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Abstract

The effect of high amounts of dietary carbohydrates on digestive and absorptive processes was studied in juvenile gilthead sea bream. Two trials were conducted over 14 weeks. In the first trial, sea bream were fed with isoproteic diets with lipid replaced with starch and in the second with isolipidic diets with protein replaced with starch; in both wheat starch was included at above the established maximum levels for this fish species. At the end of each trial, intestinal alkaline protease, α -amylase and lipase activities were measured post-feeding, and alkaline phosphatase and leucine aminopeptidase activities and nutrient absorption capacities were measured under basal conditions. When lipid (23 to 17%) was replaced with starch (12 to 28%) in the 46% protein diet, a decrease in trypsin and chymotrypsin activities was found without modifying the trypsin/chymotrypsin ratio as dietary starch increases. Moreover, the pH of the intestinal content in the pyloric caeca increased and alkaline phosphatase activity and D-glucose absorption capacity decreased. Thus, compensatory mechanisms in buffer capacity, pancreatic enzyme activities and nutrient absorption capacities took place, allowing the maintenance of feed utilization and growth. Diets for the second trial were formulated with the lowest lipid content tested previously (17%) and protein (46 to 40%) was substituted with starch (19 to 39%). Lipase and trypsin activities increased as dietary starch raise, chymotrypsin activity and L-lysine absorption were not modified and amylase activity and D-glucose and L-alanine absorption decreased, but these digestive compensatory mechanisms were not sufficient, and there was a significant increase in FCR and a decrease in voluntary feed intake and SGR. This study suggests that sea bream are able to modulate the digestive process when protein requirements are met.

Keywords	Brush border enzymes; brush border membrane vesicles; digestive enzymes; starch
Taxonomy	Carbohydrate in Animal Nutrition, Animal Digestion
Manuscript category	Vertebrate Nutrition
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FIG1.DOCX.docx [Figure]

FIG2.DOCX.docx [Figure]

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FIG5.DOCX.docx [Figure]

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Barcelona 16th July, 2019

Dear Editor,

Enclosed with this letter we have submitted the manuscript entitled **“High dietary carbohydrate inclusion by both protein and lipid replacement in gilthead sea bream. Changes in digestive and absorptive processes”**, which we would like to be considered for publication in *Aquaculture*.

The aim of the present research was to test the effect of dietary carbohydrates on digestive and absorptive processes in sea bream fed with isoproteic diets where the lipid was replaced with starch, or isolipidic diets where the protein was replaced with starch; using wheat starch to above the maximum levels established for this fish species. Summarizing, when lipid was replaced with starch in 46% protein diets, compensatory mechanisms involving changes in intestinal pH, pancreatic and intestinal digestive enzymes and nutrient absorption capacities took place, allowing the maintenance of feed utilization and growth, and suggesting that animals are able to modulate the digestive process when protein requirements are met. In contrast, when dietary protein was replaced with starch in 17% lipid diets, the digestive compensatory mechanisms were not sufficient and a significant increase in FCR and a reduction in voluntary feed intake and SGR were found.

On behalf of all the authors, I declare that we do not have any conflict of interest, that this work has not been published or submitted for publication elsewhere, and that all authors have read and approved the final manuscript. We hope you will find this manuscript interesting and worthy to be published in *Aquaculture*.

Yours sincerely,

A handwritten signature in blue ink, reading "Irene García Meilán".

Irene García Meilán

Comments from the editors and reviewers:

-Reviewer 1

- no further comments.

-Reviewer 2

- Most suggestions have been considered, but some questions must be revised:

1. Model of extrusion equip must be included

The model of the extrusion equip was included in material and methods section 2.1 (lines 133-136)

2. Tables 1-2, 3-5 and 5-6 have the same name, then it is necessary correct it including the trial

According to the referee the trial was included in the title of the table

3. Authors have included final weight in tables, but I have not understand how diets L23 and L17 have the same SGR value, being diferent the final weight: 393 and 359 g, if the initial weigh was 115 in all treatments.

4. Likewise, in diets P46, P42 and P40, final weigh were similar, but SGR differents??

The reviewer is absolutely right; I apologize for the error. Final weight data has been corrected in the tables of both experiments (Tables 3 and 5).

1. Compensatory changes in intestinal pH, digestive enzymes and nutrients absorption occur when dietary lipid or protein are replaced by starch
2. This modulation of the digestive process allows the maintenance of feed utilization and growth, if protein and lipid requirements are met
3. If both, dietary protein and lipid are replaced by starch, digestive compensatory mechanisms are not enough, and a significant increase in feed conversion ratio and a reduction in growth is found
4. A high dietary inclusion of wheat starch allows to replace lipid, but not protein of the diet, allowing to maintain growth rates

High dietary carbohydrate inclusion by both protein and lipid replacement in gilthead sea bream. Changes in digestive and absorptive processes

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Running title: High dietary starch inclusion for sea bream

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Authors' contributions: RF, AG designed the diets and the experimental trial; all authors carried out the sampling; IGM, BOG, JMV and AG performed laboratory analyses. IGM and AG analyzed and interpreted the data. IGM and AG drafted and critically reviewed the manuscript. All authors read and approved the final paper. The authors have declared no conflict of interest.

Keywords: Amylase, brush border enzymes, brush border membrane vesicles, lipase, protease, starch

Abstract

The effect of high amounts of dietary carbohydrates on digestive and absorptive processes was studied in juvenile gilthead sea bream. Two trials were conducted over 14 weeks. In the first trial, sea bream were fed with isoproteic diets with lipid replaced with starch and in the second with isolipidic diets with protein replaced with starch; in both wheat starch was included at above the established maximum levels for this fish species. At the end of each trial, intestinal alkaline protease, α -amylase and lipase activities were measured post-feeding, and alkaline phosphatase and leucine aminopeptidase activities and nutrient absorption capacities were measured under basal conditions.

When lipid (23 to 17%) was replaced with starch (12 to 28%) in the 46% protein diet, a decrease in trypsin and chymotrypsin activities was found without modifying the trypsin/chymotrypsin ratio as dietary starch increases. Moreover, the pH of the intestinal content in the pyloric caeca increased and alkaline phosphatase activity and D-glucose absorption capacity decreased. Thus, compensatory mechanisms in buffer capacity, pancreatic enzyme activities and nutrient absorption capacities took place, allowing the maintenance of feed utilization and growth.

Diets for the second trial were formulated with the lowest lipid content tested previously (17%) and protein (46 to 40%) was substituted with starch (19 to 39%). Lipase and trypsin activities increased as dietary starch raise, chymotrypsin activity and L-lysine absorption were not modified and amylase activity and D-glucose and L-alanine absorption decreased, but these digestive compensatory mechanisms were not sufficient, and there was a significant increase in FCR and a decrease in voluntary feed intake and SGR. This study suggests that sea bream are able to modulate the digestive process when protein requirements are met.

1. Introduction

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121 53 To help minimize production costs by sparing both protein and lipid sources more than
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123 54 50% of the protein in commercial diets currently comes from plant material, given the
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125 55 low availability and high prices of fish meal (SOFIA, 2018). Some vegetable meals, such
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127 56 as wheat and corn, contain a large amount of carbohydrates (Gatlin et al., 2007), with a
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129 57 potential protein sparing effect. If their use was optimum, dietary carbohydrates would
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131 58 allow the maintenance of final product quality, unlike dietary lipid inclusion. But the
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133 59 digestion and metabolization of carbohydrates by fish seems to be limited, particularly in
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135 60 carnivorous species (Wilson, 1994), even though all the enzymatic machinery required
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137 61 for their use is present (Cowey and Walton, 1989; Dabrowski and Guderley, 2002; Enes
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139 62 et al., 2009). The digestibility of carbohydrates is affected by their molecular complexity,
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141 63 different dietary inclusion levels and feeding rate, rearing temperature and technological
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143 64 treatments applied (Enes et al., 2011). In this sense, Venou et al. (2003) demonstrated that
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145 65 wheat was a good carbohydrate source even without extrusion processes, but the same
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147 66 was not true for corn due to its higher amylose/amylopectin ratio and structure. All these
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149 67 factors make studies on the sparing effect of carbohydrates interesting, but the results are
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151 68 conflicting. Enes et al. (2011) reviewed dietary carbohydrate utilization by European sea
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153 69 bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) juveniles,
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155 70 concluding that more than 20% of digestible carbohydrates could reduce growth and feed
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157 71 utilization, whereas other studies containing more than 20% of starch had no effect
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159 72 (Fernández et al., 2007). On the other hand, in a previous study (García-Meilán et al.,
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161 73 2014) we found that sea bream fed with a commercial diet showed the same growth rate
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163 74 as sea bream fed in the morning with a diet that contained 40% of highly digestible
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165 75 carbohydrates from wheat and in the afternoon with commercial diet.
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169 76 The use of plant ingredients in the diet may modify digestive and absorptive capacities
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171 77 differently according to dietary composition, with subsequent effects on metabolism and
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178 growth. For example, the presence of vegetable ingredients depresses the activity of
179 digestive enzymes, but this can be compensated by the higher relative intestinal length in
180 fish and by up-regulating trypsin secretion, which modifies the trypsin/chymotrypsin ratio
181 (García-Meilán et al., 2014, 2013; Santigosa et al., 2011a, 2008). Moreover, increased α -
182 amylase activity was detected in a diet with a high starch content (40%) (García-Meilán
183 et al., 2014). Dietary carbohydrate inclusion could also bind protease; affecting protein
184 digestion (Storebakken et al., 1998) and unbalancing the availability of amino acids that
185 can be absorbed; while the administration of low starch diets could lead to the up-
186 regulation of D-Glc and L-Ala absorption capacities (García-Meilán et al., 2014, 2013;
187 Santigosa et al., 2011b).

201 The effectiveness of a given diet is determined by digestion and absorption processes,
202 which ultimately affect growth and food utilization (Debnath et al., 2007; Lemieux et al.,
203 1999; Mohanta et al., 2008). Digestion begins in the stomach where pepsinogen is
204 converted into active pepsin by HCl and proteins are denatured (Wu et al., 2009; Yúfera
205 et al., 2004). When the chyme reaches the pyloric caeca, cholecystokinin stimulates
206 pancreatic secretions, which include, among other substances, proproteases, α -amylase
207 and lipolytic enzymes, as well as DNAase and RNAase (Bakke et al., 2011). The activities
208 of the main digestive enzymes differ between species and may be modified in relation to
209 dietary composition and its digestibility, food quantity and the natural diet (Buddington
210 et al., 1997; Hidalgo et al., 1999; Pérez-Jiménez et al., 2009; Reimer, 1982; Santigosa et
211 al., 2011a, 2011b, 2008; Zambonino-Infante and Cahu, 2007). The final step of digestion
212 is carried out in the enterocyte brush border membrane where many enzymes, such as
213 alkaline phosphatase, leucine aminopeptidase, maltase and saccharase, which release
214 molecules small enough for their absorption, are found (Bakke et al., 2011). In fish,
215 absorption processes occur throughout the entire intestine by means of diffusion,
216

facilitated transport or active transport (Mailliard et al., 1995). The presence of Na⁺-dependent and Na⁺-independent amino acid transport systems and peptide transporters has been described (Hakim et al., 2009; Sangaletti et al., 2009; Storelli et al., 1989; Terova et al., 2009), while D-glucose and D-galactose are transported by SGTL1 (Sala-Rabanal et al., 2004). Moreover, the intestinal fatty acid binding protein (I-FABP) may be involved in peptide or protein transport processes before endocytosis (Concha et al., 2002). The aim of this study was to test the effect of dietary carbohydrates on digestive and absorptive processes in sea bream fed with isoproteic diets where the lipid was replaced with starch, or isolipidic diets where the protein was replaced with starch. Fish were fed with diets containing 65% of plant-derived feed ingredients, using wheat starch to above the maximum levels established by Enes et al. (2011) for this fish species.

2. Materials and methods

2.1. Trials

Gilthead sea bream were acclimatized to the IRTA facilities (Sant Carles de la Ràpita, Spain) for two weeks. The water salinity was 35 g·L⁻¹ and the dissolved oxygen content was 8 ppm. Animals were maintained in 400L tanks in an open system at 22°C with a 12L/12D photoperiod. After acclimatization, two trials were performed in triplicate groups of fish, each weighing about 115 g.

For the first trial, 300 sea bream were randomly distributed in 12 fiberglass tanks (3 tanks per diet and 25 fish per tank). Animals were fed with four 46% protein diets with increasing starch content as lipid content decreased (12, 16, 22, 28% and 23, 20, 19, 17%, respectively). The diets, named according to their lipid content, differences in dietary ingredients and principal composition are shown in Table 1.

In the second trial, 225 sea bream were randomly distributed in 9 fiberglass tanks (3 tanks

per diet and 25 fish per tank). Fish were fed with three isolipidic diets with decreasing protein content as starch increased (46, 42, 40% and 19, 35, 39%, respectively). The diets, named according to their protein content, differences in dietary ingredients and principal composition are shown in Table 2.

Sea bream were fed the corresponding diet to apparent satiation by hand twice a day for a 14-week period and the survival was 100% for all dietary treatments. All experimental diets, where 65% fish meal was replaced with plant ingredients, were formulated and processed by Skretting ARC (Norway) and were extruded with a Wenger twin screw extruder: TX-57.

2.2. Sampling

At the end of the growth trial, nine animals per time and treatment were anaesthetized (phenoxyethanol 100 ppm), weighed and sacrificed by severing the spinal cord at post-prandial time (7 h) and under basal conditions (24 h after the last feed). Previous studies in this species (García- Meilán et al., 2014; 2013; Santigosa et al., 2011a) reported that at 7 h, the chyme was between the pyloric caeca and proximal intestine. Under post-feeding conditions, the digestive tract was isolated and samples of pyloric caeca and proximal intestine, including the intestinal content, were collected. Under basal conditions, the proximal intestine was isolated and cut lengthwise, and washed in an iso-osmotic saline solution containing 0.1 M of protease inhibitor (phenyl-methyl-sulphonyl-fluoride). All samples were rapidly frozen in liquid nitrogen and maintained at -80 °C until required for the intestinal enzymatic studies and the nutrient absorption experiments.

All fish-handling procedures complied with European guidelines for animal care (Directive 2010/63/EU).

2.3. Feed utilization and growth performance

To evaluate weight gain and calculate the specific growth rate (SGR) and feed conversion ratio (FCR) during the experimental period, the initial and final biomass of all triplicate tanks was obtained from the IRTA facilities. The SGR was calculated as follows: $((\ln W_{fin} (g) - \ln W_{ini} (g))/t) \cdot 100$, where W_{fin} and W_{ini} represent the final and initial weights respectively, and t is the number of days of feeding. The FCR was calculated as g feed given/g live weight gain. Voluntary feed intake was expressed in: $g\ BW\ (kg)^{-0.70}\ day^{-1}$ to correct for the influence of fish size on feed consumption in gilthead sea bream (Lupatsch et al., 1998), where body weight (BW) was calculated from the initial and final body weight of fish as the geometric mean: $BW = (W_{ini} \cdot W_{fin})^{0.5}$.

2.4. Intestinal pH and digestive enzyme analysis

Samples taken from the intestinal segment were used to measure the pH of the intestinal contents (Crison, micro pH 2000) and digestive enzyme activities in the pyloric caeca and proximal intestine in the post-prandial sample. A modification of the procedure described by Santigosa et al. (2008) was used to measure alkaline protease and α -amylase activities in the intestinal samples, and to characterize trypsin-like and chymotrypsin-like activities, while lipase activity was determined following Santigosa et al. (2011a). Briefly, intestinal samples were individually homogenized (Politron 2000, Sorvall TC) at 4 °C in 50 mM TrisHCl buffer pH 7.5 to a final concentration of 250 mg mL⁻¹. Homogenates were centrifuged (1100 g, 15 min, 4 °C, Jouan CR 411) and supernatants were recovered and stored at -80 °C. For alkaline protease activity (TPA) determination, samples were allowed to react with 50 mM TrisHCl buffer containing 1% casein at the pH of the intestinal contents and 20 °C. After 30 min, the addition of trichloroacetic acid 12% stopped the reaction and samples were kept at 4 °C for one hour and then centrifuged (7500 g, 5 min, 4 °C). Individual blanks were established for each sample. Supernatant

absorbance was measured at 280 nm (UV-1603, Shimadzu). Alkaline protease activity was measured as BAEE units, and bovine trypsin (Sigma Aldrich, Spain, 12100 BAEE units/mg protein, NC-IUB, 1979) was used as the standard.

Trypsin-like and chymotrypsin-like activities were characterized by zymography in which pure trypsin and albumin were used as controls and a commercial weight marker RPN 800 (Amersham, GE Healthcare, UK, 12000-225000Da) was used to determine the molecular weight of protease active fractions from the proximal intestine. Samples were loaded in 12% polyacrylamide gels (10x10.5x0.1 cm) and electrophoresis was performed at a constant current of 15 mA per gel for 150 min (EPS 301 Power Supply, 4 °C). The gels were incubated under agitation with TrisHCl buffer containing 2% casein at the pH of the intestinal content for 30 min at 4 °C, and after that were shaken for 90 min at room temperature. Gels were washed and stained in a methanol:acetic acid:water solution (40:40:10) with 0.1% BBC R-250 (Coomassie Brilliant Blue R-250). As in previous studies in sea bream (García-Meilán et al., 2014, 2013), six proteases were detected; three presented trypsin-like activity (90, 60 and 55 KDa) and the other three showed chymotrypsin-like activity (50, 30 and 25 KDa). Zymogram results were analyzed using Quantity One 1-D Analysis Software 4.6.6 (Bio-Rad). α -Amylase activity was measured using a potato starch covalently labelled with Remazol Brilliant Blue R dye, commonly known as amylose azure. The homogenate was allowed to react with 0.5% tracer solution containing soybean trypsin inhibitor (SBTI 0.04 mg·mL⁻¹) for 30 minutes, after which the reaction was stopped by the addition of 6% acetic acid and was centrifuged (13000 g; 10 min at 4 °C). Supernatant absorbance was measured at 595 nm (UV-1603, Shimadzu). α -Amylase (Sigma Aldrich, Spain, 66 U/mg solid) was used as the standard; one unit of enzyme activity was defined as 1.0 mg of maltose released from starch in 3 min at pH 6.9 and 20 °C.

For lipase determination, homogenates were mixed with buffer containing (in mM) 20.5 Tris, 3.6 taurodeoxycholate, 0.9 deoxycholate, 0.8 tartrate, 0.12 DGGR (1,2-*o*-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 CaCl₂, 30 mannitol and 1 mg L⁻¹ colipase (pH 8.3), and the increase in absorbance was recorded at 580 nm in the linear zone. Lipase (Sigma Aldrich, Spain, 33944 U/mg protein, 22980 U/mg solid) was used as a standard. One unit hydrolyzes 1.0 micro equivalent of fatty acid from triacetin in 1 h at pH 7.4 and 20 °C.

The protein concentration of the samples was measured according to the Bradford method (1976), using bovine albumin as the standard.

2.5. Brush border membrane enzyme activities

Samples taken from the proximal intestine under basal conditions were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100). Alkaline phosphatase activity (AP, EC 3.1.3.1) was analyzed following Weiser (1973). Briefly, the homogenates reacted with a buffer solution that contained p-nitrophenylphosphate (p-NPP) (in mM: 500 TrisHCl, 0.3 ZnCl₂, 10 MgCl₂·6H₂O, 0.5 p-NPP, pH 9.4). After 15 minutes, the reaction was stopped with NaOH 0.5 N. Supernatant absorbance was measured at 420 nm (UV-1603, Shimadzu). P-nitrophenol (p-NP) (Sigma Aldrich, Spain) was used as the standard. Units of alkaline phosphatase activity were calculated as the amount of enzyme activity liberating 1 mmol of p-nitrophenol per hour.

Leucine aminopeptidase activity (LAP, EC 3.4.11.1) was measured according to Maroux et al. (1973). L-Leu-p-nitroanilide (0.1 M) was mixed with phosphate buffer (in mM: 40 K₂HPO₄, 40 KH₂PO₄) and sample. The increase in absorbance was recorded at 410 nm in the linear zone for 10 minutes. Leucine aminopeptidase (Sigma Aldrich, Spain, 25UN) was used as the standard. One unit of enzyme activity is equal to 1 micromole of L-Leucinamide hydrolyzed per minute at 25 °C and pH 8.5.

2.6. Nutrient uptake analysis

Samples from the proximal intestine were processed to obtain brush border membrane vesicles (BBMVs) according to Sala-Rabanal et al. (2004). To separate mucosa from the muscular layers, samples were homogenized in a hypo-osmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4, Osm 100). Selective precipitation of basolateral and mitochondrial membranes was achieved through the addition of MgCl_2 to a final concentration of 10 mM. Subsequent selective centrifugation enabled the purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.08 LiN_3 , pH 7.4, 320 Osm) using an insulin syringe. The enrichment in brush border membrane preparations was determined by measuring alkaline phosphatase activity, which has been described as a good marker of BBMVs (Weiser, 1973). Good enrichment was observed (8.84 ± 0.74) and based on Santigosa et al.'s (2011a, b) results, vesicular preparations were used when enrichment was higher than 4 fold. At 20 °C the absorption capacities of L-lysine, an essential amino acid, L-alanine, a non-essential one, and D-glucose were determined in vesicular suspensions according to the procedure described by Santigosa et al. (2011b). These suspensions were mixed with incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 8.16 LiN_3 , 0.15 unlabelled nutrient, 0.01 ^3H -nutrient, Osm 320, pH 7.4) for 5 sec. The reaction was stopped by adding cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4.08 LiN_3 , Osm 320, pH 7.4). The resulting mix was rapidly passed under negative pressure through 0.22- μm cellulose nitrate filters (Millipore, Bedford MA) previously soaked in cold stop buffer. Filters were washed with stop buffer and dissolved in Filtron-X scintillation liquid (Itisa S.A., Spain). Samples were counted in a scintillation counter (Packard Tri-Carb 2100 TR). Radiolabelled nutrients were purchased from Amersham Pharmacia Biotech (Barcelona, Spain).

Dietary composition modifies the vesicular volume (Santigosa et al., 2011a, 2001b), therefore the influx values have to be normalized. The vesicular volume was determined by the incubation of BBMV preparations with incubation buffer on ice and measuring the amount of L-alanine retained inside the vesicles at equilibrium (Sala-Rabanal et al., 2004). After 90 min, the reaction was stopped, and the amount of L-alanine was measured following the procedure described above.

The protein concentration of different vesicular suspensions was measured using BIORAD® reagent according to the Bradford method (1976).

2.7. Statistical analysis

Significant differences between groups were determined by one-way ANOVA followed by Tukey's or Dunnett's T3 post hoc test. Differences between intestinal segments were determined using Student's *t*-test ($P < 0.05$). The software used was SPSS Statistics 24.0 (SPSS Inc., EUA).

3. Results

Replacement of dietary lipids with starch in isoproteic diets

Table 3 shows final weight, SGR, voluntary feed intake or FCR when lipid was replaced with starch and no differences were found between experimental conditions.

The pH of the intestinal content and the activity of digestive enzymes (lipase, α -amylase and alkaline protease) from the pyloric caeca and proximal intestine were analysed at post-feeding time (Figure 1). The pH of the intestinal contents showed an increase in the pyloric caeca between L20 and L17 as dietary lipid was replaced with starch, whereas no differences were found in the proximal intestine. Despite this, it was significantly higher in the proximal intestine versus the pyloric caeca except in L17 sea bream. Although no significant changes were detected in digestive enzyme activities when dietary lipids were

replaced with starch, lipase and total protease activities tended to be lower in L23 than L17 fish. Differences in protease activity were found between those fish groups in the pyloric caeca and proximal intestine. Higher protease activity was also measured in the proximal intestine than in the pyloric caeca, which was not the case for amylase activity. Alkaline protease activity in the proximal intestine was analysed by performing zymograms. Consistent with previous studies (García-Meilán et al., 2013; 2014), 6 proteolytic bands were detected in the proximal intestine, three with trypsin-like activity (90, 60 and 55 KDa) and three with chymotrypsin-like activity (50, 30, and 25 KDa). Table 4 shows a detailed analysis of individual alkaline protease activities whereby trypsin-like activities also seem to be more sensitive to changes in lipid replacement with starch than chymotrypsin. In this sense, total trypsin activity was reduced from L23 to L20-L19 and to L17, but only total chymotrypsin activity of L17 was lower. Accordingly, 90 KDa, 60 KDa, and 55 KDa trypsin-like proteases and 50 KDa chymotrypsin-like activities were significantly reduced in sea bream fed the highest starch content (L17 animals). Thirty and 25 KDa chymotrypsin-like activities were not modified by the substitution. These modulations of individual protease activities lead to a diminution on the trypsin/chymotrypsin ratio although it was not significant.

Regarding brush border enzyme activities under basal conditions, the highest alkaline phosphatase (AP) activity was detected in fish fed the two diets with high lipid and low starch content (Figure 2), and no changes were detected in leucine aminopeptidase (LAP) activities (mean 1.72 ± 0.09 mU·mg prot⁻¹·min⁻¹).

Nutrient absorption capacities were also measured in brush border membrane vesicles (Figure 3). L-Ala and D-Glc uptakes tended to decrease with the substitution of dietary lipids with starch, although only significantly for D-Glc; while L-Lys absorption did not differ significantly when all groups were considered but was significantly higher in L19

versus L17 fish.

Replacement of dietary protein with starch in 17% lipid diets

Table 5 shows that there was a significant reduction in SGR and voluntary feed intake when protein was replaced with starch, which led to a significant increase in the FCR in P40 sea bream versus the other groups.

The pH of the intestinal content and digestive enzyme activities measured post-feeding are shown in Figure 4. When dietary protein was replaced with starch no differences were found between the pyloric caeca and proximal intestine in terms of intestinal pH, although it tended to be lower in the proximal intestine of P40 sea bream versus P46 and P42 (Figure 4). Regarding digestive enzyme activities, an increase in proximal intestine lipase activity was recorded when dietary protein was substituted with starch, and this was significant in P42 animals. In contrast, pyloric caeca α -amylase activity showed a significant decrease as dietary starch increased. Finally, there was a significant increase in both pyloric caeca and proximal intestine protease activity when the dietary protein fell to 40%; this was also recorded in the pyloric caeca in P42 fish (Figure 4).

Zymography analysis (Table 6) revealed that 60 and 55 KDa trypsin activities were more sensitive to protein replacement with carbohydrates than chymotrypsin activities, which showed no changes. In this sense, the total trypsin activity of P40 sea bream was 1.7 and 2.6 higher than that of P46 and P42 animals, respectively. Significant changes in 60 and 55 KDa trypsin activities were responsible for this. Instead, the chymotrypsin activity and trypsin/chymotrypsin ratio did not show significant differences.

The activities of the brush border enzymes alkaline phosphatase (AP) and leucine aminopeptidase (LAP) were measured under basal conditions in the proximal intestine and no significant changes were found as protein substitution increased either AP

(mean 0.223 ± 0.22 mM·mg prot⁻¹·min⁻¹) or LAP activity (mean 190.9 ± 10.8 mU·mg prot⁻¹·min⁻¹). In addition, AP activity tended to be higher in P40 versus P42 and P46 (Figure 5), and was accompanied by a slight decrease in lipase activity in the proximal intestine (Figure 4).

The nutrient absorption capacities of D-Glc, L-Ala and L-Lys were determined in brush border membrane vesicles (Figure 6). The nutrient absorption capacity of L-Ala and D-Glc decreased significantly when dietary protein was replaced with starch, while L-Lys absorption was maintained.

Discussion

Vegetable ingredients are commonly used to replace fishmeal and fish oil in aquaculture feeds due to their abundance and low price (SOFIA, 2018). They are a source of both protein and energy (García-Meilán et al., 2014; 2013), and the binding properties of starch are useful (Krogdahl et al., 2005). The protein sparing effect of dietary lipid or carbohydrates has been studied in sea bream, being well established for the former (Company et al., 1999; Vergara et al., 1999). However, there is controversy over the effect of using high amounts of carbohydrates (Enes et al., 2011; García-Meilán et al., 2014), probably due to the many factors that affect starch digestibility (Enes et al., 2011; Kokou and Fountoulaki, 2018). The regulation of digestive enzyme release and nutrient absorption capacity have been related to the protein sparing effect (García-Meilán et al., 2014).

When dietary lipids were substituted with starch in isoproteic diets, the pH of the intestinal content in the pyloric caeca increased, suggesting that the amount of dietary lipid affects the buffering capacity of sea bream. In this sense, Lawlor et al. (2005) demonstrated that dietary ingredients have different acid binding capacities, and modify

bicarbonate secretion from the pancreas, pancreatic ducts, bile and intestine (Grossel and Genz, 2006; Wilson & Grossel, 2003).

Regarding α -amylase, sea bream showed higher activities (approximately 2.85-fold higher) than in other studies that tested diets containing a similar percentage of starch ranging from 10 to 28% (García-Meilán et al., 2013; Santigosa et al., 2011a). However, in the present study, diets were formulated with wheat starch instead of wheat meal, indicating that the way in which starch is incorporated into the diet is an important factor that could affect amylase secretion. Fish fed with high lipid diets tended to up-regulate both lipase and alkaline phosphatase activities as described previously for lipase (Buchet et al., 2000; Li et al., 2012; Ma et al., 2014; Zambonino-Infante and Cahu, 1999) and alkaline phosphatase activity (Gawlicka et al., 2002; Grewal and Mahmood, 2004; Kroghdahl et al., 2003; Mozes et al., 2004; Stenson et al., 1989). In fact, the latter might be involved in lipid absorption (Narisawa et al., 2003; Zhang et al., 1996).

Alkaline protease activity is modulated by diet in carnivorous species (Buddington et al., 1997; García-Meilán et al., 2014). In several species, including Atlantic salmon (Kroghdahl et al., 2003), European sea bass (Pères et al., 1998) and sea bream (García-Meilán et al., 2013), dietary protein stimulates trypsin activities, but less is known about lipid effects in isoproteic diets. In this sense, this study showed that feeding fish with high lipidic diets also up-regulated total protease activity, suggesting that this is a compensatory mechanism as fat negatively affects proteolytic activity according to Fountoulaki et al. (2005) and Murashita et al. (2008).

As mentioned earlier, the presence of vegetable ingredients can negatively affect feed digestibility in fish species (Aslaksen et al., 2007; Storebakken, 1985), so the presence of non-starch polysaccharides such as cellulose cannot be ignored and could also explain the observed differences in protease activities.

378 A trend to diminish in the trypsin/chymotrypsin ratio was found when lipid was replaced
379 with starch and cellulose in isoproteic diets, accompanied by a slight up-regulation in L-
380 Lys absorption capacity in the proximal intestine. However, when the diet did not contain
381 fibre, as was the case for L17 fish, the capacity for L-Lys uptake decreased but growth
382 remained stable. The difference in intestinal transit rate could explain these results since
383 higher amounts of starch and fibre in the diet accelerate intestinal transit, decreasing the
384 absorption time; in contrast the absence of cellulose slows it down, increasing the
385 absorption time (Francis et al., 2001; Gatlin et al., 2007; Krogdahl et al., 2010). This was
386 reflected by LAP activity, which was slightly up-regulated when L-Lys absorption
387 decreased. Moreover, animals fed with 46% protein, 20% lipid and starch levels below
388 16% showed increased D-Glc absorption capacity, whereas L-Ala uptake barely
389 increased, suggesting that, despite the high dietary lipid content, these fish were able to
390 use more glucose if it was present in the diet, thus saving lipid without affecting growth.

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392 The replacement of protein by starch in diets with the lowest lipid content (17%) caused
393 an increase in lipase activity in order to optimize lipid digestion and allowing sparing
394 effect. Protease activity was also up-regulated by more than 30%, while α -amylase
395 activity was down-regulated. Previous studies found that α -amylase activity was more
396 constitutive than protease (García-Meilán et al., 2013 and 2016b), and also that when high
397 levels of starch were included in the diet (García-Meilán et al, 2014), as in the present
398 study, the degree of change was similar for both enzymes (31.5% for α -amylase and
399 43.6% for protease, data from the present study). Although the relationship between α -
400 amylase and starch dietary content in carnivorous species is unclear (Krogdhal et al.,
401 2005) the decline in α -amylase activity in fish fed high starch diets could also be
402 explained by the adsorption of α -amylase by starch molecules, which inhibits the enzyme

(Enes et al., 2011; Fountoulaki et al., 2005). Regarding alkaline protease activity, the up-regulation found in animals fed high starch diets (P42 and P40) suggests that it provides a compensatory mechanism to improve nutrient digestion, consistent with studies that reported that dietary starch and/or lipid could interfere with proteolytic activity (Fernández et al., 2001; Fountoulaki et al., 2005; García-Meilán et al., 2013; Hidalgo et al., 1999; Murashita et al., 2008). In this sense, trypsin activity seems to be more sensitive to dietary protein replacement with starch than chymotrypsin. Thus, the zymographic study showed that the increase observed in the P40 group was mainly due to the up-regulation of 60 and 55 KDa trypsin. The 90 KDa trypsin seems to be more constitutive according to previous studies (García-Meilán et al., 2013; 2014; Santigosa et al., 2008; 2011a). Moreover, it has been reported that the kind and the amount of ingredients and the presence of antinutritional factors could negatively affect the trypsin/chymotrypsin ratio (Francis et al., 2001; Krogdahl et al., 2010). Although in the present study Soybean Protein Concentrate (SPC) was incorporated into the diets (between 2.7 to 11.5%), there were no changes in this ratio. In this context, Van der Ingh et al. (1991) and Kaushik et al. (1995) reported that diets formulated with less than 15% SPC do not cause problems either in growth or at the digestive level. Although the only experimental difference between both trials was diets used in each, different trypsin and chymotrypsin activities were measured between experiments, but it was not possible to relate these changes to the presence of a single ingredient in diet.

The modulation of lipase activity found in the present study contrasts with previous studies, where no effects on this enzyme were found following changes in protein or carbohydrate dietary content (García-Meilán et al., 2014, 2013; Santigosa et al., 2011b). These differences in lipase activity modulation could be due to the lower lipid content in the present study (17% versus 19–26% in previous studies), which could be given to

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428 optimize the digestion of lipids, when both protein and lipid content are low. Supporting
429 this hypothesis, the up-regulation of intestinal phosphatase activity detected in sea bream
430 fed high starch and low protein diets suggests that it could be a compensatory mechanism
431 for increasing lipid uptake, in an attempt to maintain growth. Brush border enzymes carry
432 out the final stages of food degradation and assimilation (Klein and Cohn, 1998) and their
433 activity is greatly modulated by the intraluminal presence of dietary substrates (Cézard et
434 al., 1983; Kimura et al., 1978; Kuperman and Kuz'mina, 1994). However, different
435 dietary protein levels had no effect on leucine aminopeptidase activity in tilapia (Hakim
436 et al., 2006), as found in the present study for sea bream, suggesting that LAP does not
437 limit digestion.

438 It is known that sea bream are able to modulate the capacity for amino acid and glucose
439 transport in the pyloric caeca and proximal and distal intestine (Santigosa et al., 2011b)
440 and whole intestine (García-Meilán et al., 2014, 2013) by up- or down-regulation to meet
441 their needs in response to dietary composition. In this sense, in the present study, D-Glc
442 absorption capacity decreased significantly when starch availability increased. Consistent
443 with these results, other studies suggest that high levels of monosaccharides could inhibit
444 amino acid transport (Ferraris and Ahearn, 1984; Vinardell, 1990), as described for L-
445 Ala. However, L-Lys absorption capacity was not inhibited, indicating low protein
446 availability that led to poor growth and higher FCR, as seen in other studies (Cheng et al.,
447 2003; Rathore et al., 2010; Sánchez-Lozano et al., 2011; Tibaldi and Lanari, 1991; Zhou
448 et al., 2011), and probably due also to the lower voluntary feed intake.

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450 In conclusion, when lipid was replaced with starch in 46% protein diets, compensatory
451 mechanisms involving changes in intestinal pH, pancreatic and intestinal digestive
452 enzymes and nutrient absorption capacities took place, allowing the maintenance of feed

utilization and growth, and suggesting that animals are able to modulate the digestive process when protein requirements are met. In contrast, when dietary protein was replaced with starch in 17% lipid diets, the digestive compensatory mechanisms were not sufficient and a significant increase in FCR and a reduction in voluntary feed intake and SGR were found. Despite this, the SGR and FCR obtained to sea bream fed P46 were good, indicating that a reduction of lipid level in commercial diets would be possible, improving the economic conversion ratio and the productivity of this species.

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Figures

Figure 1. Intestinal pH and lipase, α -amylase and alkaline protease post-feeding activities when dietary lipids were replaced by starch in sea bream. Diets were named according to their dietary lipid content. Pyloric caeca (white bars) and proximal intestine (black bars). Values are mean $\pm$ SEM. (n=9). Differences ($P < 0.05$) between dietary conditions are shown by different letters; significant differences ($P < 0.05$) between intestinal segments are shown by *.

Figure 2. Alkaline phosphatase activity (n=9) measured in brush border membrane vesicles from proximal intestine under basal conditions when dietary lipids were replaced by starch in sea bream. Values are mean $\pm$ SEM. Differences ($P < 0.05$) between dietary conditions are shown by different letters.

Figure 3. D-Glc (black bars), L-Ala (grey bars) and L-Lys (white bars) absorption capacities (n=9) in brush border membrane vesicles from proximal intestine under basal conditions when dietary lipids were replaced by starch in sea bream. Values are mean $\pm$ SEM. Differences ($P < 0.05$) between dietary conditions are shown by different letters.

Figure 4. Intestinal pH and lipase, α -amylase and alkaline protease post- feeding activities when dietary protein were replaced by starch in sea bream. Diets were named according to their dietary protein content. Pyloric caeca (white bars) and proximal intestine (black bars). Values are mean $\pm$ SEM. (n=9). Differences ($P < 0.05$) between dietary conditions are shown by different letters; significant differences ($P < 0.05$) between intestinal segments are shown by *.

Figure 5. Alkaline phosphatase activity (n=9) measured in brush border membrane vesicles from proximal intestine under basal conditions when dietary protein were replaced by starch in sea bream. Values are mean $\pm$ SEM. Differences ($P < 0.05$) between dietary conditions are shown by different letters.

Figure 6. D-Glc (black bars), L-Ala (grey bars) and L-Lys (white bars) absorption capacities (n=9) in brush border membrane vesicles from proximal intestine under basal conditions when dietary protein were replaced by starch in sea bream. Values are mean $\pm$ SEM. Differences ($P < 0.05$) between dietary conditions are shown by different letters.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Table 1. Ingredients and proximate composition of the experimental diets when dietary lipids were replaced by starch in sea bream.

Diet		L23	L20	L19	L17
Ingredients (%)	Fish meal	20			
	Wheat Gluten	20			
	Corn Gluten	13.1			
	SPC 60%	11.5			
	Fish oil north Atlantic	17.2	15.3	13.3	11.3
	Wheat starch	5.3	11.2	17.6	22.0
	Cellulose	10.8	6.8	2.4	0.0
Principal composition (dry matter)	Dry matter	93.9	93.9	92.4	92.7
	Protein	45.5	46.3	47.3	46.5
	Fat	22.8	20.3	19.1	17.0
	Starch	11.5	15.6	22.1	27.8

Diets were formulated and produced by Skretting ARC (Norway). All diets contain: 2% vitamin premix and 0.1% Yttrium premix. SPC: Soybean Protein Concentrate. Vitamin premix is a proprietary formula of Skretting (Norway).

Table 2. Ingredients and proximate composition of the experimental diets when dietary protein were replaced by starch in sea bream.

Diet		P46	P42	P40
Ingredients (%)	Fish meal	20		
	Wheat gluten	20	20	21
	Corn gluten	13.1	14.7	9.8
	SPC 60%	11.5	2.7	2.9
	Fish oil north atlantic	11.3		
	Wheat starch	14.3	29.2	32.9
	Cellulose	7.7	0	0
Principal composition (dry matter)	Dry matter	94.0	91.6	92.5
	Protein	46.2	42.1	39.6
	Fat	17.3	17.0	17.2
	Starch	18.5	35.2	38.7

Diets were formulated and produced by Skretting ARC (Norway). All diets contain: 2% vitamin premix and 0.1% Yttrium premix. SPC: Soybean Protein Concentrate. Vitamin premix is a proprietary formula of Skretting (Norway).

Table 3. Final body weight, **SGR**, voluntary feed intake and FCR for each experimental condition (n=3) when dietary lipids were replaced by starch in sea bream.

Diet	L23	L20	L19	L17
Final Body Weight	366.9 ± 11.6	378.0 ± 0.9	378.4 ± 6.3	361.9 ± 4.6
SGR	1.09 ± 0.03	1.12 ± 0.00	1.13 ± 0.02	1.08 ± 0.01
Voluntary Feed Intake (g·Kg ^{-0.7} ·day ⁻¹)	3.66 ± 0.12	3.65 ± 0.08	3.75 ± 0.07	3.63 ± 0.02
FCR	1.62 ± 0.09	1.59 ± 0.05	1.50 ± 0.04	1.63 ± 0.02

Values are presented as mean ± SEM. Different letters indicate significant differences (P<0.05) between diets.

Table 4. Trypsin and chymotrypsin activities (U·mg prot⁻¹) calculated from proximal intestine zymography analysis (n=5) when dietary lipids were replaced by starch in sea bream.

Diet	L23	L20	L19	L17
Trypsin 90KDa	1.07 ± 0.12a	0.67 ± 0.06ab	0.92 ± 0.11ab	0.62 ± 0.12b
Trypsin 60KDa	1.72 ± 0.24ab	1.69 ± 0.15a	1.80 ± 0.19a	0.89 ± 0.06b
Trypsin 55KDa	2.21 ± 0.19a	1.67 ± 0.07a	1.66 ± 0.29ab	1.16 ± 0.08b
Total Trypsin activity	5.0 ± 0.11a	4.03 ± 0.05b	4.12 ± 0.15b	2.67 ± 0.17c
Chymotrypsin 50 KDa	1.18 ± 0.11a	1.17 ± 0.07a	1.27 ± 0.11a	0.78 ± 0.09b
Chymotrypsin 30 KDa	0.69 ± 0.06	0.60 ± 0.07	0.66 ± 0.08	0.57 ± 0.05
Chymotrypsin 25 KDa	1.72 ± 0.15	1.37 ± 0.06	1.58 ± 0.18	1.28 ± 0.18
Total Chymotrypsin activity	3.59 ± 0.11ab	3.14 ± 0.05bc	3.77 ± 0.15a	2.63 ± 0.17c

Values are presented as mean ± SEM. Significant differences (P<0.05) between dietary treatments are shown by letters.

Table 5. Final body weight, **SGR**, voluntary feed intake and FCR for each experimental condition (n=3) when dietary protein were replaced by starch in sea bream.

Diet	P46	P42	P40
Final Body Weight	380.1 ± 12.5 a	348.4 ± 6.4 a	312.1 ± 3.3 b
SGR (%)	1.13 ± 0.03 a	1.05 ± 0.02 a	0.95 ± 0.01 b
Voluntary Feed Intake (g·Kg ^{-0.7} ·day ⁻¹)	4.06 ± 0.25 a	3.51 ± 0.07 ab	3.23 ± 0.04 b
FCR	1.38 ± 0.14 b	1.73 ± 0.06 b	2.15 ± 0.02 a

Values are presented as mean ± SEM. Different letters indicate significant differences (P<0.05) between diets.

Table 6. Trypsin and chymotrypsin activities (U·mg prot⁻¹) calculated from proximal intestine zymography analysis (n=5) when dietary protein were replaced by starch in sea bream.

Diet	P46	P42	P40
Trypsin 90KDa	0.41 ± 0.04	0.45 ± 0.11	0.59 ± 0.09
Trypsin 60KDa	0.70 ± 0.15 b	0.51 ± 0.07 b	1.29 ± 0.19 a
Trypsin 55KDa	0.59 ± 0.08 b	0.17 ± 0.07 c	1.03 ± 0.16 a
Total Trypsin activity	1.70 ± 0.12b	1.13 ± 0.08c	2.92 ± 0.18a
Chymotrypsin 50 KDa	0.32 ± 0.05	0.34 ± 0.09	0.46 ± 0.11
Chymotrypsin 30 KDa	0.16 ± 0.05	0.20 ± 0.03	0.21 ± 0.08
Chymotrypsin 25 KDa	0.20 ± 0.04	0.18 ± 0.06	0.15 ± 0.06
Total Chymotrypsin activity	0.67 ± 0.12	0.73 ± 0.08	0.82 ± 0.18

Values are presented as mean ± SEM. Significant differences (P<0.05) between dietary treatments are shown by letters.

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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