Modifications of intestinal nutrient absorption in response to dietary fish meal replacement by plant protein sources in sea bream (*Sparus aurata*) and rainbow trout (*Onchorynchus mykiss*).

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Abstract

Two experimental diets in which fish meal was partially (75%; diet PP75) or totally (diet PP100) replaced by plant protein sources were fed to gilthead sea bream and rainbow trout. We studied the effects of these diets on intestinal nutrient absorption in comparison to fish fed the fish meal diet (FM). A mixture of vegetal ingredients (corn gluten meal, wheat gluten, extruded peas and rapeseed meal) was used to meet the amino acid requirements of the fish. Over a 12-week trial, 3 groups of the two species were fed one experimental diet twice a day until visual satiation.

After the experimental growth trial, we measured amino acid (L-leucine, L-lysine, L-phenylalanine, L-alanine and L-proline) and D-glucose absorption at 6 h and long-term (48 h for sea bream and 36 h for rainbow trout) post-feeding in pyloric caeca, proximal intestine and distal intestine segments using brush border membrane vesicles. The absorption pattern 6 h post-feeding was modified in both species in response to fish meal replacement: total absorption capacities were generally maintained in PP75 trout because absorption was delayed from pyloric caeca to proximal and/or distal intestinal segments, while absorption uptake was significantly decreased in both sea bream groups and trout fed the 100% vegetable diet. Glucose transport capacities were increased in both experimental sea bream groups and in trout fed the PP75 diet. Long-term transport capacities showed an up-regulation for both studied species.

Results obtained in this work show that intestinal nutrient absorption is modified in response to fish meal replacement by plant protein sources and that changes are species-specific and not dose-dependent.
Keywords: fish; brush border membrane vesicles; post-prandrial; intestinal nutrient absorption; amino acid; glucose.
1. Introduction

Fish meal (FM) has been traditionally used as the main source of protein in carnivorous fish aquafeeds, but aquaculture increase in the last years has led to a limited FM availability (SOFIA, 2008). Thus, other protein sources are needed to replace FM in finfish carnivorous diets in order to permit the sustainable development of this industry, ensuring at the same time optimal growth rates and a high quality final product. In this regard, plant protein (PP) meals have been widely studied, being demonstrated that high percentages of FM can be partially replaced without compromising fish growth performance (Albrektsen et al., 2006; de Francesco et al., 2007) when diets are balanced to match fish amino acid requirements. However, vegetable sources are rich in antinutritional factors (Francis et al., 2001; Gatlin et al., 2007) that can compromise fish health (Olsen et al., 2007) and impair fish performance when not treated (Knudsen et al., 2006) or used at inadequate levels (Hansen et al., 2007). In this regard, it has been demonstrated that feeding plant protein-based diets to carnivorous fish diminishes intestinal alkaline protease activity (Lilleeng et al., 2007; Santigosa et al., 2008). The whole of these results suggest that nutrient luminal availability could be modified as a consequence of the depletion of intestinal enzymatic activity. Moreover, previous experiences have shown that feeding vegetable meals to fish provokes histological modifications in the intestine (Krogdahl et al., 2003; Santigosa et al., 2008) which might modify the capacity of the transporters immersed in the lipidic bilayer. All these modifications make indispensable the study of the effects of fish meal replacement by plant protein meals on fish intestinal nutrient absorption.

With the aim of characterizing intestinal nutrient transport in different fish species, brush border membrane vesicles have been previously used (Drai et al., 1990;
Ahearn and Storelli, 1994; Sala-Rabanal et al., 2004). This technique permits to study uptake rates with no substrate metabolism. However, other approaches such as everted sleeve technique or in vivo apparent absorption exist to determine uptake capacities (Nordrum et al., 2000; Bakke-McKellep et al., 2000; Refstie et al., 2006). Thus, wide information concerning absorption mechanisms in fish is available (Collie and Ferraris, 1995; Boge et al., 2002; Sala-Rabanal et al., 2004). In vertebrates absorption occurs by diffusion, facilitated transport or active transport (Maillard et al., 1995). Facilitated and active transports are used for the uptake of amino acids, oligopeptides and glucose. They follow asymptotic kinetics and depend on the presence of protein transporters immersed in the lipid bilayer (Palacin et al., 1998). In fish, nutrient uptake is mediated by transport proteins similar to those of mammals even if substrate specificity differences have been described between the two groups (Collie and Ferraris, 1995). Moreover, contrary to mammals, amino acid transporters in fish are present all along the intestine (Buddington et al., 1997).

On the basis of these premises, the aim of this study was to determine if nutrient absorption capacity along the intestine was modified at two different post-feeding times when a blend of vegetable meals was used to partially (75%) or totally replace fish meal in the diet of sea bream (Sparus aurata) and rainbow trout (Oncorhynchus mykiss).
2. Materials and Methods

2.1. Diets

Three experimental isoproteic and isolipidic diets for each species (Table 1) were formulated in the Institute National de la Recherche Agrobiologique (I.N.R.A., St. Pée-sur-Nivelle, France). Diet FM contained fish meal as the sole source of protein. In diets PP75 and PP100 fish meal was replaced by a blend of plant protein sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) in 75% and 100%, respectively. Diets were balanced with free crystalline amino acids to match fish nutritional requirements.

2.2. Animals and Sampling

In the Instituto de Acuicultura de Torre de la Sal (C.S.I.C., Spain) acclimated sea bream (16.5 ± 0.7 g) were randomly distributed in nine 0.5 m³ circular glassfiber tanks (90 fish per tank). Three groups of fish were fed one experimental diet twice a day until visual satiety for 12 weeks (April to July). Rainbow trout (19.0 ± 0.2 g) were randomly distributed in nine 1 m³ circular glassfiber tanks (75 fish per tank) at the INRA experimental fish farm (Donzacq, Landes, France). During 12 weeks (May to June) triplicate groups of fish were fed one of the experimental diets twice a day until visual satiety. Both trials were conducted under natural temperature and photoperiod.

At the end of each growth trial, 20 fish per experimental group were starved for 24 h, lightly anaesthetized with MS-222 (0.1 g L⁻¹) and force-fed the corresponding diet to their group (1% of their body weight) by the stomach intubation method (Schuhmacher et al., 1997) using a polyethylene catheter (0.3 mm, i.d., 10 cm. of length) and a 5 cm³ syringe. In order to get an acceptable food paste, diets were grounded and mixed with 2 parts of water. At two different post-prandial times (6 and 48 h for sea bream; 6 and 36 h for trout) fish were anaesthetized, weighed and
sacrificed by severing their spinal cord. Digestive tracts were isolated on ice and the adherent tissue was removed. Intestines were divided into pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI) according to morphological appearance. Segments were opened lengthwise, washed in isosmotic saline containing protease inhibitor phenyl-sulphonil-methyl-fluoride (PMSF 0.1M) and rapidly frozen.

2.3. Nutrient uptake studies

Obtaining of brush border membrane vesicles (BBMV) of different intestinal segments was done according to the technique described by Sala-Rabanal et al. (2004). Briefly, tissue was homogenized (in mM: 100 mannitol, 2 HEPES for sea bream; 60 mannitol, 2 HEPES for trout; pH 7.4) and baso-lateral membranes were precipitated by addition of MgCl₂ and centrifugation. Subsequent selective centrifugations allowed the purification and concentration of apical enterocyte membranes, which were vesiculated (in mM: 300 mannitol, 20 HEPES, 0.1 MgSO₄·7H₂O, 4.08 LiN₃ for sea bream; 285 mannitol, 20 HEPES, 0.1 MgSO₄·7H₂O, 4.08 LiN₃ for trout; pH 7.4) using an insulin syringe.

Validation of the BBMV suspensions: to ensure the enrichment in brush border membrane, as well as the depletion in other cellular fractions, the activity of membrane enzymes considered suitable cellular markers (Scalera et al., 1980) was measured (20°C) in the initial homogenate and in the final BBMV preparations. Thus, alkaline phosphatase activity was measured following Weiser (1973) to assure an increase in brush border membrane during vesiculation process. To rule out baso-lateral membrane contamination, Na⁺/K⁺-ATPase activity was determined using the method described by Colas and Maroux (1980) as modified by Sala-Rabanal et al.
Similarly, depletion in citrate synthase activity (Srere, 1969) discarded the presence of mitochondrial debris.

The orientation of BBMVs was studied using a modification of the approach described by Del Castillo and Robinson (1982). Thus, vesicle preparations were incubated in the presence and absence of a buffer containing 2 mM sodium deoxicolate plus 15 mM EDTA, which disrupts cellular membranes, and then total sacarase activity of the solution was measured by adding 100 μM sacarose to the preparations. An increase in sacarase activity in disrupted BBMVs versus the non-disrupted vesicles indicates the presence of vesicles in the non-physiological orientation.

*The lineal uptake zone in the obtained BBMV* was determined for 3 essential aminoacids (L-leucine, L-lysine and L-phenylalanine), 2 non essential amino acids (L-alanine and L-proline) and D-glucose as described by Sala-Rabanal et al. (2004). For the analysis, 10 μL of BBMVs were mixed with 40 μL of incubation buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 MgSO₄·7H₂O, 8.16 LiN₃, 0.15 unlabelled nutrient, 0.01 ³H-nutrient. Osm 320; pH 7.4 for sea bream; 250 NaSCN, 70 mannitol, 40 HEPES, 0.1 MgSO₄·7H₂O, 8.16 LiN₃, 0.15 unlabelled nutrient, 0.01 ³H-nutrient. Osm 305; pH 7.4 for trout). At different incubation times (1 to 10 s) reaction was stopped by adding 1 mL of cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1 MgSO₄·7H₂O, 4.08 LiN₃. Osm 320; pH 7.4 for sea bream; 285 mannitol, 20 HEPES, 0.1 MgSO₄·7H₂O, 4.08 LiN₃. Osm 305; pH 7.4 for trout). 990 μL of the resulting mix were rapidly filtered under negative pressure through 0.22 μm cellulose nitrate filters (Millipore, Bedford MA) previously wetted in cold stop buffer. Filters were washed (10 mL 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). All determinations were done at 20°C.

Determination of vesicular volume using L-alanine: since BBMVs volume changes depending on the intestinal segment studied (Sala-Rabanal et al. 2004), vesicle volume was individually determined. Thus, L-alanine retained in the inside of the vesicles at the equilibrium situation was measured incubating for 90 min in ice 10 μL of BBMVs preparation with 40 μL of the usual incubation buffer. Then reaction was stopped and counted according to the previously described protocol.

Amino acids and Glucose uptake to BBMVs: aliquots of 10 μL of each vesicular suspension were reacted for 5 s with 40 μL of the incubation buffer that contained 0.151mM (of which 0.01 ³H-labelled) of the studied nutrient: L-leucine, L-lysine, L-phenylalanine, L-alanine, L-proline or D-glucose. After the incubation time, reaction was stopped using 1 mL of stop buffer and then filtered and counted as previously described.

Quantification of proteins of the vesicular fractions: protein concentration of initial homogenate and final BBMV suspension was determined according to Bradford (1976) using BIORAD® reagent.

2.4. Statistical study of the results
To establish the existence of significant differences (p<0.05) between two parameters T-Student test was used. Differences between more than two groups were determined using the one way ANOVA test and Tuckey test. Software used was SPSS 12.0 (SPSS Inc., EUA).

2.5. Chemicals
Radiolabelled nutrients were obtained from Amersham-Pharmacia Biotech (Spain).
The rest of chemicals were purchased by Sigma-Aldrich (Spain).
3. Results

BBMVs of pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI) were obtained at two different post-feeding times (6 and 48 h for sea bream; 6 and 36 h for rainbow trout) from fish fed the experimental diets in order to study the effect of the diet on 1) amino acids and glucose uptake capacity and 2) regionalization uptake pattern along the intestinal tract.

BBMVs validation and determination of the linear uptake zone: Purity of the vesicular preparations was checked (Table 2). The increase in alkaline phosphatase activity – ranging from 3.39 to 6.56- together with the decrease in Na⁺/K⁺ ATPase (0.53 ± 0.08) and citrate synthase (0.10 ± 0.01) activities in the final BBMVs preparations when compared to the initial homogenates indicated that final suspensions were enriched in brush border membrane and impoverished in basolateral membrane and mitochondrial fractions, respectively. In all the cases, vesicles presented the physiologic orientation (99.4 ± 0.3% for sea bream; 99.0 ± 0.9% for rainbow trout; n=12), discarding the possibility of underestimating uptake capacities because of the transporters retained in the inner side of the vesicles.

Vesicular volume was determined in the different BBMV preparations types (Table 3). This parameter was modified among intestinal regions and dietary treatments. Thus, in both species the volume of PC vesicles was significantly lower when compared to BBMVs obtained from PI and DI. Moreover, only in BBMVs obtained from PC in rainbow trout vesicular volume was maintained among dietary treatments. These results prompted us to normalize influx values, which are presented and discussed as nutrient intravesicular concentration.
For the three vesicular types (PC, PI and DI) studied for each species, lineal uptake zone was determined for all the nutrients tested in this experience (Fig 1). Linearity was generally maintained until 10 seconds. Thus, 5 sec was chosen to uniform all the analyses. This incubation time also assured the maintenance of a lineal uptake even if the transport rates were up- or down-regulated in response to the experimental conditions.

Nutrient uptake to BBMV: Figures 2 and 3 show amino acid absorption capacities for sea bream and trout, respectively. In general terms, EAA absorption was higher than NEAA absorption in both species.

Sea bream fed the FM diets showed at 6 h post-feeding the maximal essential amino acid uptake in the BBMV obtained from PI and DI, and the minimal concentration in PC vesicles (Fig. 2). In sea bream fed the replaced diets PP75 and PP100, a decrease in the total uptake capacity for essential amino acids was found at this post-prandrial time. This diminution was related to a minor concentration for L-leucine, L-lysine and L-phenylalanine in PI BBMVs, and also for L-leucine in PC vesicles (Fig. 2). At 48 h post-feeding, PC vesicles showed an important up-regulation (3 to 5 fold) in transport capacities, which was more accentuated in BBMVs obtained from fish meal-replaced diets. When non essential amino acids (NEAA) absorption was studied in FM sea bream at 6 h post feeding, we found a progressive increase in uptake capacities along the intestinal tract. This pattern was not maintained in sea bream fed PP75 diet, which increased PI uptake in detriment of PC and DI transports, nor in PP100 fish, in which PC absorption was decreased and PI maintained. For this group of AA, also an increase of transport capacity was
recorded at 48 h post feeding, mainly due to a PC uptake augmentation even if this rise was minor that those recorded for the EAA previously studied in this species.

Figure 3 shows amino acid uptake capacity in rainbow trout. In this species FM-fed fish showed at 6 h post-feeding 2- to 7-fold amino acid concentration in PI and DI when compared to PC. However, it has to be taken into account that PC represent an important percentage of the gastrointestinal mass, thus its contribution to total absorption has not to be underestimate. Trout fed the two replaced diets modified these absorption capacities. PC affectation was minimal and only L-alanine uptake was decreased in BBMVs obtained from PP100-fed fish. In PP75 trout PI BBMVs an increase in uptake capacities was recorded for L-leucine, L-lysine, L-alanine and L-proline, being the modification significant for the two essential aminoacids. On the contrary, L-phenylalanine absorption was decreased in this intestinal segment when trout were fed the partially replaced diet. The effect was inversed in DI BBMVs, where L-phenylalanine concentrations where increased while the rest of amino acid concentrations decreased in comparison to FM control diet. Total replacement provoked a decrease in proximal intestine absorption for all the amino acids studied in trout fed the PP75 diet and a diminution in L-alanine and L-phenylalanine concentrations in the totally replaced group (Fig 3).

The comparison of 36 versus 6 h post-feeding nutrient uptake in this species showed an increase in PC and/or PI for the three essential amino acids and L-proline, while distal concentrations were in general diminished, with the exception of L-lysine and L-phenylalanine in PP100 trout.

D-glucose absorption at 6 h post forced-feeding was studied for both species (Fig. 4). Fish fed the FM diet showed different patterns depending on the species. Thus, in sea bream BBMVs uptake was maximal in PC and DI, versus the minimal absorption
capacity found in BBMVs obtained from this segment in rainbow trout. Moreover, in fish fed PP75 a maximal uptake capacity in PI was found, while in fish fed PP100 uptake was minimal in PC for both species being then progressively increased (sea bream) or maintained between PI and DI (trout). The response at 48 h for sea bream or 36 h for rainbow trout showed no a pattern depending on the intestinal region or the percentage of replacement studied.
Soybean meal has traditionally been used to replace fish meal in carnivorous fish aquafeeds because of a good amino acid profile and digestibility. But on the other hand, soybean meal contains important amounts of antinutritional factors, especially protease inhibitors (Francis et al., 2001; Gatlin et al., 2007), which might affect fish health (Baeverfjord and Krogdahl, 1996; Moyano et al., 1999; Kissil et al., 2000) and impair fish growth (Dabrowski et al., 1989; Venou et al., 2003). Because of this reason, we used a blend of vegetable sources (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) not containing soybean meal to partially (75%) or totally replace fish meal (FM) in the diet of sea bream and rainbow trout. This formulation is supported by previous studies in which good growth rates were achieved when extruded peas (Gomes et al., 1993), lupins (Bangoula et al., 1993) or rapeseeds (McCurdy and March, 1992) were used to replace FM in the diet of marine and freshwater fish species using adequate treatments such as dehulling or extrusion to improve nutrient utilisation.

To study the effect of the diet on intestinal nutrient absorption, brush border membrane vesicles (BBMV) obtained from pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI) were used. BBMV have previously been widely used for nutrient uptake studies in fish (Maffia et al., 1996; Bogé et al., 2002; Sala-Rabanal et al., 2004). They allow analysing transport uptake without nutrient metabolisation or further baso-lateral transport. Moreover, diffusion phenomena were minimized when working at short incubation periods (5 sec) and low substrate concentration, ruling out possible misinterpretations related to the variation of apparent diffusion capacities along the intestine described by Schep et al. (1997). In this experience we also discarded misinterpretations due to the modification of the volume of the obtained
vesicules (Sala-Rabanal et al., 2004) in response to FM replacement by normalizing data to nutrient concentration.

Results obtained in this work, with the lower amino acid concentration in BBMVs obtained from PC at 6 h post feeding in both species fed the control (FM) diets, are in contradiction with those reported for Atlantic salmon (Bakke-McKellep et al., 2000; Berge et al., 2004), where an uptake increase from pyloric caeca to distal intestine is described. In addition to the different techniques utilised (brush border membrane vesicules versus everted sleeve technique), and the fact that in this work the modifications of the vesicular volume were taken into account, divergences among experiments may arise from differences in the post feeding times studied. In this sense, the significant differences between 6 h- and long term studied times in all intestinal segments in this experience show the importance of the post prandrial moment studied. Moreover, the forced-feeding used in this experience diminished the digestion time since food was directly administered to the stomach.

Concerning FM replacement by plant protein sources, results obtained in this experience are in concordance with previous studies which have described differences in apparent amino acid and macromolecules absorption in proximal (Refstie et al., 2006; Jutfelt et al., 2007) or distal (Nordrum et al., 2000; Bakke-McKellep et al., 2000) fish intestinal segments in response to vegetable protein. In the present trial both species showed a modification of intestinal absorption pattern, being the total absorption capacities maintained on the partially replaced groups (PP75) or significantly decreased when no FM was used in the diet (PP100). These changes could be related to a different luminal nutrient availability when vegetal sources are used. Indeed, previous works concerning the use of vegetable meals support this hypothesis at different levels: 1) the use of plant ingredients in
aquafeeds has been related to a modification in the balance of digestive proteases of pancreatic origin in Coho salmon (Haard et al., 1996) or sea bream and rainbow trout (Santigosa et al., 2008). 2) Similarly, when soybean meal was used to replace FM in the diet of Atlantic salmon, a decrease in brush border enzyme activities (Bakke-Mckellep et al., 2000) was reported. 3) Moreover, free amino acids, as supplied in PP75 and PP100 diets, have higher average availability than protein-bound amino acids, leading to an asynchronous utilisation when amino acids from different origins are present in the meal (Ambardekar et al., 2009). The ensemble of these modifications could be responsible for the previously hypothesised modification of luminal nutrient abundance and the consequent variation of amino acid transport rates because of competition or inhibition phenomena, as suggested by Rosas et al., (2008). Likewise, histopathological modifications previously described in salmonids (Baeverfjord and Krogdahl, 1996; Krogdahl et al., 2003) and sea bream (Sitjà-Bobadilla et al., 2005; Santigosa et al., 2008) fed plant protein-based meals could be partially responsible for this transport modulation since transporters are immersed in the lipid bilayer and diffusion and absorption efficiency are affected by bilayer characteristics (Houpe et al., 1997; Spektor and Yorek, 1985) and enterocyte development (Smith, 1993).

Furthermore, absorption response to replacement was not identical for both species, as previously stated for marine versus freshwater species by Ferraris and Ahearn (1984). Indeed, Nordrum et al. (2000) found higher amino acid absorption rates in Atlantic salmon and trout in marine environment than in freshwater environment. On the contrary, Rosas et al. (2008) found that absorption rates in trout were 10-fold those of tuna. As previously mentioned, the use of different approaches such as the use of buffers containing individual amino acids or amino acid premixes, respectively
avoiding or evidencing the competition phenomena for the transporter active sites, may be one of the causes of the divergence of results. Concerning results obtained in this experience, one hypothesis for this differential response could reside in the specific morphological differences existing in the digestive systems between these two fish species, such as the presence of a variable number of pyloric caeca in salmonids, which is in accordance to genetic factors (Guillaume and Chourbet, 2002) versus only four blind diverticula in sea bream (Elbal and Agulleiro, 1986). Similarly, differences in relative intestinal lengths have been reported between trout and sea bream (Santigosa et al., 2008). In addition, pancreatic enzymatic activities in these fish are differentially affected by FM replacement (Santigosa et al., 2008), being the luminal concentration of nutrients differentially modified in consequence.

Despite the interspecific differences in luminal absorption at 6 h postfeeding, long-term uptake data showed for both species an up-regulation in transport capacities. This response is in concordance with a short starving situation described by Golovanova (1992), and is generally magnified in PP75 and PP100 groups when compared to control group, suggesting that these fish up-regulate transport capacities to compensate a nutritional deficit. However, even though in PP75 rainbow trout this compensation mechanism seem to be efficient because of a similar final weight when compared to control group (-11.5%; Gomez-Requeni et al., 2005), feeding PP100 diets to trout resulted in a significant decrease of SGR (-21.9%) after a 12-week trial. Similarly, the efficiency of this compensatory mechanism is doubtful in sea bream fed both replaced diets (PP75 and PP100), for which SGR of -9.4 and -19.8% were reported, respectively (Gomez-Requeni et al., 2004).
Glucose uptake was higher in sea bream than in rainbow trout, as previously described for strictly carnivorous versus occasionally omnivorous fish (Titus et al., 1991; Buddington et al., 1997). Even if this species usually do not use carbohydrates as source of energy in 'normal' physiological conditions (Collie and Ferraris, 1995), and taking into account that carnivorous fish carbohydratases are genetically programmed to be low (Cahu and Infante, 1995), data obtained in this work show that these animals are capable of modulating glucose transport capacities in proximal and distal intestinal segments in response to diet composition. Even though these results are not in accordance to those obtained by Nordrum et al. (2000) using the everted sleeve technique in freshwater fish, they are supported by previous studies in fish enterocytes (Soengas and Moon, 1998). However, existent data do not permit to establish if these modifications are related to transporter site densities variations and/or the modification of the kinetics of SGLT-1 glucose transporter.

In summary, this work shows that there is a modulation in intestinal nutrient absorption capacities when FM is replaced by plant protein sources in the diet of sea bream and rainbow trout. This modification is species-specific and not dose-dependent. These results should encourage the scientific community to get deeper inside in the mechanisms that affect the utilisation of vegetable proteins in aquafeeds, focusing on the phenomena concerning amino acid competivity for the transporter active sites in response to modified luminal nutrient availability. This knowledge is essential for the development of nutritionally-balanced sustainable aquafeeds for cultured carnivorous fish species.

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Table 1. Composition of the experimental diets for sea bream (sb) and rainbow trout (rt).

<table>
<thead>
<tr>
<th>Ingredient (g Kg⁻¹)</th>
<th>FMsb</th>
<th>PP75sb</th>
<th>PP100sb</th>
<th>FMrt</th>
<th>PP75rt</th>
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<tr>
<td>Fish meal</td>
<td>703.7</td>
<td>176</td>
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<td>637.9</td>
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<td>Mais gluten</td>
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<td>250</td>
<td>0</td>
<td>177.1</td>
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<tr>
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<td>180</td>
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<td>0</td>
<td>150</td>
<td>200</td>
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<tr>
<td>Extruded peas</td>
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<td>90</td>
<td>120</td>
<td>0</td>
<td>120</td>
<td>163.3</td>
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<tr>
<td>Rapeseed meal</td>
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<td>27.3</td>
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<td>128.6</td>
<td>151.1</td>
<td>158.7</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mineral Mix</td>
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<td>10</td>
<td>10</td>
<td>10</td>
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<td>10</td>
</tr>
<tr>
<td>Vitamin mix</td>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>CaHPO₄·2H₂O</td>
<td>0</td>
<td>30.7</td>
<td>51.1</td>
<td>0</td>
<td>37.8</td>
<td>40</td>
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<tr>
<td>L-Arginine</td>
<td>0</td>
<td>11.3</td>
<td>15.9</td>
<td>0</td>
<td>9.5</td>
<td>12.5</td>
</tr>
<tr>
<td>L- Histidine</td>
<td>0</td>
<td>3.4</td>
<td>4.8</td>
<td>0</td>
<td>2.9</td>
<td>3.9</td>
</tr>
<tr>
<td>L- Lysine</td>
<td>0</td>
<td>23.6</td>
<td>32.5</td>
<td>0</td>
<td>20.8</td>
<td>27.6</td>
</tr>
<tr>
<td>DL-Meteonine</td>
<td>0</td>
<td>3.6</td>
<td>5.4</td>
<td>0</td>
<td>3.06</td>
<td>4.1</td>
</tr>
<tr>
<td>L-Triptophane</td>
<td>0</td>
<td>2.2</td>
<td>2.9</td>
<td>0</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>L- Treonine</td>
<td>0</td>
<td>7.4</td>
<td>10.4</td>
<td>0</td>
<td>6.2</td>
<td>8.3</td>
</tr>
<tr>
<td>L- Isoleucine</td>
<td>0</td>
<td>6.2</td>
<td>8.4</td>
<td>0</td>
<td>5.3</td>
<td>7.1</td>
</tr>
<tr>
<td>L- Valine</td>
<td>0</td>
<td>8</td>
<td>11.1</td>
<td>0</td>
<td>7.0</td>
<td>9.4</td>
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</table>
Table 2. Marker enzyme enrichment factor.

<table>
<thead>
<tr>
<th></th>
<th>IS Sea bream</th>
<th>Rainbow trout</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphatase alkaline</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>4.56 ± 0.98</td>
<td>5.21 ± 0.45¹</td>
</tr>
<tr>
<td>PI</td>
<td>6.56 ± 1.36</td>
<td>3.39 ± 0.19²</td>
</tr>
<tr>
<td>DI</td>
<td>6.25 ± 0.96</td>
<td>5.15 ± 0.46¹</td>
</tr>
<tr>
<td><strong>Na⁺/K⁺ATPase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0.64 ± 0.01¹</td>
<td>0.42 ± 0.02³</td>
</tr>
<tr>
<td>PI</td>
<td>0.32 ± 0.02²</td>
<td>0.66 ± 0.02²</td>
</tr>
<tr>
<td>DI</td>
<td>0.39 ± 0.02²</td>
<td>0.80 ± 0.01¹</td>
</tr>
<tr>
<td><strong>Citrate synthase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>0.09 ± 0.05</td>
<td>0.03 ± 0.01²</td>
</tr>
<tr>
<td>PI</td>
<td>0.14 ± 0.04</td>
<td>0.12 ± 0.04¹</td>
</tr>
<tr>
<td>DI</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.03¹</td>
</tr>
</tbody>
</table>

Enrichment factor is the ratio between the specific activity measured in the final BBMV preparations and the initial homogenate. Results are presented as the mean ± S.E.M. of 12 determinations for each marker enzyme. Significant differences between intestinal segments are shown by different numbers. Intestinal Segment (IS), pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI).
Table 3. Vesicular volume referred to protein content (μL · mg⁻¹ prot) of BBMVs obtained from intestinal segments of sea bream (sb) and rainbow trout (rt).

<table>
<thead>
<tr>
<th></th>
<th>IS</th>
<th>FMsb</th>
<th>PP75sb</th>
<th>PP100sb</th>
<th>FMrt</th>
<th>PP75rt</th>
<th>PP100rt</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>1.16 ± 0.06 a,2</td>
<td>0.69 ± 0.03 b,3</td>
<td>0.41 ± 0.03 c,3</td>
<td>1.15 ± 0.05 2</td>
<td>1.20 ± 0.03 3</td>
<td>1.45 ± 0.06 3</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>1.86 ± 0.68a,1</td>
<td>1.08 ± 0.02 b,2</td>
<td>0.91 ± 0.05 b,2</td>
<td>25.79 ± 2.19 a,1</td>
<td>2.06 ± 0.10 b,2</td>
<td>6.75 ± 0.04 b,1</td>
<td></td>
</tr>
<tr>
<td>DI</td>
<td>1.64 ± 0.12 b,1</td>
<td>4.23 ± 0.13 a,1</td>
<td>1.48 ± 0.06 b,1</td>
<td>5.99 ± 0.29 a,2</td>
<td>3.10 ± 0.02 b,1</td>
<td>3.46 ± 0.06 b,2</td>
<td></td>
</tr>
</tbody>
</table>

Results are presented as the mean ± S.E.M. of 6 determinations for each condition. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by different numbers. Intestinal segment (IS), pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI). The FM diet contained fish meal as the only source of proteins; in the PP75 and PP100, 75 and 100% of fish meal was replaced by a blend of vegetable meals.
Legend of Figures

Figure 1. Time course of nutrient uptake of BBMV obtained from pyloric caeca (black circles), proximal intestine (grey circles) and distal intestine (open circles) of sea bream (A) and rainbow trout (B). Values are represented as the mean ± S.E.M of 4 determinations. $r^2$ is shown for all the conditions.

Figure 2. Intravesicular amino acid concentration in BBMVs obtained from pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI) of sea bream 6 h (grey) and 48 h (white) after forced feeding. Results are presented as the mean ± S.E.M of 8 determinations. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers; significant differences between postprandial times are shown by an asterisk. The FM diet contained fish meal as the only source of proteins; in the PP75 and PP100, 75 and 100% of fish meal was replaced by a blend of vegetable meals.

Figure 3. Intravesicular amino acid concentration in BBMVs obtained from pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI) of rainbow trout 6 h (grey) and 36 h (white) after forced feeding. Results are presented as the mean ± S.E.M of 8 determinations. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers; significant differences between postprandial times are shown by an asterisk. The FM diet contained fish meal as the only source of proteins; in the PP75 and PP100, 75 and 100% of fish meal was replaced by a blend of vegetable meals.
Figure 4. Intravesicular D-glucose concentration in BBMVs obtained from pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI) of sea bream (A) 6 h (grey) and 48 h (white) after forced feeding and rainbow trout (B) 6 h (grey) and 36 h (white) after forced feeding. Results are presented as the mean ± S.E.M of 8 determinations. Significant differences (p<0.05) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers; significant differences between postprandial times are shown by an asterisk. The FM diet contained fish meal as the only source of proteins; in the PP75 and PP100, 75 and 100% of fish meal was replaced by a blend of vegetable meals.