activities and immunological parameters

The inclusion of different levels of PP and FPH in the experimental diets did not significantly affect the specific activity of the pepsin among different experimental groups ($P > 0.05$). Regarding intestinal brush border enzymes, alkaline

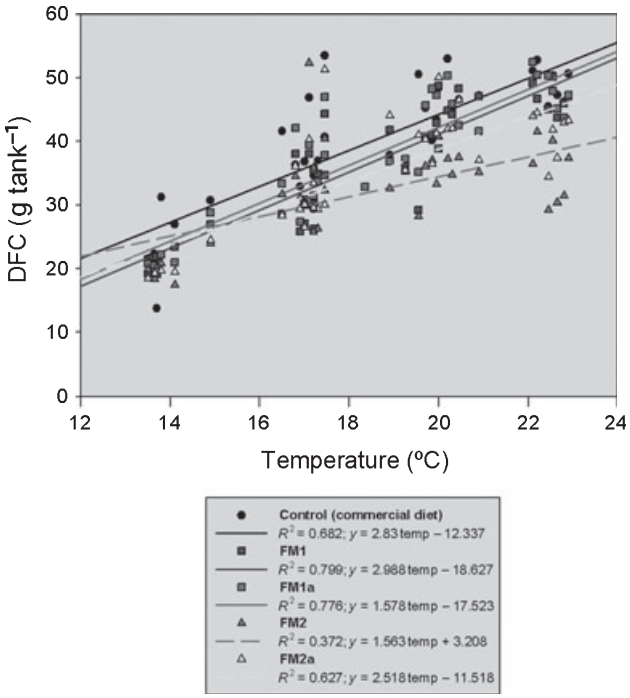

Figure 2 Average daily feed consumption (grams per tank) and regression coefficients and lines registered for each dietary group.

phosphatase and aminopeptidase N were significantly higher in the control group, being the specific activities of those enzymes registered in PP2 fish the lowest ($P < 0.05$; Fig. 3). Diet formulation had a significant effect on the activity of serum lysozyme (Fig. 4), being highest in the fish fed higher proportion of PP and the lowest recorded in the fish fed the same diet with a 50 g kg^{-1} inclusion of FPH ($P < 0.05$).

Discussion

There is a very limited knowledge on dietary nutrient requirements of meagre. Although most of the commercially available diets for meagre are formulated containing high levels of FM protein, the results of this study clearly show that PP can be used as much as 315 g kg^{-1} total protein without affecting fish growth or feed utilization. However, the nutritive value of diets for meagre may be affected when PP are used more than 380 g kg^{-1} total protein, although the inclusion of FPH may partially compensate the bad results of such high replacement of FM by PP proteins in terms of growth performance. Thus, growth was affected by the inclusion of PP, but further addition of FPH, especially at higher PP substitution levels, improved the final results. Because of changes in rearing temperatures during the experimental period, all dietary groups showed very low feeding activity and growth when temperature dropped to 13–14°C in agreement with available information (FAO 2000–2008). The decrease in growth and voluntary feed intake was more pronounced in the groups fed PP (PP1 and PP2) than in those from the control diet and fed diets containing FPH. These results might indicate that meagre is more sensitive to nutrient imbalances caused by PP inclusion in the diet at low

Table 3 Data on growth performance (body weight – BW in g, length in cm, muscle and stomach as g 100 g^{-1} BW, condition factor – CF and hepatosomatic index – HIS) and proximate composition (given as g kg^{-1} wet weight) of whole body, liver and muscle of meagre fed increased levels of plant protein for 8 weeks

Growth performance	Control	PP1	PP1H	PP2	PP2H	*P*
Initial BW	20.51 ± 0.31	20.58 ± 0.18	20.38 ± 0.57	20.28 ± 0.36	20.62 ± 0.24	
Final BW	38.11 ± 7.55^{d}	36.34 ± 6.62^{cd}	35.15 ± 6.02^{c}	30.23 ± 5.78^{a}	32.81 ± 5.61^{b}	<0.001
Final length	15.43 ± 1.05^{d}	15.15 ± 0.96^{cd}	15.03 ± 0.92^{c}	14.26 ± 0.89^{a}	4.66 ± 0.84^{b}	<0.001
CF	1.03 ± 0.06	1.04 ± 0.07	1.03 ± 0.08	1.04 ± 0.07	1.03 ± 0.07	0.158
HSI	1.66 ± 0.47	1.79 ± 0.51	1.76 ± 0.44	1.48 ± 0.43	1.73 ± 0.61	0.527
Muscle	18.00 ± 1.27^{b}	17.57 ± 1.45^{ab}	15.71 ± 2.60^{a}	16.48 ± 1.66^{ab}	16.37 ± 1.73^{ab}	0.005
Stomach	0.84 ± 0.11	0.85 ± 0.09	0.94 ± 0.43	0.88 ± 0.16	0.86 ± 0.09	0.672
Proximate composition						
Whole fish						
Protein	632.7 ± 4.7^{b}	634.7 ± 11.4^{b}	622.0 ± 7.2^{ab}	632.8 ± 8.8^{b}	619.0 ± 4.2^{a}	0.003
Fat	187.8 ± 4.9^{a}	190.4 ± 12.0^{a}	200.0 ± 12.1^{ab}	190.7 ± 2.2^{a}	212.7 ± 6.0^{b}	<0.001
Dry matter	240.4 ± 1.6	240.3 ± 9.6	230.2 ± 44.9	225.4 ± 26.9	236.4 ± 9.2	0.618
Liver						
Protein	301.5 ± 84	242.8 ± 85.9	272.3 ± 57.0	298.6 ± 34.1	243.6 ± 67.8	0.205
Fat	295.7 ± 50^{ab}	279.3 ± 76.9^{a}	412.0 ± 107.8^{b}	251.9 ± 149.8	285.0 ± 98.6	0.026
Dry matter	384.9 ± 121.6	413.4 ± 163.2	400.4 ± 56.5	343.7 ± 50.8	469.5 ± 135.4	0.511

Different superscript letters indicate significant differences (ANOVA, $P < 0.05$).
Initial fish sample: dry matter 236.8 g kg^{-1}; protein 628 g kg^{-1}, fat 184.6 g kg^{-1}.

Table 4 Data on lipid composition of the liver (g Kg^{-1} total lipids) of meagre fed increased levels of plant protein for 8 weeks

	Control	PP1	PP1H	PP2	PP2H	*P*
Total PL	43.5 ± 22.3[b]	26.0 ± 33.5[a]	19.3 ± 24.3[a]	15.6 ± 24.3[a]	4.3 ± 9.1[a]	0.035
CHOL	59.2 ± 25.1[ab]	92.2 ± 75.0[ab]	51.9 ± 23.7[ab]	117.3 ± 54.3[b]	44.4 ± 24.3[a]	0.014
TAG	534.7 ± 241.4	569.0 ± 288.5	682.0 ± 225.2	521.9 ± 31.7	605.0 ± 174.2	0.571
SE + W	83.9 ± 44.7[a]	68.0 ± 44.8[a]	21.5 ± 41.8[a]	180.8 ± 72.5[b]	88.3 ± 55.1[a]	< 0.001
MAG	47.0 ± 20.8[b]	46.0 ± 31. 3[b]	57.8 ± 12.3[b]	23.7 ± 22.2[a]	33.2 ± 5.1[b]	0.02
Total NL	956.5 ± 22.3[a]	974.0 ± 33.5[a]	980.7 ± 24.3[a]	984.4 ± 24.3[a]	995.7 ± 9.1[b]	0.035

Different superscript letters indicate significant differences (ANOVA, $P < 0.05$).
Total PL, Total polar lipids; CHOL, Cholesterol; TAG, Triacylglycerols; SE + W, Steryl esters and waxes; MAG, Monoacylglycerols; Total NL, total neutral lipids.

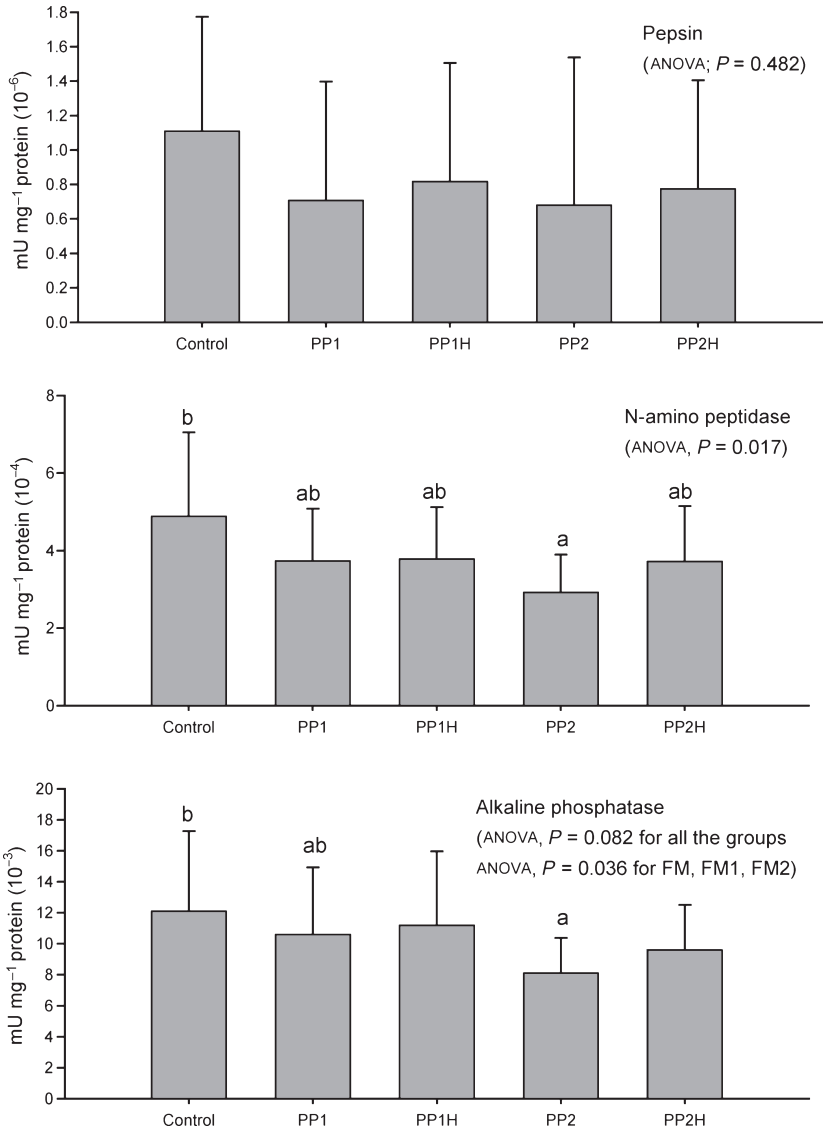

Figure 3 Enzyme activities of pepsin, N- amino peptidase and alkaline phosphatase (in mU mg^{-1} protein) obtained from the stomach and distal intestine of meagre juveniles fed plant protein–substituted diets for 8 weeks. Bars indicate standard deviations of the mean, and letters show significant differences among the groups (ANOVA, $P < 0.05$).

water temperatures, whereas under optimal conditions, those nutritional imbalances would not be so important in terms of fish performance.

Palatability may have also been affected by the level of PP inclusion in the diet. Thus, although FCR and PER were not significantly different among the diets, feed intake by PP2

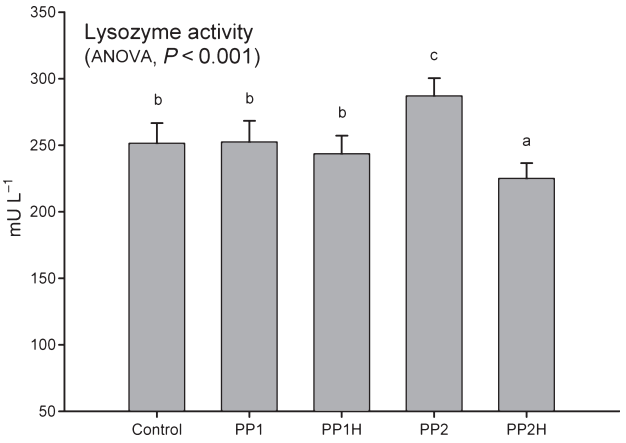

Figure 4 Lysozyme activity (in mU L^{-1}) registered in plasma of meagre fed plant protein–substituted diets for 8 weeks. Bars and letters as in Fig. 3.

group fish was lower than that in the rest of the groups, especially at high temperature, which might have affected fish growth. Robaina *et al.* (1995) did not find any difference in feed intake of gilthead sea bream fed diets with different levels of PP sources (soybean and lupin seeds). However, other authors have also indicated poor growth performance when feeding the fish with a single PP source, being this poor growth related to a reduction in feed intake (Dias *et al.* 1997; Pereira & Oliva-Teles 2002; Gómez-Requeni *et al.* 2004). This reduction in feed intake is generally associated with the lower biological value and palatability properties of PP sources, whereas in some studies, palatability was potentiated by the addition of indispensable amino acids (Dias *et al.* 1997) or FPH (Refstie *et al.* 2004; Espe *et al.* 2006, 2010, present study). The above-mentioned authors observed an increased feed intake when FPH or squid hydrolysate was included in diets based on FM or SBM with as low as 5% FM, stimulating Atlantic salmon growth. Similarly, in the present experiment, the inclusion of 50 g kg^{-1} FPH in PP1 and PP2 diets seemed to improve feed intake in meagre. Asknes *et al.* (2006a) suggested that the presence of some low molecular weight components of fish hydrolysate (taurine and anserine) can explain the growth-promoting effect of FPH. Increased palatability of FPH dietary inclusion was also observed by Asknes *et al.* (2006b) and explained also by the presence of low molecular weight substances such as free amino acids and nucleotides.

Replacing variable amounts of FM protein with different levels of PP had effects on whole meagre composition with an overall increase in the adiposity level in both muscle and liver observed especially in the diets in which FPH was added together with PP. In other studies carried out with farmed gilthead seabream, inclusion of increasing levels of plant ingredients either produced no changes in whole body lipid content (Pereira & Oliva-Teles 2002) or resulted in a reduction in muscle total lipid content as a consequence of either a reduced digestible energy or lipid intake or of specific soy factors acting on lipid metabolism and deposition (Robaina *et al.* 1995; Kissil *et al.* 2000). On the contrary, other studies with sea bass (Ballestrazzi *et al.* 1994; Kaushik *et al.* 2004; Dias *et al.* 2005) reported an augmentation in liver size, not observed in the present study, and in total lipid content when increasing levels of FM replacement by PP were used. In salmonids, increases found in whole body fat content with the use of dietary PP were explained by unbalances in amino acid concentrations (Robaina *et al.* 1995; Kaushik *et al.* 2004; Azaza *et al.* 2009a; Espe *et al.* 2010). Thus, in meagre, as well as in other marine fish species, dietary protein sources may affect the regulation of lipid metabolism, and the mechanisms responsible of this action are important to elucidate the given established trend for FM replacement in aquaculture diets.

In the case of meagre, as well as in other marine fish species and contrary to salmon, fat is primarily stored in the liver. Hepatic TAG levels have been found to be significantly reduced, and cholesteryl esters increased when soy protein concentrates or corn gluten meal was used as the major protein source for sea bass feeding, confirming the lipid-lowering effect of these ingredients (Dias *et al.* 2005). As in the case of European sea bass (Dias *et al.* 2005), the increase in hepatic fat might be a consequence of higher hepatic uptake of VLDL and that in fish neutral sterol elimination is not enhanced by soy protein feeding. Other explanation might be that dietary protein intake regulates lipid biosynthesis increasing the activity of fatty acid synthetase as indicated previously by Dias (1999) and Dias *et al.* (2005). Some studies have reported a possible cholesterol-reducing effect in salmonids by substitution of FM by SBM (Kaushik *et al.* 1995; Refstie *et al.* 1999; Romarheim *et al.* 2006; Yamamoto *et al.* 2007; Romarheim *et al.* 2008), whereas Dabrowska and Wojno (1977) found no significant effect of dietary SBM on plasma cholesterol in rainbow trout.

Digestion and absorption of nutrients depend on the activity of the digestive enzymes, in particular those located in the brush border section of the intestine, which are responsible of breaking down and assimilating the food. In salmonids, high inclusion of SBM led to a marked reduction in the activities of these enzymes as well as histopathological changes (enteritis) in distal intestine (Krogdahl *et al.* 2003; Urán *et al.* 2008), suggesting that measuring the activities of intestinal enzymes may represent a sensitive tool to study the

effects of SBM and/or other vegetable meals to ascertain tolerability to these ingredients in fish species (Tibaldi *et al.* 2006). The dietary-induced changes in the intestinal mucosa organization mentioned earlier directly affect its digestive capacity, which leads to a significant reduction in digestive enzymes located on brush border membranes of enterocytes, especially those of the distal intestine, diminishing the carrier-mediated nutrient transport/absorption ability (Nordrum *et al.* 2000) and, consequently, an impairment of growth and feed efficiency (Krogdahl *et al.* 2003). Thus, the activity of the intestinal enzymes can provide further insights into nutrient availability and the sensitivity of fish intestine function to PP. In this study, the enzymatic activities of both tested intestinal enzymes (alkaline phosphatase and aminopeptidase N) were reduced with increasing levels of PP inclusion in the diet. These results are in agreement with Santigosa *et al.* (2008) who reported a decrease in the digestive activity of pancreatic enzymes in trout and gilthead sea bream fed diets with different level of FM substitution by PP sources. In addition, changes in intestinal brush border enzymes in meagre indicated that PP substitution might cause a damage to the enterocytes, as lower values of phosphatase activity (enterocyte differentiation marker; Henning 1987) indicated, although further studies using light microscopy techniques are needed to evaluate the effects of PP in the organization of the digestive mucosa in meagre.

Cellular immunity has been described to be affected by the inclusion of vegetable oil in the diet (Montero *et al.* 2003). Changes in complement or lysozyme activity are generally associated with a response to an invading microorganism but may also be affected by nutritional factors, such as FPH or PP. In this sense, Krogdahl *et al.* (2003) observed a reduction in mucosal enzyme activities in salmon distal intestine when SBM was included in the diet as well as an inflammatory response in the intestine that may negatively affect its susceptibility to bacterial disease. Thus, the suppression of the non-specific immune capacity by high concentration of dietary SB proteins has been reported in rainbow trout (Burrells *et al.* 1999); however, other reports in Atlantic salmon (Krogdahl *et al.* 2003) have shown increased values of non-specific immune mechanisms as those found in the present study, which have been interpreted as inflammatory/hypersensitivity or immunostimulating effects of PP substitution. Other authors (Murray *et al.* 2003) did not find any changes in lysozyme activity and/or other immunostimulatory effect when using FPH in coho salmon feeding. Sea bream subjected to substitution of fish oils with linseed and soybean oils showed a significant decrease in phagocytic and complement activity especially when the replacement was of 100% and less clearly if the replacement was only up to 50% (Montero *et al.* 2008). Nevertheless, no significant changes were recorded in lysozyme activity.

Conclusions

In conclusion, up to 315 g kg^{-1} plant protein (76.2% of total protein content) can be used in practical diets for meagre without any adverse effects on growth, immunological status or feed utilization. Addition of FPH allows a better utilization of plant proteins when added in higher inclusion levels although producing a slight adiposity of the liver and the whole fish.

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