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FINE TUNING OF ON-GROWING DIETS FOR FARMED MARINE FISH SPECIES AS A KEY TOOL TO SUSTAIN THE AQUACULTURE DEVELOPMENT

Presentata da: Fulvio Mongile

Coordinatore Dottorato

Chiar.mo
Prof. Carlo Tamanini

Relatore

Chiar.mo
Prof. Pier Paolo Gatta

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Chapter 1

General introduction

Aquaculture has recorded a significant and most rapid growth among the food-producing sectors, developing into a globally robust and vital industry, capable to provide livelihoods and income to a significant share of the world's population. Moreover, aquaculture is likely to remain one of the fastest-growing animal food-producing area which, together with fisheries, is expected to exceed that of beef, pork and poultry, over the next decade (FAO, 2012).

The boost, driving up to such a growth in the aquaculture sector, should be sought under different backgrounds. First of all, population growth is mainly in emerging economies such as China, India and Brazil, whose rising incomes led to a shift in dietary patterns towards a diet dominated by protein-rich meals with meat and fish. Additionally, consumer are aware of the excellent nutritional value of fish, providing highly digestible proteins, essential vitamins and minerals in an easily accessible form and, in most species, the additional health benefits given by the high content of polyunsaturated omega-3 fatty acids EPA and DHA.

Given that global production from capture fisheries has leveled off, or in some cases has gone beyond capacity, the global growing demand for seafood cannot be met by the capture fisheries, but will mandatory rely on the aquaculture farming. Nonetheless, this has to be achieved in a sustainable manner and coping with several important challenges. In this regard, at the Nations Conference on Sustainable Development, it was stated that aquaculture sector and aqua-farmers should put more attention at the food value chain, whose improved management and efficiencies would increase food production while using fewer natural resources. It follows that a primary goal for the aquaculture sector is the optimization of fishmeal (FM) and fish oil (FO) in aquafeeds, through their highest reduction and replacement, while maintaining the important human health benefits of farmed seafood consumption. Noteworthy, FM and FO are not essential feed ingredients *per se*, but represent cost-effective providers of high quality animal protein and lipids in near ideal nutritional proportions for most aqua-cultured species (Tacon and Metian, 2008).

Fishmeal and FO production, has been relatively constant over the last 20 years, except when occurring the El Niño-Southern Oscillation phenomenon resulting in the failure capture of meal-grade fish from the wild. Therefore, there are no realistic prospect of an increased output of those raw material in the near future. Moreover, the increasing market demand of

FM and FO and the increasing global petroleum and energy costs culminated in increasing prices (FAO, 2008; IFFO, 2008a, b).

Notwithstanding the above, it is unlikely that those factors will affect aquaculture's growth. In a recent report on the State of Fisheries and Aquaculture (FAO, 2012), it was stated: "*Although the discussion on the availability and use of aquafeed ingredients often focuses on fishmeal and fish oil resource, considering the past trends and current predictions, the sustainability of the aquaculture sector will probably be closely linked with the sustained supply of terrestrial animal and plant proteins, oils and carbohydrates for aquafeeds*".

Then, in view of the aforementioned, the main focus for the aquafeed industry is to cope the growth of the sector, through the fine tuning of formulated diets, pursuing the most suitable combination between alternative ingredients with FM and FO. Indeed, despite the increases in the global consumption and because of the increased prices of marine fisheries-derived proteins and oils, aquaculture efficiency has increased consistently and is expected to further reduce his dependency on marine raw materials (Delgado *et al.*, 2003; Jackson, 2010; Kristofersson and Anderson, 2006; Naylor *et al*., 2009; Tacon and Metian, 2008). This purpose has to be pursued satisfying fish nutrient requirements, as to maximize growth performance, feed efficiency and fish welfare. Meanwhile, health, safety, quality and acceptability of the final product have to be guaranteed and maximized.

Much progress have been made in the substitution of FM and FO with plant products in aquafeeds for Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*), in terms of growth and feed utilization indices (Torstensen *et al*., 2008; Bell *et al*., 2008; Benedito-Palos *et al*., 2008; Kaushik *et al*., 2004). However, given the physiological, nutritional, environmental and compositional differences among farmed finfish, conclusions reached about the substitution level of a given species cannot be automatically applied to another one. In our case study, Chapter 2, we evaluated the suitability of plant protein ingredients as substitutes of FM and their appropriate levels to ensure good performances and fish welfare in turbot juveniles (*Psetta maxima*). Noteworthy, turbot represent the most farmed flatfish species in Europe, widely reared also in other countries, with a global production of around 70.000 tons per year (FAO FishStatJ, 2013).

Another challenge to be faced by the aquaculture sector, involves the predicted climate change and his impact on fish farming. Indeed, water temperatures are getting warmer along the coast, especially during the summers, with a direct effect on fish ingestion, evacuation rate, metabolism and growth rate. Noteworthy, the higher fish energy requirements at increasing temperature have important implications in aquaculture farming operations, since a shortage of dietary energy would lead to a lower feed and protein utilization efficiency and,

therefore, to higher nitrogenous excretion and lower productivity. In this context, the optimization of fish farming by enhancing feed efficiency and the use of specific diets represent a major factor in aquaculture and environmental sustainability (Naylor *et al*., 2009). In our case study, Chapter 3, we focused on the estimation of the most suitable dietary lipid level for the development of a specific seasonal diets for gilthead seabream (*Sparus aurata*) reared at Mediterranean summer temperature, as to make the gilthead seabream production economically and environmentally more sustainable.

Finally, the European marine aquaculture is mainly based on few species, like Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*), gilthead seabream (*Sparus aurata*) and turbot (*Psetta maxima*). In this regard, the introduction of new species would represent a key issue for the development and the sustainability of the aquaculture sector. Indeed, farming new species will strengthen and consolidate the European aquaculture industry, through the diversification of the market and generating new possibilities for growth, through the creation of new companies and the expansion of the already existing ones. Hence, the introduction on new marine species in the aquaculture panorama makes necessary the development of species-specific formulated diets. This goal shall be achieved by meeting the species-specific nutrient requirements in order to achieve the proper feed utilization and making cost-effective their farming. In our case studies, we have been focused on the further development of the nutritional knowledge regarding the on-growing of common sole (*Solea solea*) juveniles investigating the lipid requirement (Chapter 4) and the use of alternative ingredient (Chapter 5) for the exploitation of the growth potential of this species.

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Chapter 2

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.

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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 and 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 and 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 and 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 and 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 and 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 and 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.

Table 1. Ingredients and proximate composition of experimental diets.

	Experimental diets			
	FM50	FM35	FM20	FM5
Ingredients (g kg^{-1})				
Fishmeal LT	500.0	350.0	200.0	50.0
Wheat gluten	73.0	137.0	206.0	282.0
Soybean meal	100.0	130.0	170.0	200.0
Soy protein concentrate (CP 60%)	70.0	140.0	200.0	250.0
Fish oil	59.0	74.0	88.0	103.0
Wheat	188.0	159.0	114.0	74.9
DL-Methionine	0.0	0.0	0.0	1.0
L-lysine	0.0	0.0	0.0	4.6
Phosphate	0.0	0.0	12.0	24.5
Vitamin and mineral premix[1]	10.0	10.0	10.0	10.0
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)

	Experiemental diets				EAA requirements	
	FM50	FM35	FM20	FM5	*	**
Amino acids						
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)

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^- ≤ 0.2 ppm) and nitrate (NO_3^- ≤ 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 and 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 log-transformed 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.

Table 3. Growth performance and nutritional indices of turbot juveniles fed experimental diets.

	Dietary treatments			
	FM50	FM35	FM20	FM5
IBW[1]	9.7 ± 0.3	9.5 ± 0.2	9.8 ± 0.3	9.7 ± 0.1
FBW[2]	69.1 ± 4.7^{c}	57.2 ± 2.0^{b}	49.1 ± 4.8^{b}	19.9 ± 3.5^{a}
SGR[3]	3.11 ± 0.14^{c}	2.85 ± 0.05^{bc}	2.55 ± 0.20^{b}	1.12 ± 0.26^{a}
VFI[4]	38.9 ± 2.4^{c}	31.6 ± 0.8^{b}	26.8 ± 2.1^{b}	8.5 ± 2.6^{a}
FCR[5]	0.66 ± 0.01^{a}	0.66 ± 0.01^{a}	0.68 ± 0.03^{a}	0.83 ± 0.03^{b}
PER[6]	3.02 ± 0.06^{b}	3.05 ± 0.06^{b}	2.90 ± 0.14^{b}	2.32 ± 0.07^{a}
GPE[7]	43.2 ± 2.1^{b}	44.5 ± 2.5^{b}	40.9 ± 2.21^{ab}	30.1 ± 8.2^{a}
GLE[8]	64.1 ± 4.5^{b}	65.2 ± 1.4^{b}	56.2 ± 6.2^{b}	36.7 ± 4.4^{a}

[1] IBW, initial body weight (g); [2] FBW, final body weight (g); [3] SGR, specific growth rate (%/day) = 100 x (ln final body weight - ln initial body weight)/(duration of experiment, days); [4] VFI, voluntary feed intake (g $fish^{-1}$) = (total feed intake, g)/fish; [5] FCR, feed conversion ratio = (dry feed consumed, g/wet weight gain, g); [6] PER, protein efficiency ratio = (fish weight gain including weight of dead fish, g)/(total protein intake, g); [7] GPE, gross protein efficiency = 100 x ((% final body protein x final body weight) - (% initial body protein x initial body weight))/ (total protein intake, g); [8] GLE, gross lipid efficiency = 100 x ((% final body lipid x final body weight) - (% initial body lipid x initial body weight))/ (total lipid intake, g).

Each value is the mean ± SD of three replicates. Different letters (abc) in the same row denote significant ($P \leq 0.05$) differences among treatments. *P* values are also given.

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.

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

	Dietary treatments			
	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.

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.

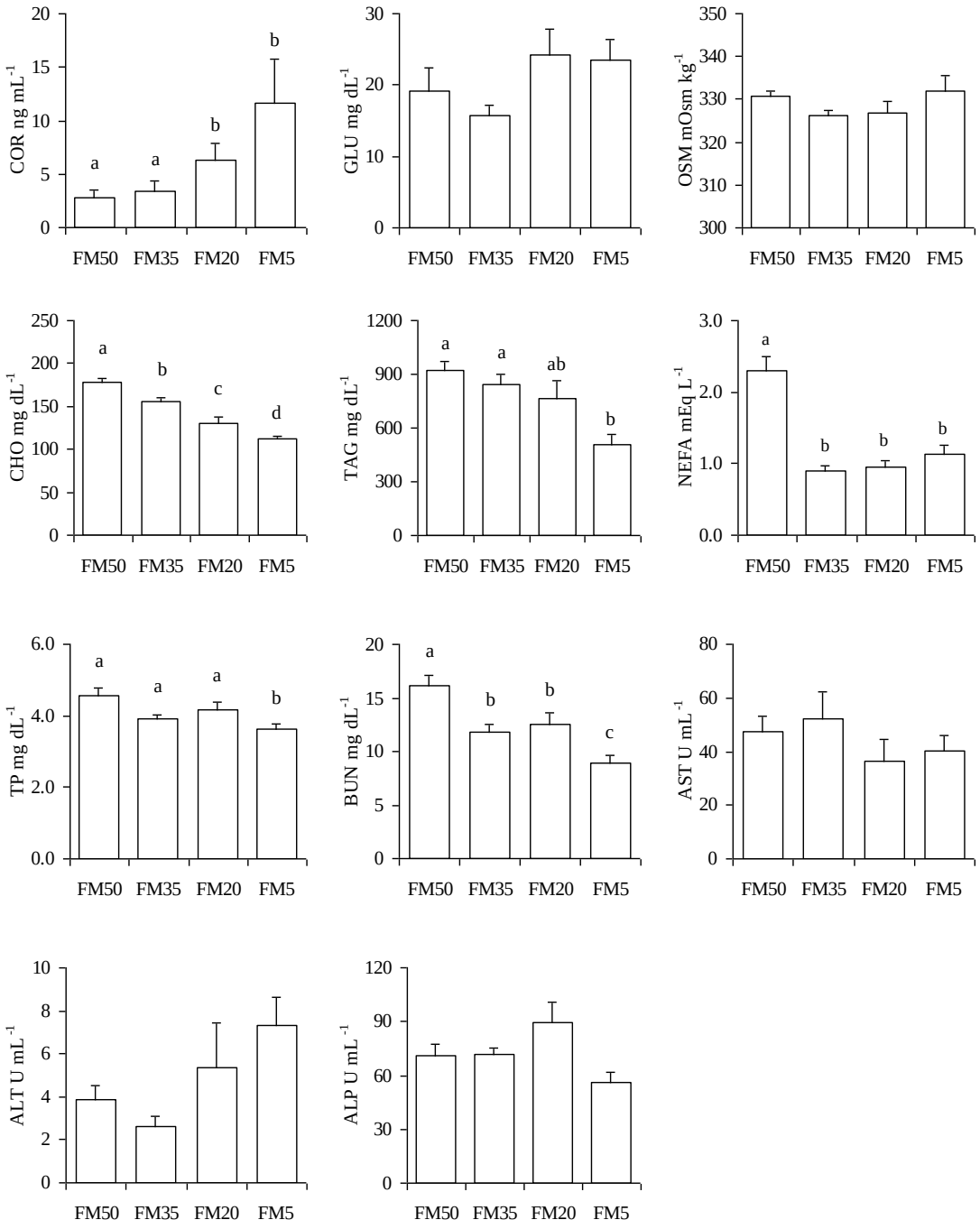

Figure 1. *Physiological parameters of turbot juveniles fed the experimental diets. Data are given as mean ± standard error of the mean. Different letters indicate significant differences among groups ($P \leq 0.05$).*

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.

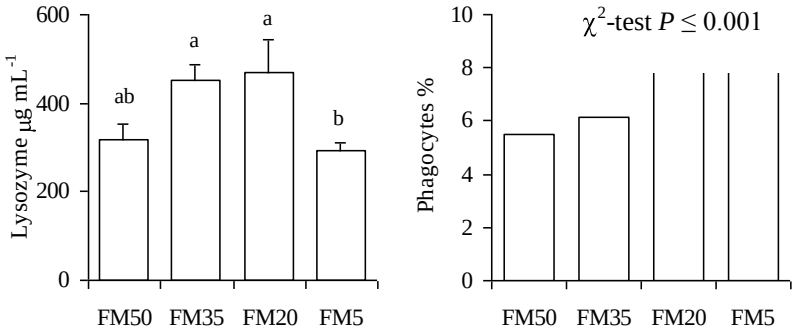

Figure 2. *Immunological parameters of turbot juveniles fed with experimental diets. Data are given as mean ± standard error of the mean. Different letters indicate significant differences among groups (P ≤ 0.05).*

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.

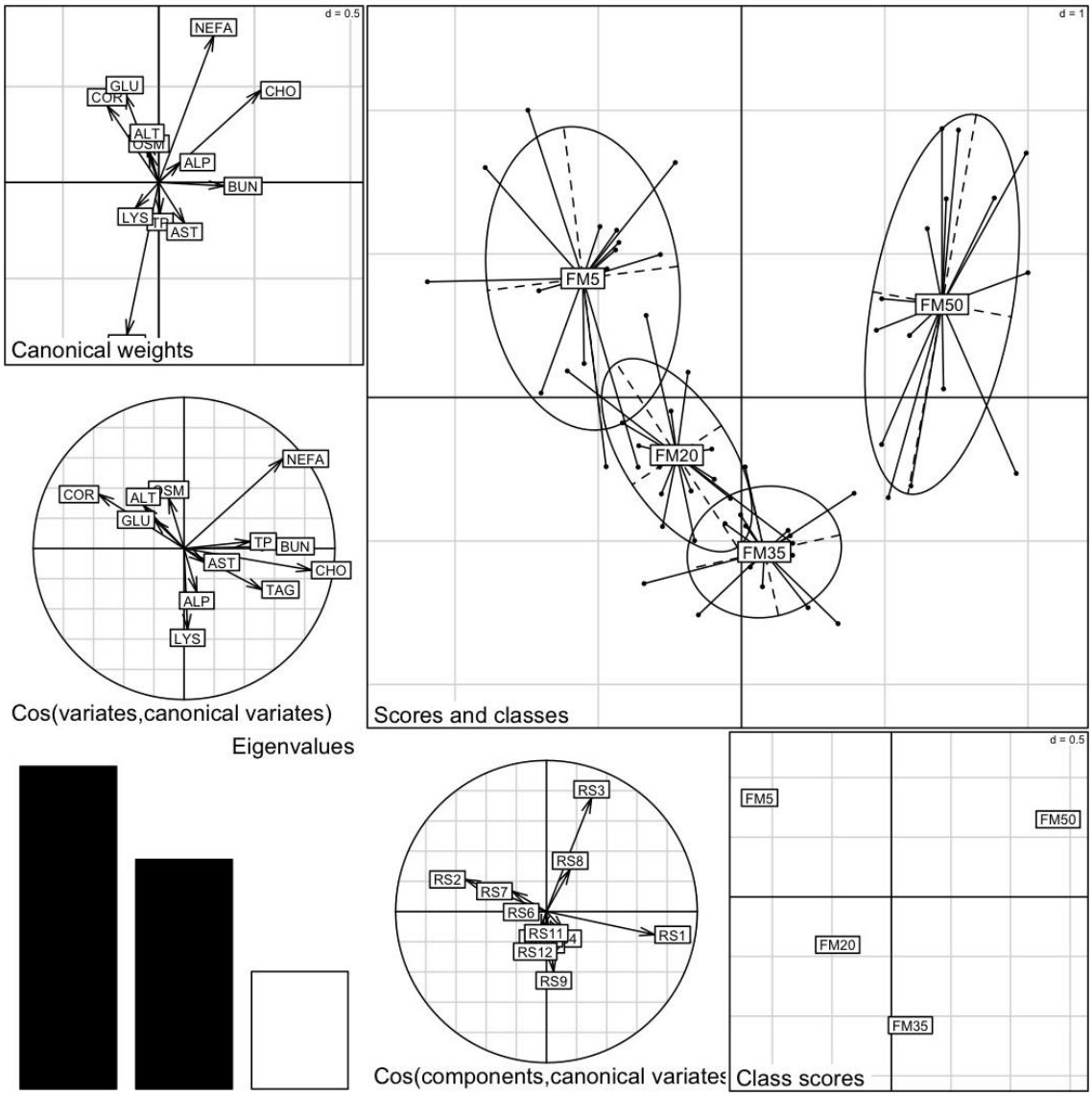

Figure 3*. Comprehensive evaluation by discriminant analysis of physiological status of turbot fed with plant protein diets (FM35, FM20, FM5) vs reference diet (FM50).*

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 and 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 and 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 and 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 (Adron and Mackie, 1978; Mackie and 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 and 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 and 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 and 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 and 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 and Bird, 2000; Roberts and 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 and 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; Burrells *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.

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Chapter 3

Effects of dietary lipid level on growth and feed utilisation of gilthead seabream (*Sparus aurata* L.) reared at Mediterranean summer temperature

Mongile F., Bonaldo A., Fontanillas R., Mariani L., Badiani A., Bonvini E., Parma L.

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Abstract

We investigated the effects of different dietary lipid levels on gilthead seabream, *Sparus aurata*, reared at Mediterranean summer temperature. Sixty fish (average weight 75 g) per tank were randomly distributed, in triplicate groups, in a recirculating rearing system (27±1°C) and fed *ad libitum* five isonitrogenous (46% dietary protein) diets with increasing lipid level (16, 18, 20, 22 and 24% named D16, D18, D20, D22 and D24, respectively), over 89 days. Specific growth rate and final body weight were not affected by dietary lipid levels. Feed conversion ratio was significantly higher (P=0.05) in D16 as compared to the other treatments, most likely due to the shortage of dietary energy supply, coped with a significantly higher voluntary feed intake. Consequently, we obtained a significantly lower protein efficiency ratio and gross protein efficiency in D16. Gross lipid efficiency was significantly higher in D16 and D18 than in the other treatments. Biometric parameters and lipase activity in gut content were not influenced by dietary treatments. In conclusion, D18 seems the most suitable diet for gilthead seabream reared at Mediterranean summer temperature, providing both the lowest fish in fish out (FIFO) ratio and a protein sparing effect, which makes gilthead seabream's production economically and environmentally more sustainable.

Introduction

Gilthead seabream, *Sparus aurata*, is a species of great interest in Europe, representing around the 51% of the total finfish marine and brackish water aquaculture production in the Mediterranean area (FAO FishStatJ, 2010). Due to the current economic downturn and the fluctuation of gilthead seabream market, aquaculture producers are focusing to improve performances and reduce costs, where feed accounts for about 60 to 80% in intensive aquaculture (Hasan *et al*., 2007).

In this context, the optimization of gilthead seabream farming by enhancing feed efficiency and the use of specific diets is a major factor in aquaculture and environmental sustainability (Bonaldo *et al*., 2010). Indeed, the optimal amount of dietary lipids would reduce the use of protein for energy production leading to a protein sparing effect, as already observed in gilthead seabream by Caballero *et al*. (1999) and to a decreased nitrogen excretion with environmental benefit (McGoogan and Gatlin, 1999). On the other hand, excess use of fish oil would lead to an increased feed price and to a worsening fish in fish out (FIFO) ratio. FIFO is defined as the efficiency at which aquaculture converts a weight-equivalent unit of wild fish into a unit of cultured fish (Merino *et al*., 2012).

Along with new feeding strategies, it is important to take into account the high water temperature of Mediterranean sea during summer period, especially as it is increasing the number of sea cages for cultured gilthead seabream. Furthermore, the average temperature of the Earth's surface has increased by about 0.8 °C over the past 100 yr for the global warming and is projected to increase by between 1.8 to 4 °C by the end of the 21st century (relative to the 1980-1999 average) (IPCC, 2007).

Most of the trials on growth performance and energy requirement in gilthead seabream have been performed between 21 and 24 °C (Aksness *et al*., 1997; Deguara, 1997; Kissil *et al*., 2000; Lupatsch *et al*., 2001, 2003; Venou *et al*., 2003; Bonaldo *et al*., 2010), though, it should be considered that water temperature of the coastal surface in the Mediterranean basin is well above this range for several weeks during summer time, reaching 26 - 27 °C or even more.

Fish are highly influenced by water temperature, which is known to affect ingestion, evacuation rate, metabolism and growth rate. The temperature at which fish growth is maximized is called the *optimum temperature for growth* and it should be noted that this optimal temperature is a few degrees lower than the temperature at which feed intake is greatest (Jobling, 1994). Over a certain temperature, rate of ingestion will decline steeply

(Jobling, 1993) with a sharply increase of fish basal metabolism and the active metabolism raise even more than the standard one (Brett 1964), generally with a consequent drop in growth (Calderer Reig, 2001). Up to a certain limit, a temperature raise, even of a few degree, has a positive effect on feed efficiency in gilthead seabream, with a much greater growth potential in spite of the increased energy requirement (Lupatsch *et al*., 2003). This latest concept has important implications in the formulations and feeding strategies in aquaculture, since a shortage of dietary energy would lead to a lower feed and protein utilization efficiency. Therefore, being lipids the main energy source in diets for carnivorous fish species (NRC, 2011), their level should be carefully assayed.

Hence, the aim of our experiment was to assess the effect of different dietary lipid levels in gilthead seabream reared at 27 ± 1 °C with the less outlay of fish oil, thus maximizing profits.

Materials and methods

Experimental diets

Ingredients and proximate composition of the experimental diets are presented in Table 1. Five isonitrogenous (46% dietary protein) diets, formulated to contain increasing fat levels (16, 18, 20, 22 and 24% named D16, D18, D20, D22 and D24, respectively), have been provided by Skretting ARC, Stavanger, Norway.

Table 1. Ingredients and proximate composition of experimental diets.

	Experimental diets				
	D16	D18	D20	D22	D24
Ingredients (g/100 g)					
Fishmeal North-Atlantic	20.00	20.00	20.00	20.00	20.00
Soy protein concentrate	20.00	20.00	20.00	20.00	20.00
Soybean meal	15.00	15.00	15.00	15.00	15.00
Wheat gluten	9.25	9.65	10.04	10.43	10.81
Sunflower meal	5.00	5.00	5.00	5.00	5.00
Corn gluten	2.00	2.00	2.00	2.00	2.00
Fish oil North-Atlantic	11.83	13.88	15.94	17.99	20.05
Wheat	15.92	13.47	11.02	8.58	6.14
Vitamin and mineral premix [1]	1.00	1.00	1.00	1.00	1.00
Proximate composition (%)					
Moisture	7.55	7.26	6.48	7.13	6.55
Crude protein	46.03	46.31	45.81	46.16	46.94
Total lipids	17.69	18.92	22.17	23.17	26.33
Ash	5.61	5.66	5.70	5.65	5.74
Gross energy (MJ/kg)	21.1	21.7	21.9	22.4	22.9
DE (MJ/kg) [2]	17.8	18.3	18.8	19.3	19.8

[1] Standard vitamin and mineral premix provided by Skretting Aquaculture Research Center, Norway.

[2] DE, digestible energy, of feed ingredients was determined according to Lupatsch *et al.* (1997), Ahmad *et al.* (2004), Kissil and Lupatsch (2004) and Lupatsch (2004).

Lipid levels were defined on the basis of a previous trial carried out in our laboratory highlighting the dietary lipid requirement in gilthead seabream fed *ad libitum*. Indeed, fish fed on 16% lipid displayed the best growth and feed utilization at 24 ± 1 °C (Bonaldo *et al*., 2010), suggesting a range of 16 to 24% lipid inclusion to cope the enhanced energy requirement at higher temperature. All feeds were produced as extruded sinking pellets with a diameter of 4 mm.

Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Science, Cesenatico, Italy. Gilthead seabream, *S. aurata*, were obtained from the Panittica Pugliese hatchery, Torre Canne di Fasano (BR), Italy. Before starting the

trial fish were acclimated at 27 ± 1 °C and fed with a mixture of the five diets for 14 d. Sixty fish per tank were weighed individually (75 ± 1.4 g initial mean body weight) and randomly assigned to 800 L square tanks with a conical bottom. Each treatment was tested in triplicate tanks. All tanks were integrated in a recirculating rearing system with a flow of 16.6 L/min/tank. The overall water renewal of the system was 5% daily. Water temperature was maintained constant at 27 ± 1 °C to simulate the water temperature of the Mediterranean Sea during summer periods. Photoperiod was held constant at a 12 h day length through artificial light (200 lx at the water surface - Delta Ohm luxmeter HD-9221, Delta-Ohm, Padua, Italy). Dissolved oxygen level was kept at 100% saturation with a liquid oxygen system connected to a software controller (B&G Sinergia snc, Chioggia, Italy).

Feed was provided to approximately 5% overfeeding. Automatic feeders distributed the feed for 7 days a week, over 89 days. The 60% of the daily ration was given in the morning between 08:30 and 09:30 and 40% in the afternoon between 15:30 and 16:30 for 1 h at a time. On Sundays, fish only get the 60% ration in the morning. During the meal, the uneaten feed was trapped by a feed collector put at the water output of tanks. In order to reduce feed leaching, collectors were emptied every 10 min. The uneaten pellets of each tank was daily gathered and dried overnight at 105 °C. Thus, the actual voluntary feed intake (VFI) was determined daily. 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 Community Council directive (86/609/ECC).

Sample collection and methods for chemical analysis

At the beginning and at the end of the experiment the fish were individually weighed and measured to determine specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (CF).

Ten fish from the initial bulk and ten fish from each tank at the end of the trial have been sampled after 1 day of starvation for chemical analyses of whole body composition and to calculate protein efficiency ratio (PER), gross protein efficiency (GPE) and gross lipid efficiency (GLE). All samples were stored at -20 °C before analysis. Moisture content was obtained by weight loss after drying samples in stove at 105 °C until constant weight. Crude protein was determined as total nitrogen (N) by using Kjeldahl method and multiplying N by 6.25. Total lipids were extracted 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, IL).

Furthermore, at the end of the experiment ten more fish per tank have been sampled for wet weight, fat viscera weight, viscera and liver weight for the calculation of fat index (FaI), viscerosomatic index (VSI) and the hepatosomatic index (HSI). Then, five fillets and two skinned fillets from each of those ten fish were sampled for the determination of fillet yield (FY) and the muscle proximate analyses, respectively.

At the end of the growth trial and all the associated samplings, the remaining groups of fish were used to determine the lipase activity from the gut content. This parameter was determined using the identical facilities and environmental conditions used in the growth trial. Gut content was collected 8 h after feeding by dissection of five animals per tank and by stripping the intestinal content into five different Eppendorf tubes (1.5 ml) respectively, prior to storage at -80 °C until quantification of lipase activity using a Lipase Assay Kit (BioVision Research Products, Mountain View, CA, USA). Briefly, lipase hydrolyzes a triglyceride substrate to form glycerol, which is quantified enzymatically by monitoring a linked change in a OxiRed probe's absorbance (λ= 500 nm). One unit of enzyme activity is defined as the amount of lipase that hydrolyzes triglyceride to yield 1 μmol of glycerol per minute at 37 °C.

Statistical analysis

Data are presented as mean ± SD of three replicate groups. All data were analyzed by one-way ANOVA with Tukey's post-hoc test. All analyses were made using the statistical package R version 2.11.1 for Windows (Revolution analytics, Palo Alto, CA, USA). Significant differences were assumed when $P \leq 0.05$.

Results and discussion

High survival (93.3 ± 0.0, 92.2 ± 2.6, 92.2 ± 4.2, 95.0 ± 4.4 and 95.6 ± 2.6% in D16, D18, D20, D22 and D24, respectively), with no significant differences, was reported in all treatments.

Water quality parameters as total ammonia nitrogen (≤ 0.1 mg/L), nitrite ($NO^{2-} \leq 0.2$ mg/L), nitrate ($NO^{3-} \leq 50$ mg/L), salinity (25 to 30 g/L) and pH (7.8 to 8.0) were held optimal and monitored daily during the whole experimental period.

The effects of different dietary lipid levels on gilthead seabream performances and nutritional indices are shown in Table 2. It is well known that at high water temperature there is an increase of fish's energy requirement, given the general effect on biochemical reactions

(Eccles 1985). In our trial the choice of setting the water temperature at 27 °C has been taken in view of the work of Rigos *et al.* (2011), whilst ensuring good growth performances (Mozes *et al.*, 2011). However, with increasing temperature there is an increase in metabolic rate and, consequently, the amount of feed required for maintenance increases. Thus, as the ratio size is gradually increased, the scope for growth is lowest at progressively higher temperature and marked increase in energy requirements for maintenance, that accompanies rising temperature, accounts for the fact that fish lose weight when fed low rations at high temperatures (Jobling, 1994). Requena *et al*. (1997) demonstrated that the metabolic rate of gilthead seabream doubled when fish were reared at 28 °C as compared to 20 °C and that a shortage of dietary energy supply would reduce growth and increase nitrogenous output as a consequence of increased protein catabolism.

In the current study, increasing dietary lipid level from 16 to 24% did not cause any significant differences in FBW and SGR. Those results are in agreement with other works (Velázquez *et al*., 2006; Bonaldo *et al*., 2010) conducted on gilthead seabream at lower temperatures, 26 and 24 °C respectively, where dietary lipid content did not exert any effect on the FBW and SGR. Conversely, VFI showed a significant increment going from the highest lipid level toward the lowest one, as if it was adjusted on the basis of the dietary lipid level. Those results are in agreement with the thesis that fish, like homeothermic animals, adapt feed intake to meet their energy requirements (Kaushik and Médale, 1994; Lupatsch *et al*., 2001; Bonaldo *et al*., 2009).

The FCR resulted significantly lower in diets from D18 to D24 as compared to the lowest lipid level diet which could be related to a deficiency of dietary lipids in the last. Moreover, this datum is confirmed by a significantly reduced PER in D16 as compared to the other groups and a significantly lower GPE in D16 when compared to D20, D22 and D24. Indeed the lower dietary lipid level in D16 led to a subsequent higher utilization of dietary protein for energy. Since protein retention is generally regulated by non-protein energy intake, PER is a good measure of the protein sparing effect of lipid (Lie *et al*., 1988). PER has been studied by several researchers in many fish species fed high energy diets where lipids represented the main energy source. In salmonids, up to 30% dietary lipid inclusion was found to improve feed and protein utilization efficiencies and to reduce N excretion (Torstensen *et al*., 2001). This effect has also been reported in tilapia (Shiau and Peng 1993), European seabass (Dias *et al*., 1998) and Atlantic halibut (Helland and Grisdale-Helland, 1998). Nevertheless, there are also some reports that have observed no protein-sparing effect of lipid in several species (McGoogan and Gatlin 1999; Ozório *et al*., 2006) including gilthead seabream (Company *et al*., 1999; Vergara *et al*., 1999; Velázquez *et al*., 2006; Bonaldo *et al*., 2010). The results of

our trial show that the increase of dietary lipids from D16 to D18 had a protein sparing effect, which did not increase further with lipid percentages beyond 18%. Consistently, significantly higher GLE values were found in fish fed the two lowest lipid diets over which any additional use of lipid resulted in a waste of energy.

FIFO ratio can be reduced both by substituting fish oil and fishmeal with plant based ingredients and/or by assessing the optimal dietary lipid level. Replacing fish oil with vegetable oil in aquafeed may affect several aspects of fish lipid metabolism as reported in several trials on gilthead seabream (Montero *et al.*, 2003; Menoyo *et al.*, 2004; Fountoulaki *et al.*, 2009). Hence, we used fish oil as the only lipid source in order to avoid any interferences or bias in data interpretation. FIFO ratio in fish fed D16 and D18 was statistically improved as compared with fish fed the two highest lipid level diets. A significant difference in FIFO ratio was also registered between D20 and D24 with the worst value for the latter. However, when considering FIFO ratio the economic efficiency ratio (ECR) should be also taken into account, as to have an economic evaluation of the diets and improving fish feeding profitability (Martínez-Llorens *et al.*, 2012). Thus, accordingly to the trend of FIFO data, fish fed D18 gave the best ECR, which resulted significantly lower as compared to fish fed D24. Our results are consistent with those obtained in previous feeding trial on the same species (Martínez-Llorens *et al.*, 2012; Bonaldo *et al.*, 2010) and on sharpsnout seabream, *Diplodus puntazzo* (Hernández *et al.*, 2007).

Table 2. Growth performance and feed utilization indices of gilthead seabream fed experimental diets.

	Dietary treatments					
	D16	D18	D20	D22	D24	P
IBW[1]	75.3 ± 1.3	74.4 ± 0.3	76.2 ± 0.7	74.9 ± 2.8	75.3 ± 0.5	-
FBW[2]	296.7 ± 12.8	303.9 ± 4.5	300.0 ± 4.1	293.8 ± 7.0	289.8 ± 12.5	NS
SGR[3]	1.54 ± 0.05	1.58 ± 0.02	1.54 ± 0.02	1.54 ± 0.03	1.51 ± 0.04	NS
VFI[4]	333.41 ± 16.17^{a}	318.91 ± 4.97^{ab}	309.73 ± 7.31^{abc}	301.50 ± 5.99^{bc}	293.41 ± 8.98^{c}	≤ 0.01
FCR[5]	1.53 ± 0.03^{a}	1.38 ± 0.03^{b}	1.37 ± 0.03^{b}	1.38 ± 0.00^{b}	1.38 ± 0.06^{b}	≤ 0.001
PER[6]	1.44 ± 0.02^{b}	1.55 ± 0.06^{a}	1.58 ± 0.02^{a}	1.57 ± 0.01^{a}	1.56 ± 0.06^{a}	≤ 0.01
GPE[7]	24.86 ± 0.58^{b}	26.23 ± 0.72^{ab}	27.33 ± 0.55^{a}	27.31 ± 0.56^{a}	26.69 ± 0.68^{a}	≤ 0.01
GLE[8]	69.86 ± 0.93^{a}	69.66 ± 3.00^{a}	56.63 ± 4.08^{b}	55.96 ± 6.27^{b}	49.76 ± 3.24^{b}	≤ 0.001
FIFO[9]	1.75 ± 0.04^{c}	1.70 ± 0.04^{c}	1.79 ± 0.04^{bc}	1.91 ± 0.00^{ab}	2.02 ± 0.08^{a}	≤ 0.001
ECR[10]	1.52 ± 0.02^{ab}	1.45 ± 0.05^{b}	1.49 ± 0.02^{ab}	1.53 ± 0.01^{ab}	1.56± 0.06^{a}	≤ 0.05

[1] IBW, initial body weight (g); [2] FBW, final body weight (g); [3] SGR, specific growth rate (%/day) = 100 x (ln final body weight - ln initial body weight)/(duration of experiment, days); [4] VFI, voluntary feed intake (g fish^{-1}) = (total feed intake, g)/fish; [5] FCR, feed conversion ratio = (dry feed consumed, g/wet weight gain, g); [6] PER, protein efficiency ratio = (fish weight gain including weight of dead fish, g)/(total protein intake, g); [7] GPE, gross protein efficiency = 100 x ((% final body protein x final body weight) - (% initial body protein x initial body weight))/ (total protein intake, g); [8] GLE, gross lipid efficiency = 100 x ((% final body lipid x final body weight) - (% initial body lipid x initial body weight))/ (total lipid intake, g); [9] FIFO, fish in fish out ratio = (fish oil content + fish meal, g)/(yield of FO from wild fish + yield of FM from wild fish, %) x FCR (Jackson, 2010); [10] ECR, economic efficiency ratio (€/kg) = ((total feed intake, g) x (feed cost, € kg^{-1}))/(weight gain, g). Each value is the mean ± SD of three replicates. Different letters (abc) in the same row denote significant ($P \leq$ 0.05) differences among treatments. *P* values are also given.

Whole-body composition, fillet composition and biometric parameters are shown in Table 3. As reported in gilthead seabream (Vergara *et al*., 1996, 1999) and in other marine fish species (Péres and Oliva-Teles, 1999; Luo *et al*., 2005), high dietary lipid levels are known to increase fat deposition in visceral cavity and muscle tissues. However, this tenet is not in accordance with our results and those of Marais and Kissil (1979), Velázquez *et al*. (2006) and Bonaldo *et al*. (2010), where no correlation was observed between dietary lipid content and fat deposition in visceral cavity and muscle tissue. Neither the fillet nor the body composition were influenced by dietary treatments and fillet proximate composition resulted consistent with that found by Testi *et al*. (2006) and Bonaldo *et al*. (2010). In the present study, a slight negative correlation between HSI and dietary lipid content was observed, where only D16 was significantly different from D22, perhaps for the restrained range of variation. However, our results are in agreement with previous findings on the same species (Venou *et al*., 2006; Couto *et al*., 2008; Bonaldo *et al*., 2010) and on other species like Sunshine bass (Hutchins *et al*., 1998) and European seabass (Peres and Oliva-Teles, 1999). This pattern may be due to the higher content of starch in low lipid diets, which could stimulate a *de novo* lipid synthesis and deposition in the liver (Evans *et al*., 2005) or increase hepatic glycogen deposition (Wilson, 1994; Couto *et al*., 2008; Coutinho *et al*., 2012). Nevertheless, the VSI was not significantly affected by dietary lipid levels even thought a slight sloping trend was observed from D20 towards D24, most likely correlate to the HSI values. The dietary treatments did not affect the CF, FaI and FY. Moreover, the FaI of our treatments was lower as compared to those found in previous trials on the same species (Martínez-Llorens *et al.*, 2007; Benedito-Palos *et al.*, 2007; Fountoulaki *et al.*, 2009; Bonaldo *et al.*, 2010) and on sharpsnout seabream (Piedecausa *et al.*, 2007), regardless of the lipid source and the inclusion level, whereas FY was consistent with those of Bonaldo *et al.* (2010) or higher as compared to Fountoulaki *et al.* (2009) and Martínez-Llorens *et al.* (2007). The lipase activity remained almost unchanged among the five treatments showing a slight inverse correlation with lipid level, as expected. Indeed, a similar pattern was found on sea bass larvae (Morais *et al.*, 2004) where it was suggested an adaptive response with lower lipase secretion in fish fed diets containing higher content of digestible lipids, whereas in Senegalese sole (Borges *et al.*, 2013) different dietary lipid levels did not exert any effect on lipase activity.

Table 3. Proximate composition of carcass and fillet and biometric parameters of gilthead seabream fed experimental diets.

	Initial carcass composition	Dietary treatments					P
		D16	D18	D20	D22	D24	
Carcass (%)							
Moisture	65.86 ± 0.31	62.38 ± 0.28	62.85 ± 0.28	62.46 ± 0.53	62.97 ± 1.10	63.18 ± 0.56	NS
Crude protein	17.26 ± 0.13	17.23 ± 0.12	16.96 ± 0.13	17.29 ± 0.18	17.32 ± 0.23	17.16 ± 0.17	NS
Total lipids	15.46 ± 0.27	17.80 ± 0.27	17.59 ± 0.14	16.87 ± 0.92	17.23 ± 1.52	17.27 ± 0.60	NS
Ash	4.44 ± 0.08	2.91 ± 0.08	2.71 ± 0.07	3.07 ± 0.18	2.75 ± 0.11	2.93 ± 0.14	NS
Fillet (%)							
Moisture		69.69 ± 0.50	68.62 ± 0.04	69.47 ± 0.94	69.28 ± 0.65	69.09 ± 0.66	NS
Crude protein		20.55 ± 0.50	20.40 ± 0.02	20.08 ± 0.18	20.15 ± 0.28	20.26 ± 0.52	NS
Total lipids		8.64 ± 0.87	9.47 ± 0.34	8.64 ± 0.83	9.45 ± 1.16	9.37 ± 1.22	NS
Ash		1.35 ± 0.04	1.36 ± 0.02	1.38 ± 0.03	1.36 ± 0.04	1.39 ± 0.02	NS
Biometric parameters							
CF[1]		1.77 ± 0.17	1.79 ± 0.15	1.76 ± 0.21	1.77 ± 0.17	1.79 ± 0.17	NS
VSI[2]		5.50 ± 0.74	5.47 ± 0.89	5.50 ± 0.96	5.26 ± 0.84	5.14 ± 0.90	NS
HSI[3]		1.40 ± 0.32[a]	1.37 ± 0.31[ab]	1.34 ± 0.31[ab]	1.18 ± 0.31[b]	1.19 ± 0.29[ab]	≤ 0.05
Fal[4]		1.47 ± 0.67	1.65 ± 0.68	1.61 ± 0.92	1.58 ± 0.82	1.40 ± 0.73	NS
FY[5]		48.97 ± 3.57	50.33 ± 2.73	49.89 ± 2.72	49.46 ± 2.87	48.70 ± 3.59	NS
Lipase activity[6]		0.70 ± 0.36	0.54 ± 0.24	0.51 ± 0.26	0.40 ± 0.24	0.50 ± 0.25	NS

Carcass moisture, protein, lipid and ash (% wet weight), n= one pool of ten fish per tank.

Fillet moisture, protein, lipid and ash (% wet weight), n= one pool of two fish per tank.

[1] CF, condition factor = 100 x(body weight, g)/(body length, cm)^3, n= one pool of 60 fish per tank.

[2] VSI, viscerosomatic index (%) = 100 x (viscera weight, g)/(whole body weight, g), n= ten fish per tank.

[3] HSI, hepatosomatic index (%) = 100 x (liver weight, g)/(whole body weight, g), n= ten fish per tank.

[4] Fal, fat index (%) = 100 x (visceral fat weight, g)/(whole body weight, g), n= ten fish per tank.

[5] FY, fillet yield (%) = 100 x (fillet weight, g)/(whole body weight, g), n= ten fish per tank.

[6] Lipase activity (one unit of enzyme activity is defined as the amount of lipase that hydrolizes trygliceride to yeld 1 μmol of glycerol per minute at 37 °C) , n= five fish per tank.

Data are shown as mean ± SD. Different letters (ab) in the same row denote significant ($P \leq 0.05$) differences among treatments. *P* values are also given.

Conclusion

In conclusion, D18 seems the most suitable diet for gilthead seabream fed *ad libitum* at Mediterranean summer temperature. Indeed, our results show that D16 and D18 allow a reduction in the FIFO ratio but an increase of lipids from 16% to 18% exerted a protein sparing effect making gilthead seabream's production economically and environmentally more sustainable.

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Chapter 4

Growth and feed utilization of common sole (*Solea solea* L.) juveniles fed diets with increasing lipid levels

Mongile F., Bonvini E., Bonaldo A., Parma L., Gatta P.P.

Abstract

Knowledge on the nutritional requirements of common sole (*Solea solea*) is limited and no information about the optimal dietary lipid level is available, yet. This study was undertaken to assess the growth response and the feed utilization of common sole juveniles fed graded dietary lipid levels. Four isonitrogenous (59% protein) pelletized diets with increasing dietary lipid levels (8%, 12%, 16% and 20% named L8, L12, L16 and L20, respectively) were fed to triplicate fish groups of 80 individuals (13.8 g) to apparent satiation, over 150 days. At the end of the trial, fish samples were taken to assess growth performance, whole body composition, feed utilization indices, biometric parameters and serum metabolites. One-way ANOVA with Tukey's *post hoc* test were used to analyze data ($P \leq 0.05$), while a linear regression analysis was conducted to analyze growth performance and feed utilization indices against the dietary lipid inclusion. At the end of the trial, final body weight resulted significantly higher in fish fed L8, followed in a decreasing order by those fed L12, L16 and L20, respectively. Specific growth rate was higher in fish fed L8 and L12 as compared to the other treatments, with the lowest value in fish fed L20. Feed intake linearly decreased by increasing dietary lipid level, with all treatments differing one to another. Feed conversion rate of fish fed L20 was significantly higher as compared to the other groups. Consistently, protein efficiency ratio and gross protein efficiency resulted significantly lower in fish fed L20 while no significant differences were observed among the other treatments. Gross lipid efficiency was linearly related to the dietary lipid level, where L8 and L12 groups resulted significantly different between them and as compared to the other groups. Viscerosomatic index resulted significantly higher in fish fed L16 and L20 than in those fed L8 and L12, while no differences were found in the hepatosomatic index. In conclusion, the results of this trial evidenced the low dietary lipid requirement of common sole juveniles. Increasing dietary lipid level over the 8% led to a substantial decline in growth performance and feed utilization indices. Those results should be taken into consideration when formulating specific practical diets for this species.

Introduction

Common sole (*Solea solea*) is a promising flatfish species for marine farming, especially due to its high market value, good flesh quality and increasing demand by consumers (Parma *et al*., 2013). Over the last years, various aspects of its culture have been developed and optimized, particularly concerning weaning techniques and larvae-culture (Bonaldo *et al*., 2011; Lund *et al*., 2008; Parma *et al*., 2013). Several studies on common sole juveniles and adults have been carried out in order to investigate on optimal growth temperature (Schram *et al*., 2013), stocking density (Lund *et al*., 2013; Schram *et al*., 2006), feeding behavior (Reig *et al*., 2003), dietary n-3 long-chain fatty acid and stress (Logue *et al*., 2000), as to reach a large scale production of this species. Still, the poor growth performance remains one of the most important constraints for the farming feasibility of common sole (Mas-Muñoz *et al*., 2011). The main reason, is the lack of specific formulated diets for the on-growing stage, which make difficult to achieve a proper feed utilization in this species.

The protein requirement for maximum growth in common sole juveniles has been estimated at 57% (Gatta *et al*., 2011) but, to our knowledge, no studies concerning the optimal dietary lipid level have been carried out. Some authors investigated the lipid requirement of Senegalese sole (*Solea senegalensis*), a common sole close related species, showing no effect on growth and feed utilization when fed high lipid diets (Dias *et al*., 2004; Valente *et al*., 2011) or even leading to a depression of performance parameters beyond a certain dietary lipid inclusion (Borges *et al*., 2009). Still, it is inappropriate to rely on data from other sole species because of the differences on growth performance, optimal thermal regimen, broodstock behavior and ecological niche (Imsland *et al*., 2003; Palazzi *et al*., 2006).

Thus, since dietary lipid and protein are recognized as key factors influencing both adequate fish nutrition and feeding costs (Watanabe, 2002) and considering the lack of specific knowledge on dietary lipid requirement on common sole juveniles, the effects of graded dietary lipid levels on growth and feed utilization have been investigated.

Materials and methods

Experimental diets

Four isonitrogenous (59%) diets were formulated to contain 8% (L8), 12% (L12), 16% (L16) and 20% (L20) dietary lipid level. Increasing dietary lipids were obtained by increasing

fish oil inclusion and lowering the amount of wheat meal. The mineral-vitamin mix formulation was the same used on Senegalese sole by Guerreiro *et al*. (2012).

Ingredients were mixed and pelleted dry without steaming, using a laboratory pelleting machine (La Monferrina, Asti, Italia) with a 1.0 mm die. Moreover, diet L20 was further greased apart. The diets were dried at 40 °C for 24 h and stored in a refrigerator (4 °C) until use.

Table 1. Ingredients and proximate composition of experimental diets

	Experimental diets			
	L8	L12	L16	L20
Ingredients (%)				
Fishmeal	35.0	35.0	35.0	35.0
Wheat meal	18.8	14.8	10.8	6.8
Mussel meal	5.0	5.0	5.0	5.0
Fish oil	0.0	4.0	8.0	12.0
Pea protein concentrate	20.0	20.0	20.0	20.0
Wheat gluten	20.0	20.0	20.0	20.0
Vitamins/ mineral mixture[1]	1.0	1.0	1.0	1.0
Choline	0.2	0.2	0.2	0.2
Proximate composition (%)				
Crude protein	60.2	59.6	59.8	57.8
Total lipid	8.0	12.6	15.5	19.4
Ash	7.5	7.4	7.4	7.0
Moisture	3.8	4.5	4.5	3.9
Gross energy (KJ/g)	19.9	21.1	22.2	23.6

[1] Vitamins (mg kg^{-1} diet or specified): retinol acetate, 18 000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 400; inositol, 400, choline chloride (50%), 2000. Minerals (mg kg^{-1} diet or specified): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).

Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Science, University of Bologna, Italy. Common sole (*Solea solea*) juveniles were obtained from natural spawning of a captive broodstock maintained at the above mentioned facilities. Before the beginning of the trial, fish were kept at experimental condition for 3 months, being fed a commercial fishmeal based diet (Aller Futura ex, Denmark, crude protein 64%, total lipids 12%). Before starting the experiment, fish have been fed with a mixture of the 4 experimental diets for one week. At the beginning of the trial, 80 fish (initial mean weight: 13.8 ± 0.4 g) per tank were randomly distributed into twelve 500 liter square flat bottom tanks (bottom surface: 0.64 m^2). Each diet was administered to triplicate groups, assigned in a completely random manner, over 150 days. Tanks were provided with natural seawater and connected to a closed recirculating system (overall water volume capacity 7000 liter). The rearing system consisted of a mechanical sand filter (0.4 m^3 of silica sand, 0.4-0.8 mm. PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain) an ultraviolet lights (PE 25mJ/cm^2: 16m^3 h^{-1}, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain). The water exchange rate per tank was 100% every 2 h while the overall water renewal of the system was 5% daily. During the trial, temperature was kept constant at 20.0 ± 1.0 °C and photoperiod was maintained at 12 h light : 12 h dark through artificial light (50 lx at the water surface, Delta Ohm lightmeter HD2302.0; Probe LP 471 PHOT; Delta Ohm, Padua, Italy). Oxygen level was kept constant (7.5 ± 1.0 ppm) by a liquid oxygen system connected to a software (B&G Sinergia snc, Chioggia, Italy), while a strong aeration (200 l min^{-1}) was applied in the stock tank to remove CO_2. Ammonia (total ammonia nitrogen ≤ 0.1 mg L^{-1}), nitrite (≤ 0.2 mg L^{-1}) and salinity (20 g L^{-1}) were daily monitored spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany). Sodium bicarbonate was added on a daily basis to keep pH constant at 7.8 - 8.0.

During the first 35 days, fish were hand fed twice a day for 90 min a meal and once on Sundays, to apparent satiation. After the 35th day, fish were fed by automatic feeders over 20 h per day, until the end of the trial. The daily ration was equally divided into 2 meals (9.00–17.00 and 17.30-5.30 h). On Sundays, fish only get the 70% ration, over 12 h (9.00-21.00 h). The feed ration was daily adjusted on the basis of feed losses, in each tank. When uneaten feed pellet was observed at the end of the meal, the daily ration was reduced by 10%, until no feed losses were recorded. When no feed losses were observed at the end of the meal, the daily ration was fixed for 4 days and then augmented by 10%, till the end of trial (Borges *et al*., 2009). Uneaten feed, when present at the end of the meal, was removed and estimated by

counting each pellet then multiplying the number with the mean weight of a single pellet, the which result was deducted from the daily feed intake of the tank.

Sample collection and chemical analysis

At the beginning and at the end of the experiment, fish were individually weighed (g) and total length (cm) was recorded. Total biomass was also determined at day 35, 70 and 105 by bulk weighing.

Carcass proximate composition was determined on a pooled sample of thirty fish before the start of the experiment and on a pooled sample of ten fish per tank at the end of the trial. Furthermore, at the end of the trial, wet weight, viscera and liver weight were individually recorded from ten fish per tank to determine viscerosomatic and hepatosomatic indexes (VSI and HSI, respectively). All fish sampled were killed by an overdose of phenoxyethanol.

In order to minimized stress caused by manipulation and to have a normally metabolizing fish, the remaining animals in each tank were fed their respective diets for 3 more days for the blood sampling. Thus, blood samples were collected from the caudal vein on 7 fish per tank 5 h postprandial. Blood samples of each tank were pooled into one sample (3 per treatment) then allowed to clot before being centrifuged (3000 g for 10 min at 4 °C). Serum aliquots were stored at 4 °C and analyzed the same day for the determination of cholesterol and triglycerides concentrations.

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.

Diets and whole body samples were analyzed after homogenization for proximate composition. Moisture content was obtained by weight loss after drying samples in stove at 105 °C until constant weight. Crude protein was determined as total nitrogen (N) by using Kjeldahl method and multiplying N by 6.25. Total lipids were extracted 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, IL, USA).

Calculations

Data on initial and final body weight, feed consumption, proximate composition of diets and carcass were used to calculate the average body weight (ABW), specific growth rate

(SGR), feed conversion ratio (FCR), feed intake (FI), VSI, HSI, protein efficiency ratio (PER), gross protein efficiency (GPE) and gross lipid efficiency (GLE). The survival rate (SR) was calculated as a percentage of the initial number of fish.

The growth performance indices were calculated as follow: ABW (g) was calculated as ABW = (final body weight + initial body weight) / 2; SGR (% day^{-1}) was calculated as SGR = 100 × (ln W_{fin} − ln W_{in}) / t, where W_{fin} and W_{in} represent final and initial body weights (tank means), respectively, and t the duration of the experiment, expressed in days; FI (% day^{-1}) was calculated as FI = 100 x (feed consumed / ABW / 2 / days).

The feed utilization indices were calculated as follow: the FCR was calculated as FCR = feed consumed, g / weight gain, g; PER was calculated as PER = (weight gain, g / total protein intake, g); GPE (%) was calculated as GPE = 100 x [(% final body protein × final body weight, g) − (% initial body protein × initial body weight, g)] / total protein intake, g; GLE (%) was calculated as GLE = 100 x (% final body lipid × final body weight, g) - (% initial body lipid × initial body weight, g)] / total lipid intake, g.

The somatic indices were calculated as follow: VSI (%) was calculated as VSI = 100 x (viscera weight, g / body weight, g); HSI (%) was calculated as HSI = 100 x (liver weight, g / body weight, g).

Statistical analysis

All data are presented as mean ± standard deviation (SD) of three replicate groups. All data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. All statistical analyses were performed using the statistical package R version 2.15.3 for Windows (Revolution analytics, Palo Alto, CA, USA). The differences between treatments were considered significant at $P \leq 0.05$. A linear regression analyze was conducted to analyze growth performance and feed utilization indices according to the dietary lipid level and judged by the coefficient of determination (R^2).

Results

High survival with no statistical differences was observed in all treatments.

Data on growth performance and feed utilization are summarized in Table 2. At the end of the 150 days feeding trial, a linear tendency for an overall growth improvement was observed

in fish fed lower dietary lipid level. The FBW resulted significantly higher in fish fed L8 (40.7 ± 1.7 g), followed by those fed L12, L16 and L20 (35.1, 27.9 and 22.1 g, respectively).

Table 2. Growth performance and feed utilization indices of common sole juveniles fed experimental diets.

	Experimental diets						
	L8	L12	L16	L20	Linear regression line	R^2	P
IBW[1]	14.0 ± 0.0	13.5 ± 0.4	13.6 ± 0.4	14.0 ± 0.2			
FBW[2]	40.7 ± 1.7^{d}	35.1 ± 1.2^{c}	27.9 ± 2.5^{b}	22.1 ± 0.3^{a}	Y = 53.58 - 1.582x	0.96	< 0.0001
SGR[3]	0.71 ± 0.03^{c}	0.63 ± 0.03^{c}	0.48 ± 0.05^{b}	0.30 ± 0.01^{a}	Y = 1.015 - 0.035x	0.94	< 0.0001
FI[4]	0.88 ± 0.01^{d}	0.78 ± 0.02^{c}	0.69 ± 0.02^{b}	0.63± 0.01^{a}	Y = 1.037 – 0.021x	0.97	< 0.0001
FCR[5]	1.32 ± 0.05^{a}	1.35 ± 0.04^{a}	1.63 ± 0.11^{a}	2.66 ± 0.29^{b}	Y = 0.233 + 0.108x	0.72	0.0003
PER[6]	1.24 ± 0.05^{b}	1.26 ± 0.05^{b}	1.11 ± 0.10^{b}	0.82 ± 0.03^{a}	Y = 1.601 – 0.0352x	0.71	0.0004
GPE[7]	21.97 ± 0.91^{b}	22.18 ± 1.10^{b}	19.16 ± 2.02^{b}	12.95 ± 1.88^{a}	Y = 29.59 – 0.752x	0.70	0.0004
GLE[8]	66.37 ± 7.29^{c}	50.43 ± 8.42^{b}	27.31 ± 2.65^{a}	30.01 ± 1.17^{a}	Y = 89.80 – 3.305x	0.77	0.0001

[1]IBW, initial body weight (g); [2]FBW, final body weight (g); [3]SGR, specific growth rate (% day^{-1}) = 100 x (ln final body weight - ln initial body weight) / (duration of experiment, days); [4]FI, feed intake = 100 x (feed consumed, g / ABW / 2 / duration of experiment, days); [5]FCR, feed conversion ratio = (feed consumed, g / weight gain, g); [6]PER, protein efficiency ratio = (weight gain g) / (total protein intake, g); [7]GPE, gross protein efficiency = 100 x ((% final body protein x final body weight) - (% initial body protein x initial body weight)) / (total protein intake, g); [8]GLE, gross lipid efficiency = 100 x ((% final body lipid x final body weight) - (% initial body lipid x initial body weight)) / (total lipid intake, g). Each value is the mean ± SD of three replicates. Different superscript letters (abcd) in the same row denote significant ($P \leq 0.05$) differences among treatments. Linear regression lines (where Y is the response and x is the dietary lipid level in diets, ranging from 8 to 20); R^2 and P are also given.

The SGR resulted significantly higher in L8 and L12 (0.71 and 0.63%, respectively) as compared to L16 and L20 (0.48 and 0.30%, respectively). The FI was inversely related to the lipid level, with the higher values corresponding to the lower lipid level treatments. The FCR ranged from 1.32 (L8) to 2.66 (L20) with no significant differences between fish fed the three lower lipid diets.

Regarding the protein efficiency, PER and GPE resulted statistically higher in L8, L12 and L16 fed groups as compared to L20 one. The GLE resulted significantly affected by the dietary lipid levels, with L8 statistically higher than the other treatments, followed by L12, while L16 and L20 showed the statistically lower values among treatments.

Data on VSI, HSI and whole body composition are shown in Table 3. The VSI varied from 3.95 to 4.75% resulting significantly higher in fish fed the high-lipid diets (L16 and L20) than in those fed the low-lipid ones (L8 and L12). No differences were found in the HSI. Whole body protein content was significantly lower in L20 and there were no statistical differences between L8, L12 and L16. Whole body lipid content ranged between 5.4% (L16) and 7.3% (L20), with the two highest dietary lipid treatments statistically different as compare to each other and to L8 (6.2%) and L12 (6.8%).

Table 3. Somatic indices and whole body composition of common sole juveniles fed experimental diets.

	Experimental diets			
	L8	L12	L16	L20
Somatic indices				
VSI[1]	4.04 ± 0.36[a]	3.95 ± 0.32[a]	4.57 ± 0.19[b]	4.55 ± 0.23[b]
HSI[2]	1.68 ± 0.15	1.46 ± 0.16	1.63 ± 0.17	1.54 ± 0.26
Whole body composition (%)				
Protein	17.4 ± 0.19[a]	17.2 ± 0.41[a]	17.0 ± 0.38[a]	16.3 ± 0.57[b]
Lipid	6.2 ± 0.55[b]	6.8 ± 0.67[cb]	5.4 ± 0.34[a]	7.3 ± 0.25[c]
Moisture	73.5 ± 0.22[b]	72.6 ± 0.37[c]	72.7 ± 0.64[c]	74.5 ± 0.41[a]
Ash	2.3 ± 0.09[b]	2.7 ± 0.13[a]	2.1 ± 0.16[b]	2.9 ± 0.31[a]

[1] VSI, viscerosomatic index (%) = 100 x (viscera weight, g)/(whole body weight, g), n= ten fish per tank.

[2] HSI, hepatosomatic index (%) = 100 x (liver weight, g)/(whole body weight, g), n= ten fish per tank.

Data are shown as mean ± SD. Different letters (abc) in the same row denote significant ($P \leq 0.05$) differences among treatments.

Serum cholesterol and triglycerides concentration (g/l) are shown in Figure 1. No significant differences were detected among the serum metabolites selected, with the cholesterol level ranging between 2.29 to 2.78 g/l, whereas triglycerides ranged between 7.83 to10.15 g/l, with the lowest value registered in the L20 treatment.

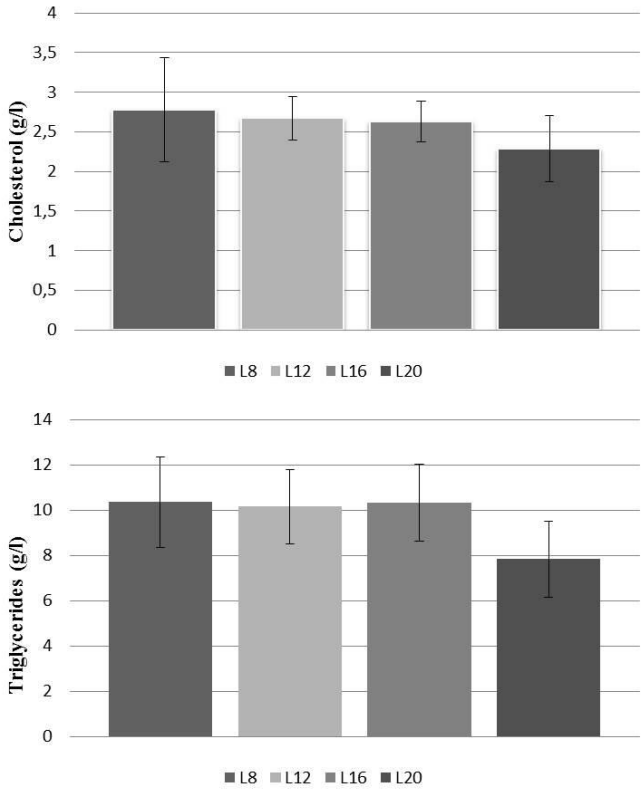

***Figure 1**. Serum triglycerides and cholesterol levels 5 h postprandial. Data are given as the mean ± SD of triplicate groups.*

Discussion

In our trial, increasing lipid levels over 8% resulted in poor growth and feed utilization. Growth depression, when fish are fed high-lipid diets, has been previously reported on other flatfish species like on Senegalese sole (Borges *et al*., 2009), turbot (*Psetta maxima*) (Regost *et al*., 2001) and halibut (*Hippoglossus hippoglossus*) (Hamre *et al*., 2003). Still, Dias *et al*. (2004) reported no negative effect on Senegalese sole fed 11% and 21% dietary lipid level, respectively. Nowadays, increasing the non-protein energy sources in aquafeed has become a common practice, as to spare protein for retention purposes, like for parr (*Salmo salar*) (Nordgarden *et al*., 2002), Pacific bluefin tuna juveniles (*Thunnus orientalis*) (Biswas *et al*., 2009) and gilthead seabream (*Sparus aurata*) (Mongile *et al*., 2014).

One of the main reason leading to a poor growth at increasing dietary lipid levels should be ascribed to a decreased FI. Different authors reported palatability of feed as a major factor determining feed acceptance (Glencross *et al*., 2007; de la Higuera, 2001). Indeed, ingredients' palatability is to be considered of major importance because, irrespective to the nutrient digestibility of a particular feedstuff, if this latest acts as a repellent by reducing the FI, it would result of limited use in feed formulation (Glencross *et al*., 2007; de la Higuera, 2001). Accordingly to our results, Reig *et al*. (2003) stated that fish oil may act as a repellent due to its strong organoleptic features, for a species like sole. Common sole trophic profile, indeed, is mainly based on polichaete, mollusks and crustaceans while fish result uncommon in his natural diet (Cabral 2000). Conversely, Borges *et al*. (2009) in a feeding trial on Senegalese sole reported a good acceptability of diets where lipid fraction was mostly represent by fish oil till a 16% dietary inclusion. A further possible explanation of the decreased FI at increasing dietary lipid levels, might be sought on the thesis that fish, like homoeothermic animals, adapt feed intake to meet their energy requirements (Kaushik and Médale, 1994; Lupatsch *et al*., 2001; Bonaldo *et al*., 2009; Mongile *et al*., 2014). Though, it has not been possible to assess the energy intake through a digestibility test, due to the difficulties of collecting sole's feces, which are semiliquid and dissolve very fast in water (Borges *et al*., 2009; Guerreiro *et al*., 2012). Meanwhile, rely on the use of caloric conversion coefficients to assume the digestible energy intake in fish is strongly discouraged, since may lead to serious error (Jobling, 1983). For future similar studies, according to Sáenz de Rodrigáñez *et al*. (2011), an *in vitro* species-specific digestibility test for the estimation of the protein nutritional quality of raw ingredients could be an alternative solution.

As well as for a reduction of FI, the lower growth at increasing dietary lipid levels has to be ascribed to the poorer feed utilization. The significantly negative effect on feed utilization in fish fed L20 was clear, having marred all indices in comparison with the other groups. PER and GPE resulted significantly improved when fish were fed below the 20% dietary lipid level and the GLE showed the best values in fish fed L8, over which any additional use of lipid resulted in a progressive waste of energy. Those results are in agreement with a previous study on Senegalese sole, where high-lipid diets negatively affected growth performance and feed utilization (Borges *et al*., 2009). Conversely, no negative effects were detected on similar trials were Senegalese sole were fed diets with low and high lipid diets, respectively (Dias *et al*., 2004; Borges *et al*., 2013). Those author, demonstrated that lipids are not efficiently used for energetic purposes and protein sparing, both when dietary protein content is kept constant either when dietary protein is below the requirement of this species. Consistently, Regost *et al*. (2001) reported that turbot fed diets over 15% dietary lipid inclusion performed poorly

without any protein sparing effect. However, no negative effects were reported in Atlantic halibut juveniles when fed diets beyond 14% lipid inclusion (Martins *et al*., 2007), while in plaice (*Pleuronectes platessa*), another flatfish species, high level of lipids in the diet led to even greater growth and protein utilization (Cowey *et al*., 1975).

The whole body composition was significantly affected by dietary treatments. Protein content resulted significantly lower in L20 as compared to the other treatments, providing additional evidence that dietary lipids do not favors protein accretion in this species. Common sole is considered a lean fish with a low whole body lipid content (Parisi *et al*., 2013), mostly attributable to his quite passive behavior and the low daily metabolic budget, which might also explain the poor lipid utilization (Borges *et al*., 2013). In this study, fish fed L8 and L12 showed a whole body lipid content in agreement with previous study on common sole juveniles (Gatta *et al*., 2011; Mongile *et al*., unpublished data), while the values obtained in fish fed L16 and L20 are controversial, showing the lowest and the highest values among treatments. In a recent similar trial on Senegalese sole, Borges *et al*. (2009) found that whole body lipid content was directly related to the experimental fed diets. Such effect, has been widely reported for several fish species like salmonids (Hillestad and Johnen 1994), European sea bass (*Dicentrarchus labrax*) (Peres and Oliva-Teles 1999), gilthead seabream (Vergara *et al*., 1999) and Atlantic halibut (Aksnes *et al*., 1996; Berge and Storebakken 1991; Martins *et al*., 2007). Consistently, in this trial L20 showed the highest body lipid content as compared to the other treatments.

The unaffected HSI among treatments, is in accordance with a previous experiment on Senegalese sole, where fish fed 4 and 17% lipid level diets did not have any changes at the HSI level (Borges *et al*., 2012). Concerning the VSI, the higher values found in the two higher lipid level treatments, may be explained as an increased periviceral fat accumulation. Accordingly, a similar trend was observed in Senegalese sole fed low and high dietary lipid diets (Borges *et al*., 2012). Moreover Borges *et al*. (2009) reported a two fold increase in intestinal lipid content in Senegalese sole fed 16 and 20% dietary lipid levels as compared to those fed 4% lipid diets.

Selected serum metabolites (cholesterol and triglycerides) values did not indicate any significant effect on lipid metabolism, even if L20 group showed the lowest values as compared to the other treatments. The lower serum triglycerides concentration may be explained as a sign of decreased lipolytic activity in stress-sensitive fish (Vanraaij *et al*., 1996; Van den Thillart *et al*., 2002), as already reported on common carp (*Ciprinus carpio*) juveniles fed high dietary lipid level compared to those fed lower lipid level diets (Poleksić *et*

al., 2013). Conversely, in an experimental trial on Senegalese sole, plasma triglyceride concentrations resulted significantly higher at 5 h postprandial in fish fed 17% lipid diet as compared to those fed a diet containing 5% dietary lipid (Borges *et al*., 2013). Anyway, serum metabolites values ranged within previously reported levels on Senegalese sole fed high and low lipid diets (Valente *et al*., 2011; Borges *et al*., 2013).

Conclusion

In conclusion, the results of this trial evidenced a low lipid requirement by common sole juveniles. Dietary lipids do not seem to be a good energy source for promoting growth and there is no evidence of a protein-sparing effect by increasing dietary lipids over the 8%. Furthermore, increasing dietary lipid level led to a substantial decline in performances and this should be taken into consideration when formulating specific practical diets for this species.

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Chapter 5

Mussel meal as alternative ingredient for the exploitation of common sole (*Solea solea* L.) juvenile's farming

Mongile F., Mandrioli L., Mazzoni M., Pirini M., Zaccaroni A., Sirri R., Parma L., Gatta P.P., Sarli G., Bonaldo A.

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Abstract

Common sole, *Solea solea*, farming in Europe, although potentially very promising, is still a little reality owing to the poor performances of sole when fed fishmeal based diets. Mussel meal, characterized by high palatability and good nutritional value, may represent an effective alternative ingredient in the formulation of a commercial feed for this species. The present study was carried out in order to determine the effect of graded level of mussel meal dietary inclusion on growth performance, fatty acid composition and liver histology of common sole juveniles. Four isoproteic (53%) and isolipidic (11%) pelletized diets were formulated to contain graded levels of mussel meal (0%, 25%, 50% and 75% named respectively MM0, MM25, MM50 and MM75) to replace fishmeal. Seventy sole juveniles (initial body weight 13.1 g) per tank were randomly distributed in twelve flat bottom 500 L square tanks (temperature 20 ± 1 °C and dissolved oxygen above 7.5 mg L^{-1}). Animals were hand-fed twice a day to apparent satiation, over 91 days. Performance, feed utilization, fatty acid composition and liver histology were investigated. One-way ANOVA and Newman–Keuls *post hoc* test were used to analyze data ($P \leq 0.05$), while a linear regression analysis was conducted to analyze growth performance and feed utilization indices against the mussel meal dietary inclusion. Diets containing mussel meal (MM25, MM50 and MM75) gave significantly higher specific growth rate and feed intake and lower feed conversion rate when compared to the fishmeal based diet (MM0). Carcass proximate composition was not influenced by dietary treatments with exception of the significantly lower lipid content in MM75 group. The protein efficiency was significantly improved by the mussel meal inclusion, as confirmed by the protein efficiency ratio and the gross protein efficiency which increased linearly with the mussel meal inclusion. A significant decrease in viscerosomatic index was observed in fish fed MM50 and MM75 in comparison to MM0. Hepatosomatic index of fish fed MM0 and MM25 was higher than that of fish fed MM75, even though in all the experimental groups the histological examination of liver parenchyma showed uniformly an abundant accumulation of lipid droplets. Carcass fatty acid composition was significantly affected by dietary treatments reflecting dietary fatty acid profile. According to those results, mussel meal represent an

effective alternative ingredient for enhancing growth performance, feed palatability and feed utilization in sole irrespectively to the inclusion levels used in this trial.

Introduction

The common sole (*Solea solea*) represents an interesting species for the European aquaculture diversification, due to its high price and market demand (Imsland *et al*., 2003). However, despite the considerable research effort to reach a large-scale production of this species, further improvement are still needed to overcome the poor performance at the on-growing stage (Mas-Muñoz *et al*., 2011). Above all, pursuing the ideal feed consumption and an adeguate nutrient utilization is of particular relevance to succeed in the aquaculture production of any given species. With regards to the common sole, the lack of a specific diet for the on-growing stage makes it difficult to achieve a proper feed consumption and growth, for farming this species in affordable times.

Since fish does not represent the main natural prey of common sole, conventional fishmeal (FM) based diets result unattractive for this species (Reig *et al*., 2003). Indeed, common sole trophic profile is mainly represented by polychaetes, mollusks and crustaceans (Cabral 2000), whose detection and selection is mediated by specific water-diffusible chemicals, reacting with sole's chemosensory cells (Mackie *et al*., 1980).

The evaluation of alternative protein and lipid sources, mimicking the organoleptic characteristics of sole's natural feed, would be of particular interest in the formulation of a specific diet, which could be able to fully exploit the growth potential of this species (Parisi *et al*., 2013). In this regard, the use of mussel meal (MM) as an alternative ingredient for animal feeds, as previously suggested for other fish species (Berge and Austreng, 1989; Grave *et al*., 1979; Kikuchi and Furuta, 2009; Kikuchi and Sakaguchi, 1997; Kitamura *et al*., 1981), would be further advisable for a species like sole. Indeed, sole are particularly fond of mollusk meat, as already reported by Mackie *et al*. (1980) and Fonds *et al*. (1989). Moreover, MM is characterized by a well balanced amino acids (AA) profile, avoiding possible deficiencies due to FM replacement.

The availability of mussels as a new ingredient for aquafeed, can result from different backgrounds. Likewise, the management of mussel's by-products from canning factories, cooking plants and dispatch centers in Galicia or Chile, may be expected to be used to produce meals (Iribarren *et al*., 2010). Kikuchi *et al*. (2002) and Kikuchi and Furuta (2009) suggested such use of mussels, through their removal from

cooling water system in electric power plants along the coast of Japan. In addition, recent studies recommend the use of mussel farming for combating eutrophication of the Baltic Sea, the realization of which would result in the disposal of the product towards the production of meals, because the animal size would be too small to be marketed as seafood (Gren *et al*., 2009).

Hence, the aim of the study was to investigate the viability of using MM as a new ingredient to substitute FM in formulated diets for common sole juveniles.

Materials and Methods

Experimental diets

Four isoproteic (53%) and isoenergetic (11%) pelletized diets were formulated to contain 0% (MM0), 25% (MM25), 50% (MM50) and 75% (MM75) of MM in replacement of FM as a protein source. The MM was manufactured using steamed, de-shelled deep frozen mussels (*Mytilus chilensis*), obtained from Fys Chile SA (Chiloé, Chile), then freeze dried and finely ground to obtain a flour. Fish oil (FO) was used to balance the lipid levels of the experimental diets. The mineral-vitamin mix formulation was the same used on Senegalese sole (*Solea sengalensis*) by Guerreiro *et al*. (2012). Ingredients were mixed and pelleted dry without steaming, using a laboratory pelleting machine (La Monferrina, Asti, Italia) with a 1.0 mm die. The diets were dried at 40 °C for 24 h and stored in a refrigerator (4 °C) until use.

Ingredients, proximate composition (%) and AA profile of the experimental diets, FM and MM are shown in Table 1. Fatty acid profile (% fatty acid methyl esters, FAME) of FM, MM, FO and experimental diets are shown in Table 2.

Table 1. Ingredients, proximate composition and amino acid profile of experimental diets and protein sources.

	Protein sources		Experimental diets			
	FM	MM	MM0	MM25	MM50	MM75
Ingredients (g kg^{-1})						
Fishmeal			800.0	600.0	400.0	200.0
Mussel meal			0.0	250.0	500.0	750.0
Wheat meal			142.5	97.5	47.5	2.5
Fish oil			35.0	30.0	30.0	25.0
Vitamins/ mineral mixture[1]			12.5	12.5	12.5	12.5
Carboxymethyl cellulose			10.0	10.0	10.0	10.0
Proximate composition (%)						
Dry matter	92.24	91.43	96.65	95.18	95.41	96.03
Crude protein	66.66	52.44	53.78	53.06	53.19	52.00
Total lipids	10.97	9.78	11.44	9.74	10.53	9.94
Ash	16.15	10.64	13.52	12.08	10.88	9.57
Gross energy (MJ/kg)	18.76	18.20	19.60	18.90	19.80	19.90
Amino acids (g 16 g $^{-1}$ N)						
Aspartic acid	10.31	10.53	10.28	10.43	10.54	10.92
Glutamic acid	14.14	12.88	14.24	13.92	13.57	13.45
Alanine	8.33	6.94	8.34	8.06	7.75	7.35
Arginine	5.98	7.31	5.80	5.80	6.84	7.08
Phenylalanine	4.39	4.18	4.41	4.44	4.55	4.36
Glycine	7.04	7.10	6.82	6.94	6.96	7.16
Hydroxyproline	1.26	0.77	1.31	1.12	1.01	0.72
Isoleucine	3.42	3.47	3.63	3.53	3.50	3.40
Histidine	3.80	3.59	3.76	3.51	3.07	2.59
Leucine	7.84	6.47	7.87	7.63	7.44	7.22
Lysine	7.42	7.86	7.81	7.63	7.89	7.45
Proline	5.01	4.69	4.43	4.93	4.41	5.00
Serine	4.92	6.09	4.85	5.19	5.46	5.81
Tyrosine	3.20	4.46	3.19	3.79	3.58	3.80
Threonine	4.81	5.65	4.92	4.93	5.30	5.42
Valine	4.09	3.57	4.37	4.06	3.98	3.76

	Protein sources		Experimental diets			
	FM	MM	MM0	MM25	MM50	MM75
Cysteine and Cystine	0.87	1.68	0.81	1.02	1.18	1.51
Methionine	2.36	1.78	2.38	2.27	2.12	2.08
Tryptophan	0.81	0.99	0.77	0.82	0.86	0.93

[1] Vitamins (mg kg^{-1} diet or specified): retinol acetate, 18 000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 400; inositol, 400, choline chloride (50%), 2000. Minerals (mg kg^{-1} diet or specified): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).

Table 2. Fatty acid profile of protein sources, fish oil and experimental diets.

	Protein sources		Fish oil	Experimental diets			
	FM	MM	FO	MM0	MM25	MM50	MM75
Fatty acid profile (% FAME)							
14:0	6.12	3.29	5.92	6.41	5.70	4.78	4.33
15:0	0.38	0.37	0.51	0.42	0.40	0.37	0.38
16:0	17.78	16.91	15.21	17.29	16.83	16.20	16.47
18:0	4.30	3.24	2.82	3.55	3.47	3.39	3.31
Σ SFA	28.58	23.80	24.45	27.66	26.39	24.74	24.50
14:1n-5	0.10	ND	ND	0.13	0.10	0.09	0.08
16:1n-7	6.81	9.17	8.71	7.17	7.33	7.45	8.03
18:1n-9	6.55	0.89	8.31	7.01	5.91	4.96	3.78
18:1n-7	2.83	2.65	2.99	2.76	2.72	2.73	2.67
20:1n-11	ND	0.94	ND	0.08	0.22	0.36	0.49
20:1n-9	0.70	1.94	1.58	1.01	1.13	1.36	1.55
20:1n-7	0.21	2.01	ND	0.19	0.49	0.82	1.13
22:1n-6	0.31	ND	ND	ND	ND	ND	ND
Σ MUFA	17.20	17.60	21.59	18.35	17.90	17.79	17.74
16:2n-4	1.36	1.50	1.44	1.37	1.36	1.37	1.55
16:3n-4	1.58	3.61	1.44	1.57	1.20	0.96	0.80
Σ PUFA n-4	2.95	5.11	2.88	2.94	2.57	2.32	5.52
18:2n-6	0.92	0.57	1.09	1.37	1.22	1.05	0.87
20:2n-6	0.14	0.30	0.17	0.13	0.15	0.19	0.22
20:3n-6	0.13	0.20	0.13	0.16	0.17	0.18	0.18
20:4n-6	1.73	3.52	0.91	1.39	1.83	2.26	2.59
22:4n-6	0.13	0.47	ND	0.35	0.18	0.24	0.31
22:5n-6	0.46	ND	0.13	0.33	0.29	0.21	0.14
Σ PUFA n-6	3.51	5.06	2.44	3.73	3.85	4.11	4.31
18:3n-3	0.64	0.31	1.00	0.72	0.64	0.63	0.56
18:4n-3	1.51	1.46	4.17	2.18	2.12	2.17	2.08
20:4n-3	0.72	0.40	1.22	0.85	0.79	0.73	0.66
20:5n-3	19.49	24.95	20.31	19.75	20.98	22.86	23.19
22:5n-3	3.55	1.53	1.88	3.20	2.95	2.57	2.16
22:6n-3	15.06	5.94	12.48	14.18	13.10	11.48	9.40

	Protein sources		Fish oil	Experimental diets			
	FM	MM	FO	MM0	MM25	MM50	MM75
Σ PUFA n-3	40.96	34.61	41.06	40.88	40.58	40.43	38.04
20:2 Δ 5.11	ND	1.21	ND	ND	0.26	0.47	0.65
20:2 Δ 5.13	ND	0.66	ND	ND	0.12	0.24	0.35
22:2 Δ 7.13	ND	0.23	ND	ND	ND	ND	ND
22:2 Δ 7.15	ND	2.96	ND	ND	0.62	0.95	2.05
Σ NMID	ND	5.06	ND	ND	1.00	1.66	3.05
Σ PUFA	47.42	49.83	46.39	47.55	48.00	48.52	50.94
Unknown	6.47	8.77	7.57	10.02	7.71	8.95	10.01
n-3/n-6	11.67	6.84	16.82	10.97	10.55	9.83	8.82
DHA/EPA	0.77	0.24	0.62	0.72	0.62	0.50	0.41
EPA/ARA	11.25	7.09	22.40	14.17	11.43	10.12	8.97

ND = not detectable.

Fish and feeding trial

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Science, University of Bologna, Italy.

Common sole (*Solea solea*) juveniles were obtained from natural spawning of the broodstock adapted to captivity at the above-mentioned laboratory. From the larval stage, fish were cultured in a 3000 liter circular tank (conical bottom). Larvae were reared using the standard protocol adopted by Bonaldo *et al*. (2011) and Parma and Bonaldo (2013) while post-larvae and juveniles were fed a commercial diet (Biomar, Denmark, crude protein 63%, total lipids 14%). Before the initiation of the trial, fish have been divided into 12 groups of 80 individuals and distributed into each of twelve 500 liter square tanks (bottom surface: 0.64 m^2) in a completely random manner, to acclimatize experimental condition for 41 days. During this period, fish were fed by automatic feeders for 12 h a day with a mixture of the 4 experimental diets.

The tanks were provided with natural seawater and connected to a unique closed recirculation system consisting of a mechanical sand filter (0.4 m^3 of silica sand, 0.4–0.8 mm, Astral pool PTK 1200 model filter, Servaqua S.A. Barsareny, Spain), an ultraviolet light (PE 25 mJ/cm2: 16 m^3 h−1, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200,

Astral Pool, Servaqua S.A. Barsareny, Spain). The water exchange rate per tank was 100% every 2 h while the overall water renewal of the system was 5% daily. Temperature was maintained constant at 20 ± 1 °C throughout the experiment. The photoperiod was held constant at a 12-h day length through artificial light (200 lx at the water surface Delta Ohm luxmeter HD-9221, Delta-Ohm, Padua, Italy). Oxygen level was kept constant (7.5 ± 1.0 mg L^{-1}) by a liquid oxygen system connected to a software (B&G Sinergia snc, Chioggia, Italy). Ammonia (total ammonia nitrogen 0.5 - 0.1 mg L^{-1}), nitrite (NO_2^- < 0.2 mg L^{-1}) and nitrate (NO_3^- < 50 mg L^{-1}) were determined spectrophotometrically daily (Spectroquant Nova 60, Merk, Lab business, Darmstadt, Germany) at 12:00 p.m. At the same time, pH (7.8-8.2) and salinity (20 g L^{-1}) were also determined.

Each experimental diet was fed to triplicate groups (13.1 ± 2.3 g initial mean body weight), assigned in a completely random manner, over 91 days. Fish were hand fed twice a day for 1 h a time (at 9:00 a.m. and 4:00 p.m.) for 6 days a week and once on Sundays (9:00 a.m.), to apparent satiation. The meal distribution was made following a daily different tank order and as to even distribute feed pellets over the surface area. Uneaten feed, when present at the end of the meal, was removed and estimated by counting each pellet then multiplying the number with the mean weight of a single pellet, the which result was deducted from the daily feed intake of the tank.

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.

Sample collection and chemical analyses

At the beginning and at the end of the trial, fish were individually weighed and measured. Intermediate biomass estimation was also made at day 28 and 56 by bulk weighing. Ten fish at the beginning and ten fish at the end of the trial have been sampled for the whole body proximate analysis to calculate protein efficiency ratio (PER) and gross protein efficiency (GPE). Ten more fish at the end of the trial have been sampled to investigate the fatty acid composition of the carcass. Additionally, at the end of the trial

viscera and liver weight was recorded from 5 fish per tank to calculate the viscerosomatic (VSI) and the hepatosomatic index (HSI).

Diets and whole body samples were analyzed using the following procedures: dry matter was determined after drying to constant weight in a stove at 105 °C for 24 h; crude protein (N x 6.25) was determined by the Kjeldahl method; total lipids were determined according to Bligh and Dyer (1959) extraction; 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, Moline, IL, USA). Amino acid analysis of FM, MM and diets was conducted using the method of Cunico *et al*. (1986), except for tryptophan, which was analyzed by the method of Garcia and Baxter (1992). Regarding FM, MM, diets and whole body samples, FAME were prepared by acid-catalyzed transmethylation of total lipids (Christie, 1989). Afterwards, FAME of all samples including FO were separated and quantified as described by Pirini *et al*. (2010). Briefly, FAME were analyzed on a Varian 3380 gas chromatograph (Walnut Creek, California, USA) equipped with a fused silica capillary column DB-23 J&W Scientific (30 m x 0.25 mm), a split injector at 230 °C and a flame ionization detector at 300 °C. The carrier gas was nitrogen at a flow rate of 1.2 ml min^{-1}. The oven temperature was set in a programmed mode from 150 °C to 230 °C at 5 °C min^{-1} and final isotherm. Data were processed using a Varian Star Chromatography Workstation (Walnut Creek, California, USA). Fatty acid identification was accomplished by comparing the retention times of unknown FAME with those of known FAME standard mixture (Sigma–Aldrich Corp., St. Louis, MO, USA; PUFA No. 1, Marine Source, and PUFA No. 3, Menhaden Oil, SUPELCO, Inc., Bellefonte, PA, USA).

Calculations

Specific growth rate (SGR, % day^{-1}) was calculated as $SGR = 100 \times (\ln W_{fin} - \ln W_{in}) / t$, where W_{fin} and W_{in} represent final and initial weights (tank means), respectively and t the number of feeding days. FCR was calculated as FCR = g feed given / g live weight gain. Condition factor (CF) was calculated as $CF = 100 \times (\text{body weight} / \text{total length}^3)$. VSI (%) was calculated as VSI = 100 x (viscera weight / body weight). HSI (%) was calculated as HSI = 100 x (liver weight / body weight). The nutritional formulae employed

were: PER, calculated as PER = (body weight gain / protein intake); GPE, calculated as GPE = (% final body protein × final body weight) − (% initial body protein × initial body weight) / total protein intake fish × 100).

Liver histology

At the end of the trial, three fish per tank were sampled for histology. A sample of the liver was fixed in 10% buffered formalin, dehydrated in a graded ethanol series and embedded in paraffin. Histological sections of 4 μm were stained with haematoxylin and eosin (H&E) and checked under light microscope for potential histopathological changes. A graduation scheme of liver lipid accumulation was used to assess the intracytoplasmic content according to Gatta *et al*. (2011). Three sections per treatment were also stained by the Periodic Acid Schiff (PAS) method for the detection of hepatocyte glycogen (Luna, 1968).

Statistical analysis

All data are presented as mean ± standard deviation (SD). All data were analyzed by one-way ANOVA followed by a *post-hoc* multiple comparison test (Newman–Keuls test), using the statistical package R version 2.11.1 for Windows (Revolution analytics, Palo Alto, CA, USA) assuming a significant difference when $P \leq 0.05$. A linear regression analysis was conducted to analyze growth performance indices and feed utilization indices against to the MM dietary inclusion.

Results

No mortality among groups was registered for the whole experimental period. Data on growth performance and feed utilization are presented in Table 3. For all growth parameters and feed utilization indices, the linear regression produced a R^2 equal or higher than 0.69. Final body weight was significantly lower in MM0 group (32.4 g) as compared with MM25, MM50 and MM75 (42.2, 46.0 and 46.4 g respectively). The same increasing trend was observed for the SGR where MM0 showed the significantly lowest value (0.98%

body weight day^{-1}) in comparison with the other treatments (1.27, 1.38 and 1.40% body weight day^{-1} in MM25, MM50 and MM75, respectively). The FI significantly increased with the lowest addition of MM, without any further significant increase by increasing the inclusion level of MM. Concerning the feed utilization indices, fish fed MM0 showed a significantly higher FCR (1.52) than those fed MM25, MM50 and MM75 (1.09, 1.00 and 0.98 respectively).

Both PER and GPE linearly increased with the MM inclusion level. PER was significantly lower in MM0 as compared to the MM based diets, while MM25 was significantly different from MM75. Yet, GPE values in MM0 and MM25 were statistically different between them and as compared to MM50 and MM75.

Table 3. Growth performance and feed utilization indices of common sole juveniles fed experimental diets.

	Dietary treatments						
	MM0	MM25	MM50	MM75	Linear regression line	R^2	P
IBW[1]	13.2 ± 0.4	13.2 ± 0.3	13.1 ± 0.7	13.0 ± 0.3	N.A.		
FBW[2]	32.4 ± 3.4[a]	42.2 ± 0.9[b]	46.0 ± 1.3[b]	46.4 ± 3.0[b]	Y= 34.91 + 0.182 x	0.71	≤ 0.001
SGR[3]	0.98 ± 0.11[a]	1.27 ± 0.01[b]	1.38 ± 0.06[b]	1.40 ± 0.05[b]	Y= 1.06 + 0.005 x	0.72	≤ 0.001
FI[4]	2.28 ± 0.18[a]	2.58 ± 0.01[b]	2.75 ± 0.12[b]	2.79 ± 0.08[b]	Y = 2.351 + 0.007 x	0.70	≤ 0.001
FCR[5]	1.52 ± 0.13[b]	1.09 ± 0.01[a]	1.00 ± 0.04[a]	0.98 ± 0.02[a]	Y= 1.40 – 0.007 x	0.69	≤ 0.001
PER[6]	1.29 ± 0.12[a]	1.76 ± 0.01[b]	1.89 ± 0.06[bc]	1.95 ± 0.08[c]	Y= 1.4 + 0.01 x	0.77	≤ 0.001
GPE[7]	25.29 ± 1.85[a]	33.38 ± 0.89[b]	35.96 ± 1.36[c]	36.59 ± 1.05[c]	Y= 27.3 + 0.15 x	0.75	≤ 0.001

[1]IBW, initial body weight (g); [2]FBW, final body weight (g); [3]SGR, specific growth rate (% day^{-1}) = 100 x (ln final body weight - ln initial body weight) / (duration of experiment, days); [4]FI, feed intake = 100 x (feed consumed, g / IBW / days); [5]FCR, feed conversion ratio = (feed consumed, g / weight gain, g); [6]PER, protein efficiency ratio = (fish weight gain g) / (total protein intake, g); [7]GPE, gross protein efficiency = 100 x ((% final body protein x final body weight) - (% initial body protein x initial body weight)) / (total protein intake, g). Each value is the mean ± SD of three replicates. Different letters (abc) in the same row denote significant ($P \leq 0.05$) differences among treatments. Linear regression lines (where Y is the response and x is the MM dietary inclusion level in diets, ranging from 0 to 75); R^2 and *P* are also given. N.A. = not applicable.

Data on carcass proximate composition and biometric parameters at the end of the trial are shown in Table 4. No significant effect of MM inclusion level was observed in the carcass proximate composition, with the exception of total lipid content, which was statistically lower in MM75 (5.58%) as compared to the other treatments (6.54, 6.64 and 6.51% in MM0, MM25 and MM50, respectively).

Table 4. Carcass proximate composition and biometric parameters of common sole juveniles fed experimental diets.

	Dietary treatments			
	MM0	MM25	MM50	MM75
Carcass (%)				
Moisture	73.14 ± 0.42	72.82 ± 0.12	72.96 ± 0.24	73.65 ± 0.44
Crude protein	17.80 ± 0.13	17.85 ± 0.40	17.95 ± 0.18	17.80 ± 0.57
Total lipids	6.54 ± 0.32^{b}	6.64 ± 0.07^{b}	6.51 ± 0.14^{b}	5.58 ± 0.26^{a}
Ash	2.37 ± 0.02	2.23 ± 0.07	2.26 ± 0.09	2.36 ± 0.11
Biometric parameters				
CF[1]	1.17 ± 0.17^{a}	1.22 ± 0.16^{ab}	1.23 ± 0.28^{b}	1.17 ± 0.14^{a}
VSI[2]	4.18 ± 0.47^{b}	4.00 ± 0.36^{ab}	3.71 ± 0.48^{a}	3.72 ± 0.45^{a}
HSI[3]	2.01 ± 0.35^{b}	2.00 ± 0.26^{b}	1.88 ± 0.32^{ab}	1.68 ± 0.30^{a}

[1] CF, condition factor = 100 x (body weight, g)/(body length, cm)^{3}, n= 50 fish per tank

[2] VSI, viscerosomatic index (%) = 100 x (viscera weight, g)/(whole body weight, g), n= five fish per tank

[3] HSI, hepatosomatic index (%) = 100 x (liver weight, g)/(whole body weight, g), n= five fish per tank

Different letters (ab) in the same row denote significant ($P \leq 0.05$) differences among treatments. Data are shown as mean ± SD.

The CF varied from 1.17 to 1.23 and was significantly lower in MM0 and MM75 (1.17 either) than in MM50 (1.23). The VSI ranged between 4.18 to 3.71%, with a significant difference of MM0 (4.18%) as compared to MM50 and MM75 (3.71 and 3.72%, respectively). The HSI decreased with the inclusion level of FM, ranging between 2.01 and 1.68%, with a significant difference between MM0 and MM25 groups (2.01 and 2.00%, respectively) as compared to MM75 (1.68%). At the histology observation, in all the liver sections, a moderate/severe intracytoplasmic clear content arranged in droplets was present within the hepatocytes. PAS stain did not reveal a conspicuous glycogen content within the cytoplasm; the optically empty vacuoles were interpreted as lipid.

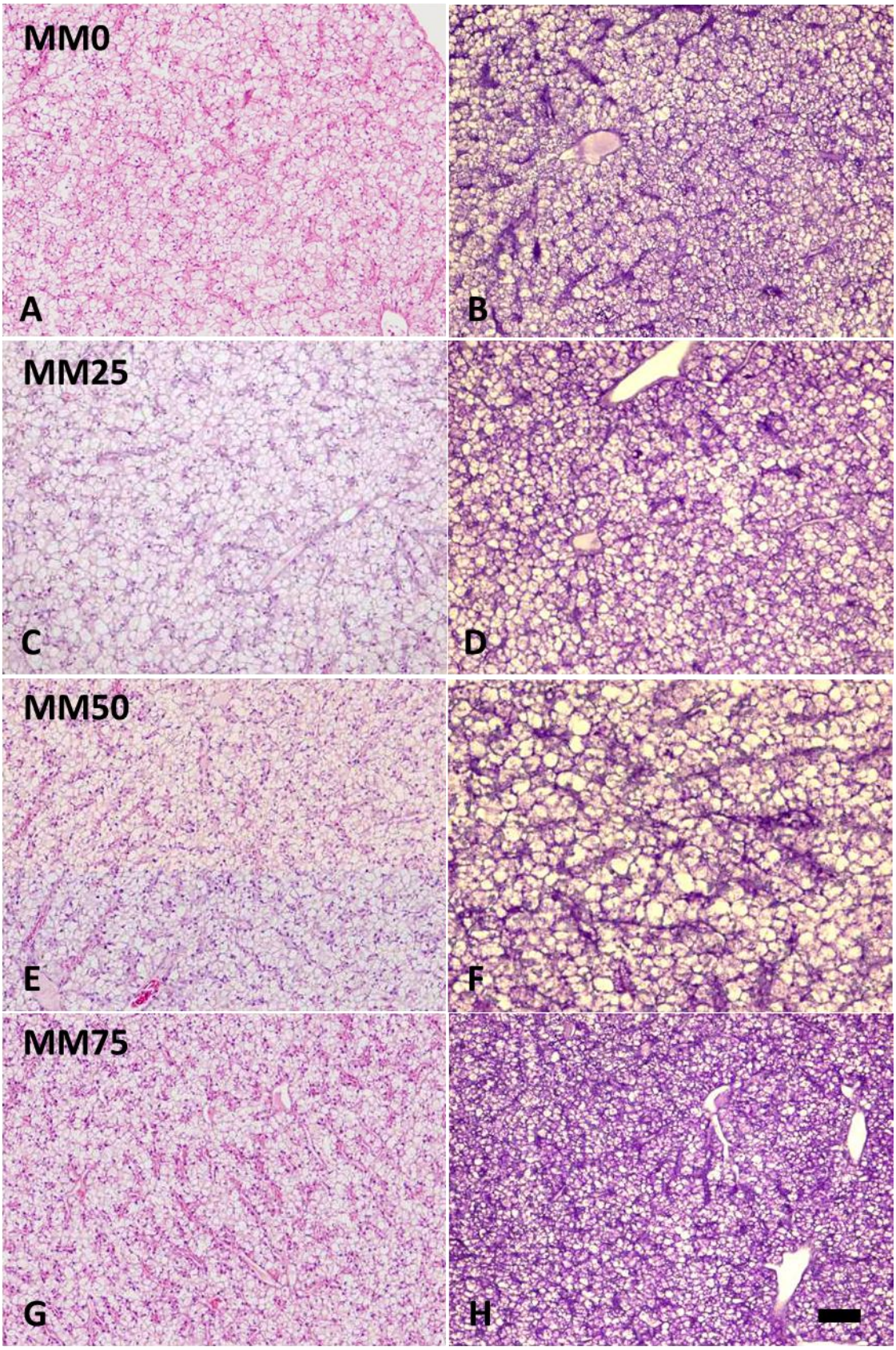

Figure 1. *(A, C, E, G) Histological sections of liver from the four diets. In all cases the cytoplasm contains a clear, optically empty content often arranged in large droplets (lipid vacuoles) (H&E). (B, D, F, H) PAS stain did not reveal glycogen as the main cytoplasmic content (10x lens; bar=50µm).*

Carcass fatty acid composition was significantly affected by dietary treatment. The polyunsaturated fatty acids (PUFA) n-3 and the monounsaturated fatty acids (MUFA) represented the main groups, followed by saturated fatty acids (SFA) and PUFA n-6 (Table 5). The PUFA n-3 significantly decreased from fish fed MM0 to those fed MM75, whereas MUFA content increased. No significant differences in SFA among groups were detected. PUFA n-6 were statistically lower in carcasses of MM25, MM50 and MM75 groups as compared to MM0. Docosahexaenoic acid (22:6 n-3; DHA) decreased from fish fed MM0 to those fed MM75, whereas no significant differences in eicosapentaenoic acid (20:5 n-3; EPA) were detected. As a consequence, the DHA/EPA ratio was significantly decreased in MM50 and MM75 carcasses in comparison with the other groups. Among PUFA n-6, arachidonic acid (20:4 n-6; ARA) increased from fish fed MM0 to those fed MM75. EPA/ARA ratio decreased from MM0 to MM75. Non-methylene interrupted dienoic fatty acids (NMID) were not detectable in carcass of fish fed MM0 whereas increased with the inclusion of MM in the other groups.

Table 5. Carcass fatty acid profile of common sole juveniles fed experimental diets.

	Dietary treatments			
	MM0	MM25	MM50	MM75
Fatty acid profile (% FAME)				
14:0	4.55^{b}	4.27^{ab}	4.08^{a}	4.09^{a}
15:0	0.36^{b}	0.34^{a}	0.36^{b}	0.40^{b}
16:0	16.31^{a}	16.80^{b}	17.23^{b}	17.64^{b}
18:0	3.98	4.20	4.28	4.29
Σ SFA	25.19	25.61	25.95	26.42
16:1n-7	6.65^{a}	7.02^{a}	7.67^{b}	8.62^{c}
18:1n-9	15.40^{a}	17.12^{b}	16.98^{b}	16.20^{b}
18:1n-7	3.15	3.15	3.25	3.31
20:1n-11	0.06^{a}	0.12^{b}	0.23^{c}	0.29^{d}
20:1n-9	1.27^{a}	1.26^{a}	1.41^{ab}	1.53^{b}
20:1n-7	0.22^{a}	0.39^{b}	0.63^{c}	0.83^{d}
22:1n-6	0.75^{b}	0.65^{ab}	0.56^{a}	0.54^{a}
Σ MUFA	27.49^{a}	29.71^{b}	30.72^{bc}	31.32^{c}
16:2n-4	0.90	0.88	0.86	0.80
16:3n-4	0.94^{d}	0.84^{c}	0.76^{b}	0.67^{a}
Σ PUFAn-4	1.83^{c}	1.72^{bc}	1.62^{b}	1.48^{a}
18:2n-6	2.42^{b}	1.97^{a}	1.77^{a}	1.79^{a}
20:2n-6	0.19	0.17	0.18	0.20
20:3n-6	0.12	0.10	0.11	0.10
20:4n-6	0.95^{a}	1.02^{b}	1.11^{c}	1.23^{d}
22:4n-6	0.35^{d}	0.24^{a}	0.28^{b}	0.31^{c}
22:5n-6	0.50^{c}	0.49^{c}	0.44^{b}	0.37^{a}
Σ PUFAn-6	4.54^{b}	3.98^{a}	3.90^{a}	4.00^{a}
18:3n-3	0.74^{b}	0.64^{a}	0.61^{a}	0.58^{a}
18:4n-3	1.31^{c}	1.19^{b}	1.14^{b}	1.08^{a}
20:4n-3	0.79^{d}	0.73^{c}	0.67^{b}	0.61^{a}
20:5n-3	5.97	5.59	5.56	5.53
22:5n-3	8.24^{b}	7.83^{b}	7.69^{b}	6.92^{a}
22:6n-3	17.59^{b}	16.61^{b}	15.10^{a}	14.51^{a}
Σ PUFAn-3	34.64^{c}	32.59^{b}	30.77^{ab}	29.23^{a}
20:2 Δ 5,11	ND	0.16^{a}	0.25^{b}	0.34^{c}

	Dietary treatments			
	MM0	MM25	MM50	MM75
20:2 Δ 5,13	ND	0.07[a]	0.12[b]	0.17[c]
22:2 Δ 7,15	ND	0.26[a]	0.62[b]	0.63[b]
Σ NMID	ND	0.49[a]	1.00[b]	1.14[b]
Σ PUFA	41.01[c]	38.77[b]	37.29[ab]	35.85[a]
Unknown	6.31	5.90	6.18	6.41
n-3/n-6	7.64[ab]	8.19[b]	7.89[b]	7.30[a]
DHA/EPA	2.95[b]	2.97[b]	2.72[a]	2.63[a]
EPA/ARA	6.25[d]	5.49[c]	4.99[b]	4.49[a]

Different letters (abcd) in the same row denote significant ($P \leq 0.05$) differences among treatments. ND = not detectable.

Discussion

At the end of the growth trial, all the treatments have more than tripled the initial mean body weight, with the exception of group MM0. To our knowledge, the MM inclusion in the present trial allowed a higher SGR than those registered in the previous recent trials on common sole (Gatta *et al*., 2011; Lund *et al*., 2013; Overton *et al*., 2010; Palazzi *et al*., 2006; Piccolo *et al*., 2008; Schram *et al*., 2006).

Common sole is known as a fastidious feeder on pelleted FM based diets (Bromley 1977). In fact, fish is not commonly present in the natural diet of sole, being his natural trophic profile mainly composed of polychaetes and mollusks as well as crustaceans (Cabral 2000). Possibly, this may explain the low growth of sole in culture conditions, which remains one of the most important economic constraints for commercial sole in aquaculture (Mas-Munõz *et al*., 2011).

The enhancement of feed intake for this species, might be sought by the formulation of a specific diet reflecting, as far as possible, common sole's natural prey items. In the wild, common sole detects and selects his prey through chemosensory cells with high chemical specificity for some substances. These chemical compounds have been investigated between fish, mollusk and crustaceans extract by Carr *et al*. (1996). The results of that study show the similar content of low-molecular-weight substances-free in crustaceans and mollusks and their consistently lower concentration in teleost extract, resulting 57% lower as compared to the former ones. At this regard, the most efficient diffusible chemical compounds mediating

feed selection and feeding behavior in juvenile common sole have been indicated by Makie *et al*. (1980) as glycine-betaine with glycine or alanine, whom, consistently, result among the most important components in mollusks and crustaceans extracts (Carr *et al*., 1996). Mussel meat is rich in the AA glycine, alanine and the non-AA betaine (Nagel *et al*., 2013), thus representing a proper ingredient in the formulation of a specific diet for common sole. In the present experiment, the addition of MM in substitution to FM was found to significantly improve common sole FI. Still, a further MM addition over 250 g kg^{-1} did not result in any significant effect. Similar results of an upper limit were highlighted in a previous trial with tiger puffer, *Takifugu rubripes*, fed soybean meal based diets (270 g kg^{-1}) (Kikuchi and Furuta 2009). In this study no further positive effect on growth and feed utilization was observed over the 100 g kg^{-1} MM inclusion. The same effect has been also reported in Japanese flounder, *Paralichthys olivaceus*, where increasing dietary levels of mussel extract over 5% inclusion, corresponding to a dietary addition of MM equal to 25 g kg^{-1}, did not led to further feeding and growth improvement (Kikuchi *et al*., 2002). However, the attractivity potential of MM seems to be species related, there being species-specificity for feeding stimulants (de la Higuera, 2001). In a feeding trial on Japanese flounders, MM inclusion (50 g kg^{-1}) effectively increased dietary palatability of the soybean meal (250 g kg^{-1}) based diet, improving growth by increasing feeding activity and feed utilization over the control group (Kikuchi 1999). The MM was also used as a feed attractant (80 g kg^{-1}) in a trial on turbot, *Psetta maxima*, fed diets with increasing level of rapeseed (RPC) (> 454 g RPC kg^{-1}) with a significant improvement on FI, thus of dietary palatability (Nagel *et al*., 2013). Conversely, the use of MM (40-80 g kg^{-1}) in a potato protein concentrate-based diets, provided unaffected daily FI when fed to rainbow trout, *Oncorhynchus mykiss* (Tusche *et al*., 2011). In gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) fingerlings, the use of MM mixed with a commercial feed (71 g kg^{-1}) allowed a higher total feed intake only in seabream, yet deteriorating the FCR values in both species (Anagnostidis *et al*., 2013).

Noteworthy, the increased FBW and SGR of common sole fed MM25, MM50 and MM75 is due not only to a higher FI, but also to an improved FCR. An increased palatability of diets can exert by itself an improvement at digestive and metabolic level. According to Giduck *et al*. (1987), the presence of feeding stimulant (FS) in the diet activates a cephalic reflex, creating a feed forward response in the animals. This kind of effect has been suggested also on striped bass, *Morone saxatilis*, where the addition of 20-40 g kg^{-1} FS led to a significant improvement of the FCR, indicating an increased efficiency of the diets (Papatryphon and Soares 2000). Consistently, Takii *et al*. (1986a, 1986b) reported a significantly higher activity of several digestive and hepatic enzymes in eels, when fed a FS supplemented diet. A further

element to take into account are the higher GPE and PER registered in fish fed MM-based diets. Being the AA profile of the two protein sources very similar one to another, we would discard the hypothesis of a shortage in one or more indispensable AA, moreover as the indispensable AA dietary composition reflects the ideal profile suggested by Silva *et al*. (2009) for Senegalese sole, *Solea senegalensis*. A different digestibility of MM and FM should be considered. In sole, acid digestion and proteolysis in the stomach seems to be residual and the digestion occurs primarily in the long intestine in a slightly alkaline environment (Yúfera and Darias 2007). According to Nankervis and Southgate (2006), MM protein is more soluble than that of FM (18.66% and 2.89%, respectively), being the digestibility of the soluble protein in an alkaline environment particularly higher than that of the insoluble fraction (Tonheim *et al*., 2007). Therefore, we assume that sole can better digest MM in comparison with FM. Furthermore, Sáenz de Rodrigáñez *et al*. (2011) reported that the hydrolysis of MM by digestive enzyme of Senegalese sole *in vitro*, presents a slower degradation kinetics in comparison with FM resulting in a maintained release of AA at the intestinal level, thus preventing the saturation of transporters in the brush border membrane of enterocytes.

Data on carcass proximate composition are in line with previous results on the same species (Gatta *et al*., 2011) and on Senegalese sole fed 56% protein and 12% lipid level diets (Borges *et al*., 2009; Rema *et al*., 2008).

In the present trial, HSI and VSI were slightly higher as compared to other experiments on sole juveniles (Borges *et al*., 2009; Dias *et al*., 2004; Gatta *et al*., 2011). The increasing HSI from MM75 towards MM0, could be related to the rising dietary starch levels, as already reported in common sole Gatta *et al*. (2011), Senegalese sole (Dias *et al*., 2004), gilthead seabream and European seabass (Bonaldo *et al*., 2008; Dias *et al*., 1998; Moreira *et al*., 2008); however PAS stain revealed that glycogen was not the main cytoplasmic content. Higher dietary digestible carbohydrate can enhance *de novo* lipid synthesis and deposition in the liver (Evans *et al*., 2005) and this can be associated with a higher HSI (Krogdahl *et al*., 2004). Thus, the abundant intracytoplasmic lipid content observed in all treatments, might be related to the tendency of sole to store lipids in the liver, as previously observed on the same species (Gatta *et al*., 2011) and on Senegalese sole (Fernandes *et al*., 2012; Rueda-Jasso *et al*., 2004; Valente *et al*., 2011).

Dietary treatments directly influenced the fatty acid profile of carcasses', as already described for other flatfish and marine fish (Bell *et al*., 2004; Martins *et al*., 2007; Sargent *et al*., 2002; Tocher, 2003; Turchini *et al*., 2009). Studies on juvenile and sub-adult marine fish

indicate that the essential fatty acids requirements cannot be met by C18 PUFA and that the highly unsaturated fatty acid (HUFA) n-3, EPA and DHA, are required (Tocher, 2010). On the other hand, similar to freshwater fish, the quantitative requirement for HUFA n-6, has not been fully determined in marine fish (Bell and Sargent, 2003) although ARA seems to be essential. To our knowledge, there is a lack of information in specific dietary fatty acid requirements for optimal growth, in common sole at juvenile stage. However, sole's natural diet is likely characterized by substantially higher EPA levels than DHA, which differs from the dietary regime of piscivorous carnivores fish (Morais *et al*., 2012). Indeed, polychaete (*Nereis diversicolor*), which forms an important part of sole's diet, is a rich source of EPA (5–10 mg/g DW), with only residual amounts of DHA (0.5–1 mg g^{-1} DW) (Luis and Passos 1995). Recent studies on Senegalese sole demonstrated that this species is capable of synthesizing DHA from EPA (Morais *et al*., 2012). In our study, the lower level of DHA in the MM-based diets, did not seem to negatively affect growth. On the other hand, the higher amount of EPA in MM as compared to FM was not entirely reflected in the carcasses at the end of the trial, suggesting that this fatty acid was preferentially catabolized than DHA (Bell *et al*., 2002) or readily converted to DHA (Valente *et al*., 2011; Morais *et al*., 2012).

The ARA content showed an increasing pattern related to MM dietary inclusion. The effects of different levels of ARA in sole species were observed in larvae and broodstock, on pigmentation and reproduction (Lund *et al*., 2007; Norambuena *et al*., 2012), respectively; however, there is no information about the requirement of this fatty acid for growth. Interestingly, the dietary inclusion of MM determined the presence of NMID in the carcass of sole. These fatty acids are synthesized by various shellfish and present anti-inflammatory properties in mammals (Trigari *et al*., 2001; Berger *et al*., 2002).

Conclusion

In conclusion, the substitution of FM by MM improved performance and feed utilization, demonstrating that a FM based diet is not the reference for determining the growth potential of this species. MM could mimic the characteristic of sole’s natural preys, in terms of attractiveness and nutrient utilization. Further studies are needed to combine MM and different protein sources to obtain a valid sustainable feed formulation for this species.

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Chapter 6

General discussion

This thesis, from a nutritional point of view, aimed to investigate on some important key factors to support the prospected growth of the aquaculture sector. Indeed, fish nutrition is becoming increasingly important both for reducing costs, strictly linked to the rising demand for FM and FO, either to exploit growth performances, feed utilization and welfare of new farmed fish species.

Yet, further efforts are needed to reduce the aquaculture dependence from marine feedstuff and thereby enhance the sustainability of the sector. Till now, plant feedstuff aroused the main interest among alternative aquafeed ingredients being widely available, economically viable and environmentally friendly, as well as for their suitable nutritional characteristics (Gatlin III *et al*., 2007). Still, different species with the same carnivorous trophic profile, perform differently when fed plant-based aquafeeds, given physiological and metabolic differences. In our case study, Chapter 2, it was highlighted that increasing the 15% FM replacement by a mixture of plant protein sources in turbot has lead to a worsening of growth performances. Such a negative effect was confirmed by a decline in health and welfare of fish, as attested through the metabolic stress and the immune response, at hematological level.

In the past few years, the research of novel dietary formulations capable to counteract the effects of high temperatures, on aquacultured fish species, is getting more and more attention. Aquafeed industry has recently developed new on-growing diets counter high temperature for various fish species, such as Atlantic salmon, European seabass and rainbow trout (*Oncorhynchus mykiss*). Hence, the study of such a diet for a species like gilthead seabream results strategic, representing around the 51% of the total finfish marine and brackish water aquaculture production in the Mediterranean area (FAO FishStatJ, 2010). In our case study, Chapter 3, we defined the 18% lipid inclusion as the most suitable dietary level for gilthead seabream reared at Mediterranean summer temperature, able to spare protein and making the farming of this species economically and environmentally more sustainable.

The attention toward the introduction of new farming species in the European aquaculture, represents an important challenge such as to increase the supply, thus filling still empty market segments. The common sole has been recognized as a possible candidate for the European aquaculture farming. In our case study, Chapter 4, we established that feeding

common sole juveniles over the 8% dietary lipid inclusion would affect growth performance and feed utilization indices. The low dietary lipid requirement in this species is mostly attributable to his quite passive behavior and the low daily metabolic budget. Further data regarding the histological status of gut mucosa are still under evaluation.

In the last case study, Chapter 5, the mussel meal has been evaluated as alternative ingredient in diet for common sole juveniles. Mussel meal has proved to be a very suitable ingredient in diets for common sole juveniles, enabling for the first time to deeply exploit the growth potential in this species. Indeed, mussel meal positively affected growth performance, feed palatability and feed utilization in sole irrespectively to the inclusion levels used in our trial. In the future, further investigations will be needed to find the most suitable combination between mussel meal and plant protein ingredients.

In view of the results in this thesis, it is clear that to sustain the aquaculture sector further developments are needed toward the formulation of more specific diets. Future studies should be based on the crucial need to find alternative ingredients viable to substitute fishmeal and fish oil in aquafeeds. This goal must be pursued taking into account animal welfare, which is strictly related to the growth performances and which is becoming of increasing interest for the consumer acceptability of the product. Moreover, the fine tuning of on-growing diets, according to species-specific seasonal requirements, would further help the productions and the sustainable management of marine raw materials. Finally, formulating specific diets for new marine aquacultured species would represent a fundamental boost to start their production on an industrial level.

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