Pea protein concentrate in diets for sharpsnout sea bream (*Diplodus puntazzo*): effects on growth and health status.

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Abstract

Four diets for sharpsnout sea bream juveniles (14 g) with four levels of air-processed pea protein (PPC) (0,160,320and487 g/kg) were tested in triplicate.

The experimental diets were isonitrogenous (43%CP) and isolipidic (19%EE), and the fish were fed to satiation twice a day. After 125 days, fish growth was found to be negatively affected by the inclusion of PPC. Feed conversion did not show significant differences in any treatment. Neither the body analyses nor the protein and individual essential amino-acid retention efficiencies were affected by the inclusion of PPC in the diet. However, histological gut examinations revealed noticeable differences. Fish fed the diet with the highest inclusion level of PPC presented the longest villous length and the most goblet cells, and the width of the *lamina propria* increased in the anterior intestine. Although no negative changes in nutritive parameters were detected, these alterations might affect nutrient transport, with negative consequences for fish growth. We conclude that the PPC in the amounts tested here is an inappropriate substitute for

fishmeal in diets for sharpsnout sea bream juveniles.

Keywords: Feed formulation; fishmeal; performance; pea protein; replacement; *Diplodus puntazzo*.

1. Introduction

Sharpsnout sea bream (*Diplodus puntazzo*) is one of the alternative species that has been proposed to diversify aquaculture in the Mediterranean. As an omnivorous fish, it could be given feed with a high content of vegetable protein, thereby decreasing the cost of fish diets and diminishing aquaculture's current dependence on fishmeal. The prices of these meals have risen by more than 260 % over the last decade, which has caused a price increase. Moreover, the production of gilthead seabream, which is one of the most commonly produced Mediterranean fish, has decreased due to low market prices, thus diminishing the profit of fish farms. Studies on substitution of fishmeal with vegetable protein sources in diets for sharpsnout sea bream are scarce. Soybean meal (Rondán et al. 2004; Hernández et al.2007), alfalfa protein concentrate (APC) (Chatzifotis et al. 2006) and sunflower meal (SFM) (Nogales-Mérida et al. 2010; Nogales-Mérida et al. 2011) have been explored as substitutes for fishmeal in sharpsnout seabream, but good growth was obtained only at low levels in the diet.

Over the last decade, pea protein concentrate (PPC) has been considered as an alternative protein source to replace fish and soybean meal because of its protein content (420–700 g/kg CP), amino-acid profile (which is similar to that of soybean meal; Øverland et al. 2009), relatively low quantities of anti-nutritional factors, and good binding properties and palatability. This alternative protein has been tested in some marine species, such as *Sparus aurata* (Sánchez-Lozano et al. 2009; Sánchez-Lozano et al. 2011), *Oncorhynchus mykiss* (Thiessen et al. 2003) and *Salmo salar* (Carter and Hauler 2000; Øverland et al. 2009; Penn et al. 2011), with good growth results obtained below 200 g/kg PPC. Additionally, Schulz et al. (2007) observed good growth of *Oreochromis niloticus* fed diets containing150 g/kg of PPC or less.

However, PPC assays indicate that the process used for protein extraction might be crucial to successful use in fish diets (Schulz et al. 2007). A dry process of grinding dehulled peas increased the levels of anti-nutritional factors such as protease inhibitors, phytic acid and alpha-galactosides. However, wet processing methods, such as acidic washing or thermal processing, produced a protein with lower levels of anti-nutritional factors (ANFs). Despite these differences, and the ANFs contained in air-processed pea protein, Thiessen et al. (2003) reported that diets with 200 g/kg of air-processed pea protein inclusion showed a higher concentration of protein with superior digestibility relative to whole, dehulled or extruded peas and were thus highly desirable for rainbow trout feeding.

Furthermore, there has been some research on alternative meals and their effects on the fish gut and liver, especially soybean meal (Krogdahl et al. 2003; Bonaldo et al. 2008; Urán et al. 2009) and PPC (Øverland et al. 2009, Penn et al. 2011). Most reported alterations in the gut and liver caused by ANFs or amino-acid (AA) deficiencies are associated with alternative vegetable meals. To the best of our knowledge, the only study carried out on sharpsnout sea bream fed alternative proteins (Nogales-Mérida et al. 2010) found that sunflower meal affected gut and liver morphology. Some of these gut alterations have negative consequences for the digestive process (Baeza- Ariño et al. 2014), with possible reductions in nutrient absorption, particularly in the absorption of some EAA. The lower amino-acid availability usually entails a reduction in the efficiency of AA retention and is accompanied by poor growth. The aim of this study was to determinate the feasibility of including air-processed PPC in experimental diets for sharpsnout sea bream from the perspectives of growth, nutritional efficiency and gut histology.

2. Material and Methods

1.1. Ethics statements

The experimental protocol was reviewed and approved by the Committee of Ethics and Animal Welfare of the Universitat Politècnica de València (UPV), following the Spanish Royal Decree 53/2013 on the protection of animals used for scientific purposes.

1.2. Experimental conditions, fish and feeding regime

The experiment was carried out at the Aquaculture laboratory of the Animal Science Technological Institute of Polytechnic University of Valencia, (Spain). *D. puntazzo* juveniles were brought from an Italian commercial hatchery and acclimatized to laboratory conditions foreight weeks. The fish were fed a standard commercial diet (480 g/kg crude protein (CP), 23 % ether extract (EE), 11 % ash, 2.2 % crude fibre (CF), and 14 % nitrogen free extract, NFE) (dibaq-Diproteg, S.A., Spain).

Four cylindrical fiber glass (750 l) tanks were used inside a circulating marine water system (30 m^3 capacity). Throughout the experimental period, the average temperature was 22.2 ± 2°C, the dissolved oxygen was 6.3 ±0.4 mg/l, and the salinity was 31 ±5 g/l. The pH was 7.6 ±1.0. The $NH_4^+$and NO_2^-valueswere 0.02 ± 0.1 and 0.1 ± 0.06 mg/l, respectively, and the photoperiod was natural.

Initially, 216 fish were collected (average weight 14 g) and distributed into 12 cages (three cages per tank) with a capacity of 98l. Triplicate groups of fish were fed by hand until apparent satiation for 125days, in two separate meals on 6 days per week. Fish were weighed individually every three weeks, after fasting for 24 hand anesthesia with clove oil containing 87 % eugenol (Guinama®, Spain).

At the start of the growth trial, a pool of 10 fish was collected for initial body analysis. At the end of the experiment, five fish per tank were collected for measurement of biometric parameters and stored at -30 ºC for analyzing their whole-body composition. Additionally, three fish per tank were collected for histologic analysis. Their guts and livers were removed and kept in formaldehyde [10 % v/v].

1.3. *Experimental diets*

The composition of the experimental diets is shown in Table 1. Four isonitrogenous (450 g/kg CP) and isolipidic diets (200 g/kg EE) were formulated with air-processed pea protein (PPC) in place of fishmeal at 0, 16, 32 and 48 % (0, 16, 32 and 48 diet,

respectively). The PPC amount was calculated to replace 0, 20, 40 and 60 % of the fishmeal protein, based on previous results (Sánchez-Lozano et al. 2011).The 16, 32 and 48 diets were supplemented with L-methionine (1, 3 and 4 g/kg, respectively) (Table 1).The wheat-meal content was reduced in parallel to adjust carbohydrate levels. Additionally, soybean oil was included at 50 g/kg in all diets following results obtained in a previous trial (Piedecausa et al. 2007). Diets were prepared by cooking-extrusion processing with a semi-industrial twin-screw extruder (CLEXTRALBC 45, Firmity, St.Etienne, France).

[Table 1 near here].

1.4. Biometric analysis

At the end of the experimental period, three animals from each cage were sacrificed to collect data on fish length, total weight and liver and mesenteric fat weights. These data were used to calculate these indices according to the following formulae:

Condition factor, CF [%] = body weight [g] / total length [cm]^3 •100

Hepatosomatic index, HSI (%) =liver weight [g] / body weight [g] • 100.

Mesenteric fat index MFI [%] = mesenteric fat weight [g] / body weight [g] • 100.

Intestinal somatic index, ISI [%] = intestine weight [g] / body weight [g] •100.

Viscerosomatic index, VSI [%] = visceral weight [g] / fish weight [g] •100.

Dress-out percentage DP [%] = (total fish weight [g] – visceral weight [g] –head weight [g]) / fish weight [g]) • 100.

Protein productive value, PPV [%] = Increase in body protein [g] • 100 / ingested protein [g]

Energy productive value, EPV [%] = [%] Increase in body energy [kJ] • 100 / ingested energy [kJ]

To determine the amino-acid (AA) retention, the following formula was applied:

AA Retention [%] =Increase in body AA [g] • 100 / ingestion of AA [g]

To evaluated the dietary AA profile for *Diplodus puntazzo,* the amino-acid ratio (Cerezo et al. 2013) was calculated as [AAR, %] = AA diets [%] / AA whole body [%] • 100.

1.5. *Chemical analysis*

Dietary ingredients, diets and fish composition were analyzed using the procedures of the Association of Official Analytical Chemists AOAC (1990): dry matter was analyzed after drying in an oven at 105 ºC until a constant weight was reached; ash was obtained by incineration in a muffle furnace at 550 ºC for 5h; CP (N x 6.25) was analyzed by the Kjeldahl method after acid digestion (Kjeltec 2300 Auto Analyser, Tecator Höganas, Sweden); and EE was examined using diethyl-ether extraction in a Soxhlet1043 extraction unit (Tecator). CF analyses of raw materials and diets were performed following the Ankom system (Fibertec System M., 1020 Hot Extractor, Tecator).

Amino-acid (AA) analyses of raw material, diets and fish were done using 25 g of CP. AAs were analyzed in a Waters HPLC system (Waters 474, Waters, Milford, MA, USA) consisting of two pumps (Model 515, Waters), an auto sampler (Model 717, Waters), a fluorescence detector (Model 474, Waters) and a temperature-control module. Aminobutyric acid was added as an internal standard control before hydrolyzation. The amino acids were derivatized with AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). Methionine and cysteine were measured separately as methionine sulphone and cysteic acid after oxidation with performic acid. AAs were separated with a C-18 reverse-phase column Waters Acc. Tag (150 mm × 3.9 mm) and then transformed to methionine and cystine.

1.6. *Histological methodology*

The intestines were divided into three parts: 1) anterior intestine (AI), close to the pyloric caeca, 2) mid intestine (MI) in the middle, and 3) posterior intestine (PI), one

centimetre before the anus; then, 0.5 cm of each was cut off. Tissue samples were fixed in phosphate-buffered formalin (4%, pH 7.4) and then transferred to 70% ethanol for storage until processing. All formalin-fixed tissues were dehydrated in ethanol, equilibrated in Ultraclear™ and embedded in paraffin according to standard histological techniques. Eighteen longitudinal sections (approximately 5 μm thick) of each section per treatment were cut and stained with hematoxylin and eosin (HE). Processing of the tissues took place at the Histology Section of the Animal Science Department of Polytechnic University of Valencia (Spain). Blinded histological examination was performed by light microscopy (Nikon Phase Contrast 0.90 Dry JAPAN). Tissue alterations were examined according to Baeverfjord and Krogdahl (1996) and Lundstedt (2004). The villous length, villous width, *lamina propria* width, muscular thickness and were measured in μm, and the goblet cells were counted per unit area (125,000 μm^2).

The criteria of Mc Fadzen et al. (1997) were used to record possible alterations in the hepatocytes or hepatic cell morphology. Additionally, hepatocytes were quantified. A number from 1 (normal), 2 (some alterations) and 3 (the most affected cells) was assigned, depending on the hepatocyte size, nucleus deformity, hepatic vacuolization, and deformities in pancreatic acinar cells. Degeneration of hepatocytes and acinar cells was also evaluated.

1.7. Statistical analysis

Differences in growth and nutritive parameters were examined using multifactor analysis of variance (ANOVA), with the initial live weight used as a covariate. The Newman-Keuls test was used to assess specific differences among diets at a significance level of ($p < 0.05$) (Statgraphics, Statistical Graphics System, Version Plus 5.1, Herndon, VA, USA).

Differences in length and the width of the mucosa fold and the number of mucus cells were analyzed using a simple ANOVA. Histological analyses ofhepatocytesandacinarcellswereevaluatedbycrosstabulationandchi-square contrast (χ^2) in the Statgraphics program.

2. **Results**

With respect to the AA composition (Table2), except for His, the EAA content increased with increasing PPC as a consequence of the slight increases in crude protein in the diets (from 425 g/kg in the 0 diet to 436 g/kg in the 48 diet).

[Table 2 near here].

At the end of the growth trial, the fish survival rate was above 91 % in all dietary groups. Mortality occurred when fish jumped out of the tanks.

The final fish weight was negatively associated with high levels of dietary PPC (Table 3). Sharpsnout sea bream fed the 48 diet had the lowest final body weight, SGR and TCG. However, feed efficiency parameters (FCR, FIR, DFI and PER) did not exhibit significant differences across PPC levels.

[Table 3 near here].

Concerning the biometric parameters (Table 3), the fish fed the 48 diet showed a decreased HSI (0.66%) compared to the fish fed the 0 diet (0.96%). At the end of the growth trial, whole-body composition was not affected by fish-meal substitution (Table 4). Similarly, the protein productive value (PPV) and energy productive value (EPV) did not show any significant differences among the diets.

[Table 4 near here].

In relation to whole-body amino-acid composition (Table 5), no significant differences were observed for any AA or in the efficiency of the retention of ingested EAA (Table 6).

[Table 5 and 6 near here].