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Published Version:
Feeding turbot juveniles Psetta maxima L. with increasing dietary plant protein levels affects growth performance and fish welfare / Bonaldo, A.; Di Marco, P.; Petochi, T.; Marino, G.; Parma, L.; Fontanillas, R.; Koppe, W.; Mongile, F.; Finoia, M.G.; Gatta, P.P.. - In: AQUACULTURE NUTRITION. - ISSN 1353-5773. - STAMPA. - 21:4(2015), pp. 401-413. [10.1111/anu.12170]

Availability:
This version is available at: https://hdl.handle.net/11585/515078 since: 2016-07-18

Published:
DOI: http://doi.org/10.1111/anu.12170

Feeding turbot juveniles *Psetta maxima* L. with increasing dietary plant protein levels affects growth performance and fish welfare

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Running title: Growth and welfare of turbot fed plant proteins

Keywords: Turbot, *Psetta maxima*, growth, protein, welfare, fishmeal replacement

Abstract

A 9-week feeding trial was performed to evaluate the effects of fishmeal (FM) replacement by a mixture of plant proteins (PP) on growth performance and welfare of turbot juveniles (initial weight 9.7 ± 0.2 g). Four isonitrogenous and isolipidic diets containing FM at 50% (FM50), 35% (FM35), 25% (FM20) and 5% (FM5) were tested. A decreased feed intake was the more relevant effect observed in FM35, FM20 and FM5 groups. Feed conversion rate was lower in FM5 group. Specific growth rate was significantly reduced in FM20 and FM5 groups, whereas protein and lipid utilization and proximate whole body composition were significantly different in FM5 group. Serum cortisol significantly increased in FM20 and FM5 groups whereas cholesterol, triglycerides, NEFA, total protein and urea concentrations significantly decreased. Serum lysozyme and blood phagocytes increased in FM20 and FM5 groups. FM35 ensured growth close to FM50, without significant effects on health and welfare of animals. FM20 and FM5 groups displayed reduced growth, metabolic stress and an immune response with effects on health and welfare. Results highlighted the consistency between growth performance and welfare status, suggesting the usefulness of their combined assessment for evaluating the suitability of PP and to improve dietary formulation for turbot.

Introduction

In the last two decades, substantial efforts have been made in exploring the use of plant proteins (PP) as fishmeal (FM) substitutes in many finfish species and it is now consensual that PP sources are valid ingredients in aquafeeds (Gatlin *et al.* 2007; Tacon & Metian 2008; Conceição *et al.* 2012). Many experiments have been made to develop low-FM diets leading to optimal fish performance, high feed efficiency and acceptable quality of final product, whereas less attention has been paid to fish health and welfare (Conceição *et al.* 2012; Waagbø *et al*. 2013). The relationship between feed formulation and fish welfare is receiving increasing attention for sustainability of feed industry and ethics of aquaculture productions (Damsgård 2008; Li *et al.* 2009; Kaushik & Seiliez 2010; Oliva-Teles 2012). According to literature, the use of PP diets may have different implications on fish health and welfare depending on species and developmental stages as well as origin, processing, nutritional composition, anti-nutritional factors (ANFs) and amount of plant ingredients, highlighting the complexity of this topic (Glencross *et al.* 2007; Krogdahl *et al.* 2010). Adverse effects on stress tolerance, metabolic functions, immune response, gut integrity and disease resistance have been reported, although the physiological and molecular mechanisms involved are still not completely known (Sitja-Bobadilla *et al.* 2005; Olsen *et al.* 2007; Panserat *et al.* 2009; Ye *et al.* 2011; Laporte & Trushenski 2012; Tacchi *et al*. 2012). These aspects have been poorly investigated in turbot *Psetta maxima* (Bonaldo *et al.* 2011; Yun *et al.* 2011; Nagel *et al.* 2012), the most important cultured flatfish species in Europe, widely reared also in other countries such as East Asia, with a global production of around 70.000 t/year (FAO FishstatJ 2013). Compared to other marine fish, turbot has a high dietary protein requirements (Lee *et al.* 2003), and current practical diets are still based on FM as the main dietary protein source (Bonaldo *et al.* 2011).

In this study, the effect of increasing FM replacement by a mixture of PP sources in experimental diets, on growth performance and welfare of turbot juveniles was evaluated. The PP mixture consisted of wheat gluten (WG), soybean meal (SBM) and soy protein concentrate (SPC). To our knowledge, such a combination of ingredients is utilized for the first time in a feeding trial on turbot and was chosen to reduce the potential negative effects of ANFs and to provide a more adequate amino acid (AA) profile (Fournier *et al.* 2004) than when a single ingredient is utilized.

An integrated approach by assessing growth, nutritional indices, stress, metabolic and immune parameters, was used to give a comprehensive evaluation of the suitability of PP ingredients as substitutes of FM and their appropriate levels to ensure good performance and fish welfare.

Material and methods

Experimental diets

Four experimental diets were manufactured by Skretting Aquaculture Research Centre (Stavanger, Norway) using extrusion technology and common feed ingredients. A control diet (FM50) was formulated with practical ingredients to contain 50% crude protein and 16% crude fat. Fish meal percentage was 50% and the inclusion level of PP was 15%. This level was chosen in order to guarantee an optimal growth and was based on previous studies on FM substitution in turbot juvenile (Regost *et al.* 1999; Burel *et al.* 2000; Day & González 2000; Fournier *et al.* 2004; Bonaldo *et al.* 2011). The other three experimental diets were formulated in order to be isoproteic and isolipidic to the control diet containing 35% (FM35), 20% (FM20) and 5% FM (FM5) by increasing the level of WG, SBM and SPC.

These ingredients were chosen on the basis of their high protein content, necessary to reach a target protein level of 50% of diet. Wheat gluten has been already included in diet for turbot at increasing levels, showing a good potential in substituting FM (Fournier *et al.* 2004; Bonaldo *et al.* 2011). Soybean meal and SPC have also shown good results at high inclusion in previous trials on turbot (Day & González 2000, Bonaldo *et al.* 2011). On a protein basis, WG is low in lysine whereas soy products are low in methionine level when compared to turbot essential (E) AA requirements (Kaushik 1998; Peres & Oliva-Teles 2008) so they can be complementary in formulating feed. The ratio of the three ingredients was chosen in order to balance the AA content, and their increase was in the same proportion at each step. FM5 was also supplemented with methionine and lysine. In the absence of specific data on vitamin, mineral and trace mineral requirements for turbot, requirement data for other species were considered (NRC 2011) adopting the same vitamin-mineral premix formulation used in the trial by Fournier *et al.* (2004) on the same species. The ingredients, the proximate and AA composition are given in Tables 1 and 2.

Fish, experimental set-up and sampling

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, located in Cesenatico, Italy. Turbot *P. maxima* juveniles with an initial average weight 9.7 ± 0.2 g were obtained from the hatchery France Turbot, Noirmoutier, France. Before the experiment, the fish were acclimated for 4 weeks to the experimental tanks and fed commercial FM-based diets (Europa 22, Skretting, Cojóbar Burgos, Spain; crude protein 55%, crude fat 22%). At the start of the trial, 55 fish per tank were randomly distributed into twelve 500-liter square tanks (bottom surface: 0.56 m^2) to obtain four triplicate fish groups, each of which was fed one experimental diet. Tanks were provided with natural seawater and connected to a unique closed recirculation system consisting of a mechanical sand filter (Astralpool, Spain), an ultraviolet light (Philips, the Netherlands) and a biofilter (Astralpool, Spain). The water exchange rate per tank was 100% every 2 h. The overall water renewal of the system was 5% daily. Temperature was maintained constant at 18 ± 1 °C throughout the experiment; photoperiod was held constant at a 12 h day $length^{-1}$ through artificial light (200 lux at the water surface — Delta Ohm luxmeter HD-9221; Delta-Ohm, Padua, Italy). Water temperature and dissolved oxygen (≥ 7 ppm) were monitored daily in each tank. Ammonia (total ammonia nitrogen, TAN ≤ 0.1 ppm), nitrite ($NO_2^- \leq 0.2$ ppm) and nitrate ($NO_3^- \leq 50$ ppm) were determined spectrophotometrically once a day (Spectroquant Nova 60, Merk, Lab business) at 12.00 p.m. At the same time, pH (7.8–8.2) and salinity (28–33 g l^{-1}) were determined. Feeding trial lasted 9 weeks. Fish were hand-fed to apparent satiation twice a day (at 9.00 a.m. and 5.00 p.m.), 6 days per week and once on Sundays. Feed losses were minimal throughout the trial but, when necessary, remaining feed was siphoned from tank bottom and pellets were counted and deducted from the feed intake for overall calculations. At the beginning and at the end of the experiment, all the fish of each tank were individually weighed and total length was recorded. Carcass proximate composition was determined at the beginning and at the end of the trial. In the former case, one pool of ten fish was sampled to determine initial proximate composition whereas, in the latter case, one pool of five fish per tank was collected to determine final proximate composition. Furthermore, at the end of the trial, wet weight, viscera and liver weight were individually recorded from five fish per tank to determine visceral (VSI) and hepatosomatic (HSI) indices. All experimental procedures were evaluated and approved by the Ethical-scientific Committee for Animal Experimentation of the University of Bologna, in accordance with the European directive 2010/63/UE on the protection of animals used for scientific purposes.

Analytical methods

Analyses of experimental diets and carcasses samples were made using the following procedures: dry matter was determined after drying to constant weight in a stove at 105 °C; crude protein was determined by the Kjeldahl method; fat was determined according to Folch *et al.* (1957); ash content was made by incineration to a constant weight in a muffle oven at 450 °C; gross energy was determined by calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261, PARR Instrument, Illinois). Amino acids analysis of diets was made using the method of Cunico *et al.* (1986); tryptophan was analyzed by the method of Garcia & Baxter (1992).

Calculations

The formulae employed were calculated as follows:

Specific growth rate (SGR) (% day^{-1}) = 100 × (ln FBW − ln IBW) / days, where FBW and IBW represent final and initial weights (tank means), respectively. Voluntary feed intake (VFI) (g fish^{-1}) = feed intake / fish. Feed conversion rate (FCR) = feed given / weight gain. Protein efficiency ratio (PER) =body weight gain / protein intake. Gross protein efficiency (GPE) (%) = 100 x ((% final body protein × final body weight) − (% initial body protein × initial body weight)) / total protein intake fish. Gross lipid efficiency (GLE) (%) = 100 x ((% final body lipid × final body weight) − (% initial body lipid × initial body weight)) / total lipid intake fish. Condition factor (CF) = 100 x (body weight/total lenght^3). Viscerosomatic index (VSI) (%) = 100 x (viscera weight / body weight). Hepatosomatic index (HSI) (%) = 100 (liver weight / body weight).

Blood sampling and analysis

Blood sampling was performed at the end of the trial. After 12 h starvation, five fish per tank were quickly dip-netted and anaesthetized with clove oil (Sigma, Italy) at dose of 70 mg l^{-1}, according to Weber *et al.* (2009). Fish reached deep stage of anaesthesia within 3 min. Blood samples collected from caudal vein, were centrifuged at 3000 x ***g*** for 10 min at 4 °C and serum aliquots were stored at -80 °C until analysis. Cortisol (COR) concentration was measured by chemiluminescent enzyme immunoassay (Immulite Siemens Medical Solution Diagnostic, Los Angeles USA); glucose (GLU), triglycerides (TAG), cholesterol (CHO), non-esterified fatty acids (NEFA), total protein (TP), urea (BUN), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and ALP (alkaline phosphatase) by spectrofotometric assays (BPC Biosed, Italy; Wako Chemicals, Germany) and osmolality by crioscopic method (Fiske-Associates, USA) according to Di Marco *et al.* (2011).

Serum lysozyme activity (LYS) was assessed by agarose lysoplate method as described in Bagni *et al.* (2005). The differential blood leukocyte count was performed on blood smears fixed in methanol and stained with May-Grunwald Giemsa according to Roberts *et al.* (1995).

Statistics

Performance, nutritional indices, proximate whole body composition and biometric parameters (Tables 3 and 4) were analyzed by one-way ANOVA with *post-hoc* multiple comparisons (Newman-Keuls), using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). A significant level of $P \leq 0.05$ was adopted for all parameters. Data on blood parameters were analyzed using the SPSS 12.01 software statistical package. Data were checked for normal distribution and logtransformed when necessary before being analyzed statistically. One-way ANOVA and *post-hoc* multiple comparisons (Neuman-Keuls and Dunnett's T3) were performed to assess the effect of dietary treatment and significant differences of the blood parameters between groups ($P \leq 0.05$). Relationships between blood parameters were analyzed by Pearson correlation. Principal component analysis (PCA) and discriminant analysis on PCA factors were applied to data set in order to assess discrimination among groups and the associated variables. Significance of discriminant analysis was assessed by Montecarlo test. Data on differential blood leukocyte count were analyzed by χ^2-Test.

Results

Performance, nutrient utilization, whole body composition and biometric parameters

The effects of the experimental diets on turbot growth performance and nutritional utilization are shown in Table 3. Voluntary feed intake was statistically influenced by diet and fish fed FM50 showed a VFI higher than all the other groups, whereas animals fed with FM5 showed the lowest level. The same pattern was observed in the final weight, whereas the SGR of the animals fed FM50 was significantly higher than that of fish fed FM20 and FM5, the latter having the SGR lower than all the other groups. Animals fed FM5 also showed the significantly highest FCR in comparison with the other groups and lower values of PER and GLE. GPE of fish fed FM50 and FM35 was significantly higher than that of fish fed FM5. PER, GPE and GLE values showed a decreasing trend, although not statistically significant, in fish fed FM20 in comparison with those fed FM50 and FM35.Whole body composition and biometric parameters are shown in Table 4. Moisture content of fish fed FM50 and FM35 was lower than that of fish fed FM5, whereas protein content was higher. Lipid content was higher in fish fed FM50, FM35 and FM20 in comparison with FM5. CF displayed a decreasing trend with PP inclusion. Dietary treatment did not affect VSI, whereas HSI value of FM5 was significantly lower as compared to the other groups.

Physiological and immunological parameters

Dietary treatment significantly affected physiological parameters of turbot juveniles, (one-way ANOVA $P < 0.05$). Physiological changes in COR, TAG, CHO, NEFA, ALP, TP and BUN occurred at increasing level of FM replacement. Minor changes were measured in fish fed FM35, showing lower concentration of CHO, NEFA and BUN compared to FM 50 (Fig. 1). Besides these changes, a significant increase of COR level occurred both in fish fed FM20 and FM5, which showed also an increasing trend of ALT and GLU levels compared to FM50. Additionally, a significant depletion of total proteins content was observed in fish fed FM5 as well as a slight reduction of ALP enzymatic activity.

Dietary treatment significantly affected immunological parameters (one way ANOVA $P < 0.005$; χ^2-test $P < 0.001$). Serum LYS concentration was found higher in turbot fed FM35 and FM20 compared to FM50. An increase of circulating phagocytes, mainly neutrophils, was also observed in fish fed FM20 and FM5 respect to control fish (Fig. 2). The percentage of lymphocytes and trombocytes did not show any difference among groups.

Results of discriminant analysis performed on physiological variables and LYS are shown in Fig. 3. The first two discriminant functions accounted for 82.5% of the variability of data (first for 48.2% and second for 34.3%). Turbot juveniles fed FM35, FM20 and FM5 are discriminated from those fed with FM50 (Montecarlo test $P < 0.001$). Their position along the x-axis is determined by a physiological gradient according to the variables

CHO, TAG, BUN and TP and along the y-axis to LYS, COR, OSM, ALT and GLU. FM35 group is closer to the FM50 reference group.

Discussion

The growth performance registered in this trial, excluding those of fish fed FM5, are higher than those found in other studies on turbot (Regost *et al.* 1999; Burel *et al.* 2000; Day & González 2000; Fournier *et al.* 2004; Bonaldo *et al.* 2011; Yun *et al.* 2011; Nagel *et al.* 2012; Dietz *et al.* 2012). However, when comparing the different treatments, increasing the percentage of dietary PP has resulted in a decreased performance, with fish fed FM20 and FM5 growing less than those fed FM50 and FM35. This seems primarily due to a lower feed intake. The reduced feed intake commonly observed in fish fed feeds containing PP may be related to a reduced feed palatability (Bureau *et al.* 1998; Arndt *et al.* 1999). Among the PP ingredients used in this trial, only SPC has been tested in turbot as a single PP source, affecting ingestion rate only at 75% and 100% of protein FM replacement (Day & González 2000). Wheat gluten was used as a single ingredient in the another marine flatfish, Atlantic halibut **Hippoglossus hippoglossus*, without affecting feed intake up to the maximum dietary inclusion of 30%*** (Helland & Grisdale-Helland 2006). On the other hand, several studies have suggested that the poor palatability of diets containing SBM can be responsible for the limited consumption and thus reduced growth observed in many fish species (Arndt *et al.* 1999). According to Bureau *et al.* (1998), the low palatability of this ingredient is due to the undesirable taste of saponins. At this regard, the use of a PP mixture should reduce the potential inhibition of feed consumption due to the specific effect of a single ingredient (Fournier *et al*. 2004). However, as reported in other studies where mixtures of PP instead of a single ingredient were used on turbot, the maximum replacement of FM without decreasing the feed intake, did not exceed 60% (Fournier *et al.* 2004; Bonaldo *et al.* 2011). Compared to other carnivorous species, turbot seems to be more sensitive to a reduced palatability of medium or low high PP diets. In Atlantic salmon *Salmo salar,* a reduction in feed intake was observed when more than 80% of FM was replaced by PP sources (Sveier *et al.* 2001; Espe *et al.* 2006) and the reduction of the FM content up to 5% did not result in a decrease of feed intake in rainbow trout *Oncorhynchus mykiss* (Kaushik *et al.* 1995), European sea bass *Dicentrarchus labrax* (Kaushik *et al.* 2004), gilthead sea bream *Spaura aurata* (Sánchez-Lozano *et al.* 2009) or Senegalese sole *Solea senegalensis* (Silva *et al.* 2009). In fact, the degree of acceptability of a diet may vary from species to species because feeding stimulants are species-specific (de la Higuera 2001). For example, an L-AA mixture that induced a positive response in rainbow trout was ineffective for turbot, whereas a synthetic squids mixture was highly stimulatory for this species (Andron & Mackie 1978; Mackie & Mitchell 1978). Recently, the utilization of blue mussel meal improved the palatability of rapeseed protein-based diets for turbot, increasing daily feed intake and SGR, where FM protein replacement was of 75% (Nagel *et al.* 2013). As well as for a reduction of feed intake, a lower growth at high PP dietary inclusion has been associated with various factors including poorer utilization of nutrients, which can negatively influence FCR. Regarding PER, GPE and GLE, the reduction of FM dietary inclusion exerted a decline in values, although not statistically significant, in fish fed FM20 in comparison with those fed FM50 and FM35. According to Fournier *et al.* (2004), PER and N retention was similar among groups up to a FM level of 20%, whereas in Bonaldo *et al.* (2011), the reduction of FM inclusion from 55% to 35% resulted in decreased values of the nutritional indices related to protein utilization, such as PER and GPE. We hypothesized that an increased substitution of FM had led to a greater use of protein for energy production, instead of protein synthesis. In this study, such effect seems to be occurred less markedly, and this could be related to the higher protein digestibility of SPC in comparison with CG used in the previous trial. In fact, in the experiments where these two ingredients were individually used in turbot, SPC did not alter protein apparent digestibility up to the total dietary replacement of FM, whereas the increasing dietary content of CG caused a lower apparent protein digestibility already at the minimum inclusion level (Regost *et al.* 1999; Day & González 2000). However, in fish fed FM5, the influence of dietary PP inclusion was more evident and FCR significantly increased whereas GPE and GLE decreased in comparison with the other groups. The effects of FM5 on FCR and nutritional indices could be more influenced by the severely reduced feed intake than to the nutritional composition of the diet. In fact, the feed intake registered in our trial corresponded to a ration of 1.57, 1.50, 1.44 and 0.91% body weight day $^{-1}$ for fish fed FM50, FM35, FM20 and FM5, respectively. Recently, Dietz *et al.* (2012) calculated the percentage of gross energy utilized for maintenance in turbot juveniles (average initial weight 48-49 g) fed different feeding levels. It was found that, while in animals fed 1.5% body weight day^{-1}, this percentage was 13.0-19.2%, in those fed 0.9% the values raised to 20-28.3%, corresponding to a higher FCR as observed in the group fed FM5. The proximate composition of the carcass was influenced by diets, with fish fed FM5 showing a lower lipid content as compared to all other groups and a lower protein content when compared to groups fed FM50 and FM35. Similarly, the HSI of the animals fed FM5 was lower than in the other groups. A reduction in HSI was also observed in the study of Regost *et al.* (1999), where turbot were fed diets containing CG. These differences can be attributed to a reduced growth, rather than a specific effect of the ingredients, as demonstrated in the trial of Dietz *et al.* (2012), where HSI showed lower levels as feeding level and SGR decreased. The CF decreased with increasing FM substitution and this was also found in Bonaldo *et al.* (2011), where turbot were fed diets containing increasing amount of PP mixture. This data seem to be correlated to the reduced development of muscle in these animals, leading to a lower thickness of fillets.

In order to investigate effects of dietary treatment on stress, metabolism and immune response on turbot juveniles, the function-based approach to fish welfare was used, by assessing hormonal, metabolic and immune parameters (Huntingford & Kadri 2008).

A progressive decline of fish physiological status occurred with increasing level of FM replacement. Turbot fed FM35 experienced slight physiological changes compared to those fed FM50, showing a good nutritional status. A primary stress response and a greater reduction of serum nutrients were observed in turbot fed FM 20 and FM5, in agreement with the significant decrease of SGR and VFI. In particular, in turbot fed FM5 all serum nutrients were very low, suggesting a poor nutritional status. The most consistent blood chemistry responses to dietary treatment were in serum CHO, TAG, NEFA, TP, BUN concentrations. These parameters decreased proportionally in respect to the level of FM replacement, except for NEFA concentration, suggesting a direct influence of plant ingredients on lipid and protein metabolism. Decrease in plasma CHO and TAG concentration was already observed in several species when substituting FM by a high proportion of one single PP ingredient like SPC in rainbow trout and European sea bass (Kaushik *et al.* 1995; Dias *et al.* 2005; Yamamoto *et al.* 2007), extracted SBM in gilthead sea bream (Venou *et al.* 2006), or by a combination of different vegetable ingredients in cod *Gadus morhua* L. (Hansen *et al.* 2007). Similar findings were reported for turbot juveniles fed diets containing high CG levels and rapeseed protein isolate (Regost *et al.* 1999; Nagel *et al.* 2012). Biochemical and molecular studies in different species showed an interference of PP diets on CHO and fatty acids metabolism (Dias *et al.* 2005; Panserat *et al.* 2009; Lim *et al.* 2011; Tacchi *et al.* 2012; Sahlmann *et al.* 2013). In particular, turbot fed plant-based diet supplemented with CHO displayed plasma and liver CHO concentration correlated to dietary intake. The hypocholesterolemic effect found in plasma and liver was due to a decreased ability of carrying cholesterol from peripheral tissues to liver, rather than an interference on its biosynthesis. Lower conversion into bile salts was also reported as important effect of PP on CHO metabolism (Yun *et al*. 2011).

The COR stress response has been little investigated in studies on alternative PP sources in aquafeeds, although it has relevant secondary and tertiary effects on metabolism, growth and immune system in fish (Wendelaar Bonga 1997). Higher plasma COR levels, although not significant, were measured in turbot juveniles fed diet with total inclusion of rapeseed protein isolate as FM alternative (Nagel *et al.* 2012). Turbot is a low stress-responsive species and therefore little plasma COR increase, especially under chronic stressful conditions, may be physiologically important. Higher COR response after a stress challenge was observed in sunshine bass *Morone chrysops* x *M. saxatilis*, fed increasing levels of SBM, suggesting a reduced stress tolerance, even in the absence of a significant growth impairment (Laporte & Trushenski 2012). The significant COR increase occurred in turbot fed FM20 and FM5 compared to FM50, is interpretable as an attempt of metabolic adjustment to cope with stress (Mommsen *et al.* 1999; Aluru & Vijayan 2009). Indeed, higher glucose level coupled with lower total proteins support this hypothesis. The model proposed by Milligan (1997) in rainbow trout on the stimulating effect of COR on proteolysis to sustain gluconeogenesis, well explains physiological results on serum GLU, ALT, TP and BUN concentration. Briefly, the model envisages the release of alanine from the muscle by branched-chain AA oxidation and utilization for gluconeogenesis in the liver, coupled with the synthesis of glutamine from ammonia and glutamate in the muscle. Glutamine in turn may be used as an oxidative substrate and/or for gluconeogenesis, rather than for BUN synthesis in the liver. According to this model, alanine and glutamine are therefore the key players in the AA metabolism and are used to meet the energetic demand required to maintain acceptable level of physiological homeostasis, in the face of metabolic stress arising from reduced VFI in turbot fed FM20 and FM5. Direct use of some EAA as energetic substrates or as carbon sources for hepatic gluconeogenesis, was also observed in fish under stressful conditions as an adaptive metabolic response to stress challenge (Costas et al. 2011a). Similar findings have been observed in Senegalese sole following feed deprivation (Costas *et al.* 2011b). Hypothesis of some interferences of PP ingredients on the lipid metabolism and the activation of compensative/integrative proteins catabolism, is supported by the decreasing trend of nutritional indices and proximate composition, observed in turbot fed both FM20 and FM5, although differences are statistically significant only in this latter group. Dietary treatment further induced significant changes in immune response of turbot juveniles. The percentage of blood phagocytes, mainly neutrophils, proportionally increased with increasing level of FM replacement in experimental diets, suggesting a cellular innate immune response mediated by COR, which appears more evident in turbot juveniles fed FM20 and FM5 (Harris & Bird 2000; Roberts & Ellis 2012). It is well reported that partial replacement of FM with vegetable ingredients affects activities of enzymes involved in fish innate immune response, as LYS and ALP (Krogdahl *et al.* 2000; Sitjà-Bobadilla *et al.* 2005; Kumar *et al.* 2010; Lin & Luo 2011; Peng *et al.* 2013). This latter has a pivotal role in maintaining the integrity and homeostasis of the intestinal barrier in fish and in higher vertebrates (Bates *et al.* 2007; Lallès 2010). In the present study, dietary treatment significantly affected both parameters (ANOVA: LYS, $P < 0.001$; ALP $P < 0.05$). Higher serum LYS and ALP concentration was found in turbot fed FM20 compared to FM50 and statistical analysis highlighted a positive correlation ($P = 0.019$). Similar findings are already reported in other species fed with graded level of vegetable meals (Kumar *et al.* 2010; Kokou *et al.* 2012), suggesting an adaptive immune response to counteract the potential risk of gut inflammation and/or hypersensitivity reaction to PP ingredients (Rumsey *et al.* 1994; Burrels *et al.* 1999; Krogdahl *et al.* 2000; Krogdahl 2011). Given that blood phagocytes are the main source of serum LYS (Ellis 1999), a direct relationship between the increase in circulating phagocytes and serum LYS concentration in fish fed

FM35 and FM20, cannot be excluded. In this case, a functional impairment of phagocytes in turbot fed FM5 could explain the decrease of serum LYS concentration measured in this group (Geay *et al.* 2011). Integration of blood parameters by means of multivariate analysis, confirmed the influence of dietary treatment on physiological status of turbot. Fish fed with FM35, FM20 and FM5 are discriminated from the FM50 group along a physiological gradient identifying a progressive welfare impairment.

Conclusion

The administration of FM35 ensured growth performance close to FM50, without significant effects on health and welfare of turbot juveniles. The FM20 dietary treatment produced sub-optimal growth performance, metabolic stress and an immune response with consequences on health and welfare status. FM5 caused a worsening of growth performance and fish welfare, probably due to an insufficient feeding and nutrients intake. However, the slight changes in serum lipids in fish fed FM35 encourage a long-lasting feeding trial in order to exclude long-term effects of this diet on physiological status.

Overall results highlighted the consistency between growth performance and welfare status, suggesting the usefulness of their combined assessment for evaluating the suitability of PP ingredients and improving dietary formulation for turbot juveniles.

Acknowledgements

This research was supported by a grant of the Italian Ministry for Agricultural, Food and Forestry Policies. We thank Marina Silvi, Sara Giuliani, Alessandro Longobardi, Valeria Donadelli and Alessandra Priori for technical assistance and Matthew Owen for the English editing. Diets were kindly provided by Skretting Aquaculture Research Centre, Stavanger, Norway.

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Figure captions

Figure 1. Physiological parameters of turbot juveniles fed the experimental diets. Data are given as mean ± standard error of the mean. Different letters indicate significant differences among groups ($P \leq 0.05$).

Figure 2. Immunological parameters of turbot juveniles fed with experimental diets. Data are given as mean ± standard error of the mean. Different letters indicate significant differences among groups ($P \leq 0.05$).

Figure 3. Comprehensive evaluation by discriminant analysis of physiological status of turbot fed with plant protein diets (FM35, FM20, FM5) *vs* reference diet (FM50).

Table 1 Formulation and proximate composition of experimental diets

Ingredient (g kg^{-1})	FM50	FM35	FM20	FM5
Fishmeal LT	500	350	200	50
Wheat gluten	73	137	206	282
Soybean meal	100	130	170	200
Soy protein concentrate (CP 60%)	70	140	200	250
Fish oil	59	74	88	103
Wheat	188	159	114	74.9
DL-Methionine	0	0	0	1
L-lysine	0	0	0	4.6
Phosphate	0	0	12	24.5
Vitamin and mineral premix[1]	10	10	10	10
Proximate composition (g kg^{-1})				
Moisture	80	78	79	78
Crude protein	509	495	504	518
Crude fat	155	160	162	160
Ash	82	69	59	51
Gross energy (MJ/Kg)	20.37	20.25	20.56	20.48

[1] As described by Fournier *et al.* (2004). Supplied the following (to provide mg kg^{-1} diet, except as noted): retinyl acetate (250,000 U/g), 0.5; cholecalciferol (240,000 U/g), 2.4; ascorbyl phosphate (25%) 200; tocopheryl acetate, 50; menadione, 10; thiamin, 1; riboflavin, 4; pyridoxine, 3; Ca-pantothenate, 20; vitamin B12, 0.01; niacin, 10; biotin, 0.15; folic acid, 1; choline, 1000; inositol, 300; magnesium carbonate, 1.24 g; calcium carbonate, 2.15 g; potassium chloride, 0.90 g; sodium chloride, 0.40 g; potassium iodide, 0.4; copper sulfate, 30; cobalt sulfate, 0.2; ferric sulfate, 0.20 g; manganese sulfate, 30; zinc sulfate, 40; dibasic calcium phosphate, 5 g; sodium fluoride: 10.

Table 2 Amino acid profile of the diets and requirements of turbot (g 16 g $^{-1}$ N)

Amino acids	FM50	FM35	FM20	FM5	EAA requirements	
					*	**
Methionine	1.77	1.90	1.65	1.67	{ 2.7	1.68
Cysteine	1.90	1.29	1.37	1.52		
Lysine	6.63	5.94	5.12	5.09	5.0	5.00
Threonine	4.34	4.24	4.00	3.54	2.9	2.37
Arginine	5.66	5.52	5.98	5.52	4.8	4.22
Isoleucine	3.24	3.06	2.94	3.01	2.6	2.59
Leucine	7.82	7.67	7.50	7.41	4.6	4.47
Valine	4.10	3.76	3.57	3.47	2.9	2.74
Histidine	3.35	2.90	2.81	2.65	1.5	1.28
Phenylalanine	4.38	4.59	4.78	4.29	{ 5.3	2.54
Tyrosine	3.00	2.93	2.88	3.22		1.90
Glycine	6.13	5.52	4.87	4.29		
Serine	5.37	5.65	5.69	5.75		
Proline	6.28	6.94	7.82	8.49		
Alanine	6.23	5.63	4.83	4.22		
Aspartic acid	9.23	9.17	8.67	8.14		
Glutamic acid	17.14	21.59	24.33	26.71		
Hydroxyproline	1.02	0.82	0.45	0.20		
Tryptophan	0.77	0.88	0.76	0.80	0.6	

* from Kaushik (1998); ** from Peres and Oliva-Teles (2008)

Table 3 Performance and nutritional indices of fish fed experimental diets

	FM50	FM35	FM20	FM5
Initial weight (g)	9.7 ± 0.3	9.5 ± 0.2	9.8 ± 0.3	9.7 ± 0.1
Final weight (g)	69.1 ± 4.7^{c}	57.2 ± 2.0^{b}	49.1 ± 4.8^{b}	19.9 ± 3.5^{a}
SGR (% day^{-1})	3.11 ± 0.14^{c}	2.85 ± 0.05^{bc}	2.55 ± 0.20^{b}	1.12 ± 0.26^{a}
VFI (g fish^{-1})	38.9 ± 2.4^{c}	31.6 ± 0.8^{b}	26.8 ± 2.1^{b}	8.5 ± 2.6^{a}
FCR	0.66 ± 0.01^{a}	0.66 ± 0.01^{a}	0.68 ± 0.03^{a}	0.83 ± 0.03^{b}
PER	3.02 ± 0.06^{b}	3.05 ± 0.06^{b}	2.90 ± 0.14^{b}	2.32 ± 0.07^{a}
GPE	43.2 ± 2.1^{b}	44.5 ± 2.5^{b}	40.9 ± 2.21^{ab}	30.1 ± 8.2^{a}
GLE	64.1 ± 4.5^{b}	65.2 ± 1.4^{b}	56.2 ± 6.2^{b}	36.7 ± 4.4^{a}

Values are given as mean ± standard deviation. Values in the same row with common superscript letters are not significantly different ($P \geq 0.05$).

SGR, Specific growth rate; VFI, Voluntary feed intake; FCR, Feed conversion rate; PER, Protein efficiency ratio; GPE, Gross protein efficiency; GLE, Gross lipid efficiency.

Table 4 Proximate whole body composition (g kg $^{-1}$ wet weight) and biometric parameters of fish fed the experimental diets

	FM50	FM35	FM20	FM5
Proximate composition				
Moisture	762 ± 4^{b}	763 ± 3^{b}	770 ± 1^{ab}	782 ± 10^{a}
Protein	146 ± 8^{b}	147 ± 5^{b}	144 ± 5^{ab}	142 ± 16^{a}
Lipid	62 ± 3^{b}	65 ± 2^{b}	58 ± 4^{b}	46 ± 3^{a}
Ash	35 ± 1^{bc}	29 ± 1^{a}	32 ± 1^{b}	36 ± 1^{c}
Biometric parameters				
CF, g $(cm^3)^{-1}$	1.95 ± 0.20^{c}	1.94 ± 0.15^{c}	1.87 ± 0.19^{b}	1.60 ± 0.24^{a}
VSI (%)	6.72 ± 0.69	6.63 ± 0.43	7.10 ± 1.07	7.06 ± 1.06
HSI (%)	1.84 ± 0.24^{b}	1.95 ± 0.22^{b}	1.91 ± 0.42^{b}	1.54 ± 0.16^{a}

Values are given as mean ± standard deviation. Values in the same row with common superscript letters are not significantly different ($P \geq 0.05$).

CF, condition factor; VSI, viscerosomatic index; HSI, hepatosomatic index.

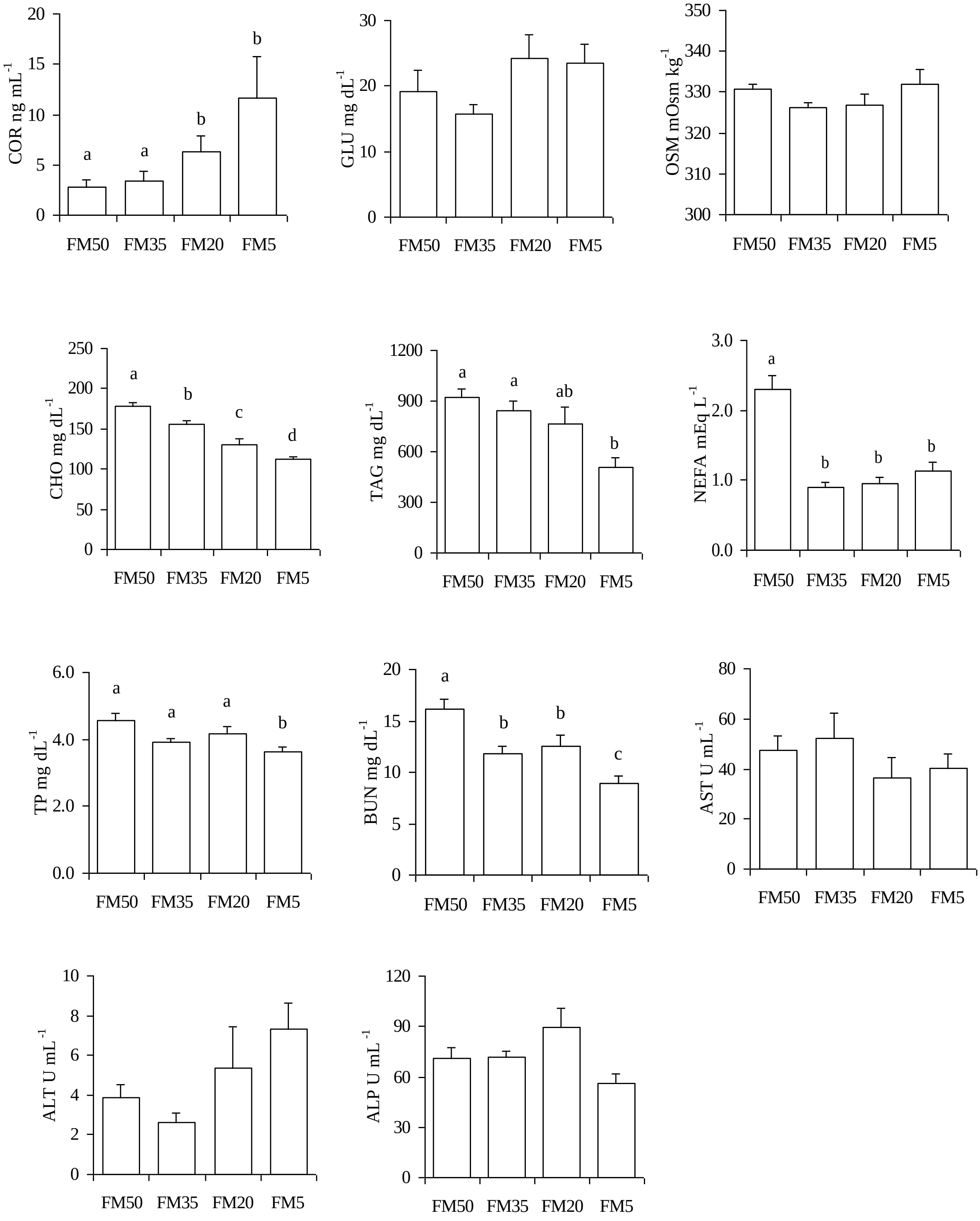
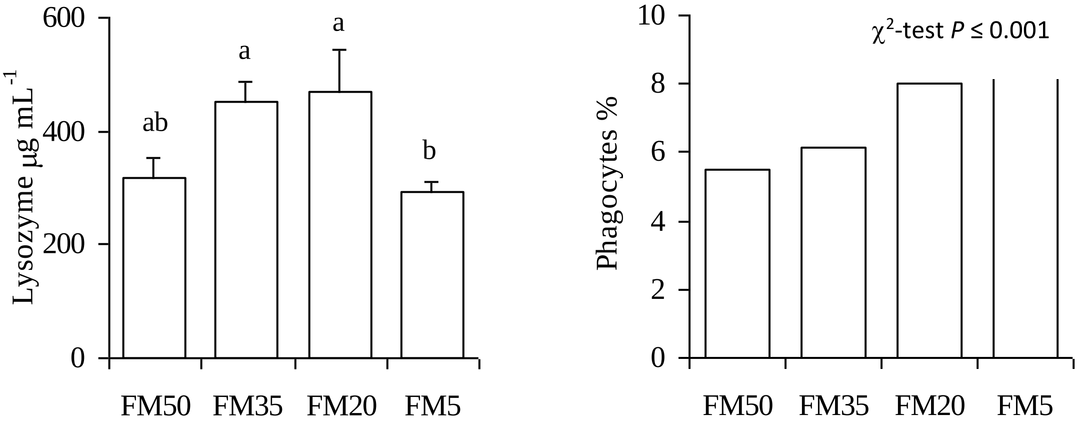


Figure 1 and Figure 2


600
400
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Lysozyme µg mL-1
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Phagocytes %
χ2-test P ≤ 0.001
FM50
FM35
FM20
FM5

Figure 3