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*).

Santigosa, E.[1], García-Meilán, I.[1], Valentin, J.M[1]., Pérez-Sanchez, J[2]., Médale, F. [3], Kaushik, S. [3], Gallardo, M.A. [1]

[1] Departament de Fisiologia, Facultat de Biologia, Universitat de Barcelona. Diagonal, 645, 08028, Barcelona, Spain.

[2] Fish Nutrition Research Laboratory, UMR, NuAGe, Inra, St-Pée-sur-Nivelle, 64310, France

[3] Instituto de Acuicultura de Torre de la Sal (CSIC), Ribera de Cabanes, Castellón, Spain.

Corresponding author: Ester Santigosa

Phone: +34 93 403 59 24

Fax: +34 93 411 03 58

Email: estersantigosa@gmail.com

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.

47 Keywords: fish; brush border membrane vesicles; post-prandrial; intestinal nutrient
48 absorption; amino acid; glucose.
49

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;

75 Ahearn and Storelli, 1994; Sala-Rabanal et al., 2004). This technique permits to
76 study uptake rates with no substrate metabolisation. However, other approaches
77 such as everted sleeve technique or in vivo apparent absorption exist to determine
78 uptake capacities (Nordrum et al., 2000; Bakke-McKellep et al., 2000; Refstie et al.,
79 2006). Thus, wide information concerning absorption mechanisms in fish is available
80 (Collie and Ferraris, 1995; Boge et al., 2002; Sala-Rabanal et al:, 2004). In
81 vertebrates absorption occurs by diffusion, facilitated transport or active transport
82 (Maillard et al., 1995). Facilitated and active transports are used for the uptake of
83 amino acids, oligopeptides and glucose. They follow asymptotic kinetics and depend
84 on the presence of protein transporters immersed in the lipid bilayer (Palacin et al.,
85 1998). In fish, nutrient uptake is mediated by transport proteins similar to those of
86 mammals even if substrate specificity differences have been described between the
87 two groups (Collie and Ferraris, 1995). Moreover, contrary to mammals, amino acid
88 transporters in fish are present all along the intestine (Buddington et al., 1997).
89
90 On the basis of these premises, the aim of this study was to determine if nutrient
91 absorption capacity along the intestine was modified at two different post-feeding
92 times when a blend of vegetable meals was used to partially (75%) or totally replace
93 fish meal in the diet of sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus*
94 *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 $\pm$ 0.7 g) were randomly distributed in nine 0.5 m^3 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 $\pm$ 0.2 g) were randomly distributed in nine 1 m^3 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 cm^3 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-metyl-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 $MgCl_2$ 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 $MgSO_4 \cdot 7H_20$, 4.08 LiN_3 for sea bream; 285 mannitol, 20 HEPES, 0.1 $MgSO_4 \cdot 7H_20$, 4.08 LiN_3 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 (20ºC) 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*.

144 (2004). Similarly, depletion in citrate synthase activity (Srere, 1969) discarded the
145 presence of mitochondrial debris.
146 The orientation of BBMVs was studied using a modification of the approach
147 described by Del Castillo and Robinson (1982). Thus, vesicle preparations were
148 incubated in the presence and absence of a buffer containing 2 mM sodium
149 deoxicolate plus 15 mM EDTA, which disrupts cellular membranes, and then total
150 sacarase activity of the solution was measured by adding 100 μM sacarose to the
151 preparations. An increase in sacarase activity in disrupted BBMVs *versus* the non-
152 disrupted vesicles indicates the presence of vesicles in the non-physiological
153 orientation.
154 *The lineal uptake zone in the obtained BBMV* was determined for 3 essential
155 aminoacids (L-leucine, L-lysine and L-phenylalanine), 2 non essential amino acids (L-
156 alanine and L-proline) and D-glucose as described by Sala-Rabanal et al. (2004). For
157 the analysis, 10μL of BBMVs were mixed with 40 μL of incubation buffer (in mM: 250
158 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled
159 nutrient, 0.01 ^{3}H-nutrient. Osm 320; pH 7.4 for sea bream; 250 NaSCN, 70 mannitol,
160 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient.
161 Osm 305; pH 7.4 for trout). At different incubation times (1 to 10 s) reaction was
162 stopped by adding 1 mL of cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1
163 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 320; pH 7.4 for sea bream; 285 mannitol, 20 HEPES,
164 0.1 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 305; pH 7.4 for trout). 990 μL of the resulting mix
165 were rapidly filtered under negative pressure through 0.22 μm cellulose nitrate filters
166 (Millipore, Bedford MA) previously wetted in cold stop buffer. Filters were washed (10
167 mL stop buffer) and dissolved in Filtron-X scintillation liquid (ITISA S.A, Spain).

168 Samples were counted in a scintillation counter (Packard TRI-CARB 2100 TR). All
169 determinations were done at 20ºC.
170 *Determination of vesicular volume using L-alanine:* since BBMVs volume changes
171 depending on the intestinal segment studied (Sala-Rabanal *et al*. 2004), vesicle
172 volume was individually determined. Thus, L-alanine retained in the inside of the
173 vesicles at the equilibrium situation was measured incubating for 90 min in ice 10 μL
174 of BBMVs preparation with 40 μL of the usual incubation buffer. Then reaction was
175 stopped and counted according to the previously described protocol.
176 *Amino acids and Glucose uptake to BBMVs*: aliquots of 10 μL of each vesicular
177 suspension were reacted for 5 s with 40 μL of the incubation buffer that contained
178 0.151mM (of which 0.01 ^{3}H-labelled) of the studied nutrient: L-leucine, L-lysine, L-
179 phenylalanine, L-alanine, L- proline or D-glucose. After the incubation time, reaction
180 was stopped using 1 mL of stop buffer and then filtered and counted as previously
181 described.
182 *Quantification of proteins of the vesicular fractions*: protein concentration of initial
183 homogenate and final BBMV suspension was determined according to Bradford
184 (1976) using BIORAD^{R} reagent.
185 2.4. Statistical study of the results
186 To establish the existence of significant differences ($p<0.05$) between two
187 parameters T-Student test was used. Differences between more than two groups
188 were determined using the one way ANOVA test and Tuckey test. Software used
189 was SPSS 12.0 (SPSS Inc., EUA).
190 2.5. Chemicals
191 Radiolabelled nutrients were obtained from Amersham-Pharmacia Biotech (Spain).
192 The rest of chemicals were purchased by Sigma-Aldrich (Spain).
193

194 3. Results

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

200

201 *BBMVs validation and determination of the linear uptake zone*: Purity of the vesicular
202 preparations was checked (Table 2). The increase in alkaline phosphatase activity –
203 ranging from 3,39 to 6,56- together with the decrease in Na^+/K^+ ATPase (0.53 $\pm$ 0.08)
204 and citrate synthase (0.10 $\pm$ 0.01) activities in the final BBMVs preparations when
205 compared to the initial homogenates indicated that final suspensions were enriched
206 in brush border membrane and impoverished in basolateral membrane and
207 mitochondrial fractions, respectively. In all the cases, vesicles presented the
208 physiologic orientation (99.4 ± 0.3% for sea bream; 99.0 ± 0.9 % for rainbow trout;
209 n=12), discarding the possibility of underestimating uptake capacities because of the
210 transporters retained in the inner side of the vesicles.

211 Vesicular volume was determined in the different BBMV preparations types (Table 3).
212 This parameter was modified among intestinal regions and dietary treatments. Thus,
213 in both species the volume of PC vesicles was significantly lower when compared to
214 BBMVs obtained from PI and DI. Moreover, only in BBMVs obtained from PC in
215 rainbow trout vesicular volume was maintained among dietary treatments. These
216 results prompted us to normalize influx values, which are presented and discussed
217 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

242 recorded at 48 h post feeding, mainly due to a PC uptake augmentation even if this
243 rise was minor that those recorded for the EAA previously studied in this species.
244 Figure 3 shows amino acid uptake capacity in rainbow trout. In this species FM-fed
245 fish showed at 6 h post-feeding 2- to 7-fold amino acid concentration in PI and DI
246 when compared to PC. However, it has to be taken into account that PC represent an
247 important percentage of the gastrointestinal mass, thus its contribution to total
248 absorption has not to be underestimate. Trout fed the two replaced diets modified
249 these absorption capacities. PC affectation was minimal and only L-alanine uptake
250 was decreased in BBMVs obtained from PP100-fed fish. In PP75 trout PI BBMVs an
251 increase in uptake capacities was recorded for L-leucine, L-lysine, L-alanine and L-
252 proline, being the modification significant for the two essential aminoacids. On the
253 contrary, L-phenylalanine absorption was decreased in this intestinal segment when
254 trout were fed the partially replaced diet. The effect was inversed in DI BBMVs,
255 where L-phenylalanine concentrations where increased while the rest of amino acid
256 concentrations decreased in comparison to FM control diet. Total replacement
257 provoked a decrease in proximal intestine absorption for all the amino acids studied
258 in trout fed the PP75 diet and a diminution in L-alanine and L-phenylalanine
259 concentrations in the totally replaced group (Fig 3).
260 The comparison of 36 versus 6 h post-feeding nutrient uptake in this species showed
261 an increase in PC and/or PI for the three essential amino acids and L-proline, while
262 distal concentrations were in general diminished, with the exception of L-lysine and
263 L-phenylalanine in PP100 trout.
264 D-glucose absorption at 6 h post forced-feeding was studied for both species (Fig. 4).
265 Fish fed the FM diet showed different patterns depending on the species. Thus, in
266 sea bream BBMVs uptake was maximal in PC and DI, versus the minimal absorption

267 capacity found in BBMVs obtained from this segment in rainbow trout. Moreover, in
268 fish fed PP75 a maximal uptake capacity in PI was found, while in fish fed PP100
269 uptake was minimal in PC for both species being then progressively increased (sea
270 bream) or maintained between PI and DI (trout). The response at 48 h for sea bream
271 or 36 h for rainbow trout showed no a pattern depending on the intestinal region or
272 the percentage of replacement studied.
273

4. Discussion

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

299 vesicules (Sala-Rabanal et al., 2004) in response to FM replacement by normalizing
300 data to nutrient concentration.
301 Results obtained in this work, with the lower amino acid concentration in BBMVs
302 obtained from PC at 6 h post feeding in both species fed the control (FM) diets, are in
303 contradiction with those reported for Atlantic salmon (Bakke-McKellep et al., 2000;
304 Berge et al., 2004), where an uptake increase from pyloric caeca to distal intestine is
305 described. In addition to the different techniques utilised (brush border membrane
306 vesicules *versus* everted sleeve technique), and the fact that in this work the
307 modifications of the vesicular volume were taken into account, divergences among
308 experiments may arise from differences in the post feeding times studied. In this
309 sense, the significant differences between 6 h- and long term studied times in all
310 intestinal segments in this experience show the importance of the post prandrial
311 moment studied. Moreover, the forced-feeding used in this experience diminished the
312 digestion time since food was directly administred to the stomach.
313 Concerning FM replacement by plant protein sources, results obtained in this
314 experience are in concordance with previous studies which have described
315 differences in apparent amino acid and macromolecules absorption in proximal
316 (Refstie et al., 2006; Jutfelt et al., 2007) or distal (Nordrum et al., 2000; Bakke-
317 McKellep et al., 2000) fish intestinal segments in response to vegetable protein. In
318 the present trial both species showed a modification of intestinal absorption pattern,
319 being the total absorption capacities maintained on the partially replaced groups
320 (PP75) or significantly decreased when no FM was used in the diet (PP100). These
321 changes could be related to a different luminal nutrient availability when vegetal
322 sources are used. Indeed, previous works concerning the use of vegetable meals
323 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.

Acknowledgements

398 This research was funded by the EU (Q5RS-2000-30068; “Perspectives of Plant
399 Protein Use in Aquaculture”, PEPPA). E. Santigosa and I. García-Meilán received a
400 fellowship from the Universitat de Barcelona.
401
402 References
403 Ahearn, G.A., Storelli, C., 1994. Use of membrane vesicle techniques to characterize
404 nutrient transport processes of the teleost gastrointestinal tract. In: Hochachka, P.W,
405 Mommsen, T.P., (editors) Biochemistry and Molecular Biology of Fishes. Pp Vol. 3:
406 513–524, Elsevier Science, Amsterdam.
407
408 Albrektsen, S., Mundheim, H., Aksnes, A., 2006. Growth, feed efficiency, digestibility
409 and nutrient distribution in Atlantic cod (*Gadus morhua*) fed two different fish meal
410 qualities at three dietary levels of vegetable protein sources. Aquaculture 261, 626–
411 640.
412
413 Ambardekara, A.A., Reigh, R. C., Williams, M. B., 2009. Absorption of amino acids
414 from intact dietary proteins and purified amino acid supplements follows different
415 time-courses in channel catfish (Ictalurus punctatus). Aquaculture 291, 179-187.
416
417 Baeverfjord, G., Krogdahl, A., 1996. Development and regression of soybean meal
418 induced enteritis in Atlantic salmon, *Salmo salar* L., distal intestine: a comparison
419 with the intestines of fasted fish. J. Fish Dis. 19, 375–387.
420

421 Bakke-Mckellep, A.M., Nordrum, S., Krogdahl, A., Buddington, R.K., 2000.
422 Absorption of glucose, amino acids, and dipeptides by the intestines of Atlantic
423 salmon (*Salmo salar* L.). Fish Physiol. Biochem. 22, 33-44.
424
425 Bangoula, D., Parent, J.P., Vellas, F., 1993. Nutritive value of white lupin (Lupinus
426 albus var Lutop) fed to rainbow trout (Oncorhynchus mykiss). Effects of extrusion
427 cooking. Reprod. Nutr. Dev. 33, pp. 325–334
428
429 Berge, G., E., Goodman, M., Espe, M., Lied, E., 2004. Intestinal absorption of amino
430 acids in fish: kinetics and interaction of the in vitro uptake of L-methionine in Atlantic
431 salmon (*Salmo salar* L.). Aquaculture 229, 265–273.
432
433 Boge, G., Roche, H., Balocco, C., 2002. Amino acid transport by intestinal brush
434 border vesicles of marine fish, Boops salpa. Comp. Biochem. Physiol. 131, 19– 26.
435
436 Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram
437 quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-
438 254.
439
440 Buddington, R. K., Krogdahl, A., Bakke-Mckellep, A.M., 1997. The intestine of
441 carnivorous fish: structure and functions and the relations with diet. Acta Physiol.
442 Scand. 161, Suppl 638: 67– 80.
443

444 Cahu, C.L., Zambonino-Infante, J.L., 1995. Maturation of the pancreatic and
445 intestinal digestive functions in sea bass (*Dicentrarchus labrax*): effect of weaning
446 with different protein sources. Fish Physiol. Biochem. 14, 431–437.
447
448 Colas, B., Maroux, S., 1980. Simultaneous isolation of brush-border and basolateral
449 membrane from rabbit enterocytes - presence of brush-border hydrolases in the
450 basolateral membrane of rabbit enterocytes. Biochim. Biophys. Acta. 600, 406-420.
451
452 Collie, N.L., Ferraris, R.P., 1995. Nutrient fluxes and regulation in fish intestine. In:
453 Hochachka, P.W, Mommsen, T.P., (editors) Biochemistry and Molecular Biology of
454 Fishes. pp Vol. 4: 221– 239, Elsevier Science, Amsterdam.
455
456 Dabrowski, K., Poczyczynski, P., Kock, G., Berger, B., 1989. Effect of partially or
457 totally replacing fish-meal protein by soybean-meal protein on growth, food utilization
458 and proteolytic-enzyme activities in rainbow-trout (Salmo-Gairdneri) - new invivo test
459 for exocrine pancreatic-secretio. Aquaculture 77, 29-49.
460
461 De Francesco, M., Parisi, G., Pérez-Sanchez., J., Gomez-Requeni, P., Médale, F.,
462 Kaushik, S., Mecatti, M. & Poli, B.M., 2007. Effect of high-level fish meal replacement
463 by plant proteins in gilthead sea bream (Sparus aurata) on growth and body/fillet
464 quality traits. Aquacult. Nutr. 13, 361-372.
465
466 Del Castillo, J.R., Robinson, J.W.L., 1982. The simultaneous preparation of
467 basolateral and brush-border membrane-vesicles from guinea-pig intestinal

468 epithelium, and the determination of the orientation of the basolateral vesicles.
469 Biochim. Biophys. Acta 688, 45-56.
470
471 Drai, P., Albertini-Berhaut, J., Lafaurie, M., Sudaka, P., Guidicelli, J., 1990.
472 Simultaneous preparation of basolateral and brush-border membrane vesicles from
473 sea bass intestinal epithelium. Biochim. Biophys. Acta 1022, 251–259.
474
475 Elbal, M.T., Agulleiro B., 1986. A histochemical and ultrastructural-study of the gut of
476 Sparus auratus (Teleostei). J. Submicrosc. Cytol. 18, 335-347.
477
478 Ferraris, R. P., Ahearn, G.A., 1984. Sugar and amino-acid-transport in fish intestine.
479 Comp. Biochem. Physiol. A 77, 397-413.
480
481 Francis, G., Makkar, H.P.S., Becker, K., 2001. Antinutritional factors present in plant-
482 derived alternate fish feed ingredients and their effects in fish. Aquaculture 199, 197-
483 227.
484
485 Gatlin, D.M., Barrows, F.T., Brown, P., Dabrowski, K., Gaylord, T.G., Hardy, R.W.,
486 Herman, E., Hu, G.S., Krogdahl, A., Nelson, R., Overturf, K., Rust, M., Sealey, W.,
487 Skonberg, D., Souza, E.J., Stone, D., Wilson, R., Wurtele, E., 2007. Expanding the
488 utilization of sustainable plant products in aquafeeds: a review. Aquac. Res. 38, 551–
489 579.
490
491 Golovanova, I.L., 1992. The characteristics of carbohydrate transport in the
492 freshwater fish intestine. Fiziol. Zh SSSR Im I M Schenova 78, 151-157.

Gomes, E.F., Corraze, G., Kaushik, S., 1993. Effects of dietary incorporation of a co-extruded plant protein rapeseed and peas. on growth, nutrient utilization and muscle fatty acid composition of rainbow trout Oncorhynchus mykiss. Aquaculture 113, 339–353.

Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J.A., Médale, F., Martin, S.A.M., Houlihan, D.F., Kaushik, S., Pérez-Sánchez, J., 2004. Protein growth performance, amino acid utilisation and somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead sea bream (Sparus aurata). Aquaculture 232, 493–510.

Gómez-Requeni, P., Calduch-Giner, J., Vega-Rubín de Celis, S., Médale, F., Kaushik, S., Pérez-Sánchez, J., 2005. Regulation of the somatotropic axis by dietary factors in rainbow trout (Oncorhynchys mykiss). Br. J. Nutr. 95, 353–361.

Guillaume, J., Chourbet, G., 2002. Fisiología digestiva y digestibilidad de nutrientes en los peces. In: Nutrition et alimentation des poissons et crustacés. (Ed.I.N.R.A.). pp 53-89.

Haard, N.F., Dimes, L.E., Arndt, R.E., Dong, F.M., 1996. Estimation of protein digestibility IV. Digestive proteases from the pyloric caeca of coho salmon (*Oncorhynchus kisutch*) fed diets containing soybean meal. Comp. Biochem. Physiol. 115B, 533–540.

518 Hansen, A.C., Rosenlund, G., Karlsen, Ø., Koppe, W., Hemre, G.I., 2007. Total
519 replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua*
520 L.) I: effects on growth and protein retention, Aquac. Res. 272, 599–611.
521
522 Houpe, K. L., Malo, C., Buddington, R. K., 1997. Dietary lipid and intestinal brush
523 border membrane phospholipid fatty acid composition and glucose transport of
524 channel catfish. Physiol. Zool. 70, 230-236.
525
526 Jutfelt, F., Olsen, R.E., Björnsson, B.Th., Sundell, K., 2007. Parr–smolt
527 transformation and dietary vegetable lipids affect intestinal nutrient uptake, barrier
528 function and plasma cortisol levels in Atlantic salmon. Aquaculture, 273, 298–311.
529
530 Kissil, G. W., Lupatsch, I., Higgs, D. A., Hardy, R. W., 2000. Dietary substitution of
531 soy and rapeseed protein concentrates for fish meal, and their effects on growth and
532 nutrient utilization in gilthead seabream Sparus aurata. Aquac. Res. 31, 595-601.
533
534 Knudsen, D., Røn, Ø., Baardsen, G., Smedsgaard, J., Koppe, W., Frøkiær, H., 2006.
535 Soya saponins resist extrusion cooking and are not degraded during gut passage in
536 Atlantic salmon (*Salmo salar* L.). J. Agric. Food Chem. 54, 6428–6435.
537
538 Krogdahl, Å., McKellep, A.M.B., Baeverfjord, G., 2003. Effects of graded levels of
539 standard soybean meal on intestinal structure, mucosal enzyme activities, and
540 pancreatic response in Atlantic salmon (*Salmo salar* L.). Aquac. Nutr. 9, 361–371
541

542 Lilleeng, E. , Frøystad, M.K., Østby, G.C., Valen, E.K., Krogdahl, Å., 2007. Effects of
543 diets containing soybean meal on trypsin mRNA expression and activity in Atlantic
544 salmon (*Salmo salar* L), Comp. Biochem. Physiol. 147A, 25–36.
545
546 Maffia, M., Acierno, R., Cillo, E., Storelli, C., 1996. Na^+-D-glucose cotransport by
547 intestinal BBMVs of the antarctic fish Trematomus bernachii. Am. J. Physiol. 271,
548 R1576–R1583.
549
550 Mailliard, M.E., Stevens, B.R., Mann, G.E., 1995. Amino acid transport by small
551 intestinal, hepatic, and pancreatic epithelia. Gastroenterology 108, 888–910.
552
553 McCurdy, S.M., March, B.E., 1992. Processing of canola meal for incorporation in
554 trout and salmon diets. JAOCS 69, 213–220.
555
556 Moyano, F.J., Martinez, I., Diaz, M., Alarcon, F.J., 1999. Inhibition of digestive
557 proteases by vegetable meals in three fish species; seabream (*Sparus aurata*),
558 tilapia (*Oreochromis niloticus*) and African sole (*Solea senegalensis*). Comp.
559 Biochem. Physiol. 122B, 327–332.
560
561 Nordrum, S., Bakke-McKellep, A.M., Krogdahl, A., Buddington, K.R., 2000. Effects of
562 soybean meal and salinity on intestinal transport of nutrients in Atlantic salmon
563 (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol.,
564 125B, 317–335.
565

566 Olsen, R.E., Hansen, A.C., Roselund, G., Hemre, G.I., Mayhew ,T.M., Knudsen, D.L.,
567 Eroldogan, O.T., Myklebust, R., Karlsen, O., 2007. Total replacement of fish meal
568 with plant proteins in diets for Atlantic cod (*Gadus morhua* L.) II- Health aspects.
569 Aquaculture 272, 612-624.
570
571 Palacin, M., Estevez, R., Bertran, J., Zorzano, A., 1998. Molecular biology of
572 mammalian plasma membrane amino acid. Physiol. Rev. 78, 969-1054.
573
574 Refstie, S., Førde-Skjærvik, O., Rosenlund, G., Rørvik, K.-A., 2006. Feed intake,
575 growth, and utilisation of macronutrients and amino acids by 1- and 2-year old
576 Atlantic cod (*Gadus morhua*) fed standard or bioprocessed soybean meal.
577 Aquaculture 255, 279-291.
578
579
580 Rosas, A., Vazquez-Duhal, R., Tinoco, R., Shimada, A., Dabramo, L.R., Viana, M.T.,
581 2008. Comparative intestinal absorption of amino acids in rainbow trout
582 (*Oncorhynchus mykiss)*, totoaba (*Totoaba macdonaldi*) and Pacific bluefin tuna
583 (*Thunnus orientalis*). Aquac. Nutr. 16, 481-489.
584
585 Sala-Rabanal, M., Gallardo, M. A., Sanchez, J., Planas, J. M., 2004. Na-dependent
586 D-glucose transport by intestinal brush border membrane vesicles from gilthead sea
587 bream (Sparus aurata). J. Membrane Biol. 201, 85-96.
588
589 Santigosa, E., Sánchez, J., Médale, F., Pérez-Sánchez, J. & Gallardo, M.A., 2008.
590 Modifications of digestive enzymes in trout (*Onchorynchus mykiss*) and sea bream

(*Sparus aurata*) in response to dietary fish meal replacement by plant protein sources. Aquaculture 252, 68-74.

Scalera, V., Storelli, C., Storellijoss, C., Haase, W., Murer, H., 1980. Simple and fast method for the isolation of basolateral plasma-membranes from rat small-intestinal epithelial-cells. Biochem. J. 186, 177-181.

Schep, L.J., Tucker, I.G., Young, G., Butt, A.G., 1997. Regional permeability differences between the proximal and distal portions of the isolated salmonid posterior intestine. J Comp Physiol 167B, 370-377.

Schuhmacher, A., Wax, C., Gropp, J., 1997. Plasma amino acids in rainbow trout (*Oncorhynchus mykiss*) fed intact protein or a crystalline amino acid diet. Aquaculture 151, 15–28.

Sitjà-Bobadilla, A., Peña-Lopez, S., Gomez-Requeni, P., Médale, F., Kaushik, S., Pérez-Sanchez, J., 2005. Effect of fish meal replacement by plant protein sources on non-specific defence mechanisms and oxidative stress in gilthead sea bream (Sparus aurata). Aquaculture, 249, 387-400.

Smith, M.W., 1993. Genetic regulation of enterocyte differentiation. Proc. Nutr. Soc. 52, 293– 300.

SOFIA, 2008. The state of world fisheries and aquaculture. FAO (Food and Agriculture Organization), Fisheries and aquaculture Department, Rome, pp. 1–196.

Soengas, J.L., Moon, T.W., 1998. Transport and metabolism of glucose in isolated enterocytes of the black bullhead Ictalurus melas: effects of diet and hormones. J. Exp. Biol. 201; 3263–3273.

Spector, A.A., Yorek, M.A., 1985. Membrane lipid composition and cellular function. J. Lipid Res. 26, 1015-1035

Srere, P. A., 1969. Citrate synthase. In: Methods in Enzymology (Colowick, S. 703 P., Kaplan, N.O.P., editors). Academic Press, London. Vol. XIII, 3rd ed., pp. 3-5.

Titus, E., Karasov, W.H., Ahearn, G.A.., 1991. Dietary modulation of intestinal nutrient transport in the teleost fish Tilapia. Am. J. Physiol. 261, R1568- R1574.

Venou, B., Alexis, M.N., Fountoulaki, E., Nengas, I., Apostolopoulou, M., Castritsi-Catharíou, I., 2003. Effect of extrusion of wheat and corn on gilthead sea bream (*Sparus aurata*) growth, nutrient utilization efficiency, rates of gastric evacuation and digestive enzyme activities. Aquaculture 225, 207-223.

Weiser, M. M., 1973. Intestinal epithelial-cell surface membrane glycoprotein synthesis. Indicator of cellular differentiation. J. Biol. Chem., 248, 2536-2541.

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

637 (rt).

Ingredient (g Kg^{-1})	**FMsb**	**PP75sb**	**PP100sb**	**FMrt**	**PP75rt**	**PP100rt**
Fish meal	703.7	176	0	637.9	159.5	0
Mais gluten	0	180	250	0	177.1	232.4
Wheat gluten	0	180	250	0	150	200
Extruded peas	0	90	120	0	120	163.3
Rapeseed meal	0	67.2	27.3	0	75	100
Sweet white lupin	0	0	6.9	0	0	0
Extruded wheat	142	30.6	15.5	203.4	42.5	0
Fish oil	124.3	149.8	158	128.6	151.1	158.7
Ligand	10	10	10	10	10	10
Mineral Mix	10	10	10	10	10	10
Vitamin mix	10	10	10	10	10	10
$CaHPO_4 \cdot 2H_20$	0	30.7	51.1	0	37.8	40
L-Arginine	0	11.3	15.9	0	9.5	12.5
L- Histidine	0	3.4	4.8	0	2.9	3.9
L-Lysine	0	23.6	32.5	0	20.8	27.6
DL-Meteonine	0	3.6	5.4	0	3.06	4.1
L-Triptophane	0	2.2	2.9	0	2.1	2.7
L- Treonine	0	7.4	10.4	0	6.2	8.3
L- Isoleucine	0	6.2	8.4	0	5.3	7.1
L-Valine	0	8	11.1	0	7.0	9.4

638

639

640 Table 2. Marker enzyme enrichment factor.

	IS	Sea bream	Rainbow trout
Phosphatase alkaline	PC	4.56 ± 0.98	5.21 ± 0.45[1]
	PI	6.56 ± 1.36	3.39 ± 0.19 [2]
	DI	6.25 ± 0.96	5.15 ± 0.46 [1]
Na^+/K^+ATPase	PC	0.64 ± 0.01 [1]	0.42 ± 0.02 [3]
	PI	0.32 ± 0.02 [2]	0.66 ± 0.02 [2]
	DI	0.39 ± 0.02 [2]	0.80 ± 0.01 [1]
Citrate synthase	PC	0.09 ± 0.05	0.03 ± 0.01 [2]
	PI	0.14 ± 0.04	0.12 ± 0.04 [1]
	DI	0.12 ± 0.01	0.09 ± 0.03 [1]

641

642 Enrichment factor is the ratio between the specific activity measured in the final
643 BBMV preparations and the initial homogenate. Results are presented as the mean ±
644 S.E.M. of 12 determinations for each marker enzyme. Significant differences
645 between intestinal segments are shown by different numbers. Intestinal Segment
646 (IS), pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI).

647 Table 3. Vesicular volume referred to protein content (μL · mg^{-1} prot) of BBMVs obtained from intestinal segments of sea bream (sb)
648 and rainbow trout (rt).

IS	FMsb	PP75sb	PP100sb	FMrt	PP75rt	PP100rt
PC	1.16 ± 0.06 ^{a,2}	0.69 ± 0.03 ^{b,3}	0.41 ± 0.03 ^{c,3}	1.15 ± 0.05 ^{2}	1.20 ± 0.03 ^{3}	1.45 ± 0.06 ^{3}
PI	1.86 ± 0.68^{a,1}	1.08 ± 0.02 ^{b,2}	0.91 ± 0.05 ^{b,2}	25.79 ± 2.19 ^{a,1}	2.06 ± 0.10 ^{b,2}	6.75 ± 0.04 ^{b,1}
DI	1.64 ± 0.12 ^{b,1}	4.23 ± 0.13 ^{a,1}	1.48 ± 0.06 ^{b,1}	5.99 ± 0.29 ^{a,2}	3.10 ± 0.02 ^{b,1}	3.46 ± 0.06 ^{b,2}

649

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

654 Legend of Figures

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

659

660 Figure 2. Intravesicular amino acid concentration in BBMVs obtained from pyloric
661 caeca (PC), proximal intestine (PI) and distal intestine (DI) of sea bream 6 h (grey)
662 and 48 h (white) after forced feeding. Results are presented as the mean ± S.E.M of
663 8 determinations. Significant differences ($p<0.05$) between diets are shown by
664 different letters; significant differences between intestinal segments are shown by
665 numbers; significant differences between postprandrial times are shown by an
666 asterisk. The FM diet contained fish meal as the only source of proteins; in the PP75
667 and PP100, 75 and 100% of fish meal was replaced by a blend of vegetable meals.

668

669 Figure 3. Intravesicular amino acid concentration in BBMVs obtained from pyloric
670 caeca (PC), proximal intestine (PI) and distal intestine (DI) of rainbow trout 6 h (grey)
671 and 36 h (white) after forced feeding. Results are presented as the mean ± S.E.M of
672 8 determinations. Significant differences ($p<0.05$) between diets are shown by
673 different letters; significant differences between intestinal segments are shown by
674 numbers; significant differences between postprandrial times are shown by an
675 asterisk. The FM diet contained fish meal as the only source of proteins; in the PP75
676 and PP100, 75 and 100% of fish meal was replaced by a blend of vegetable meals.

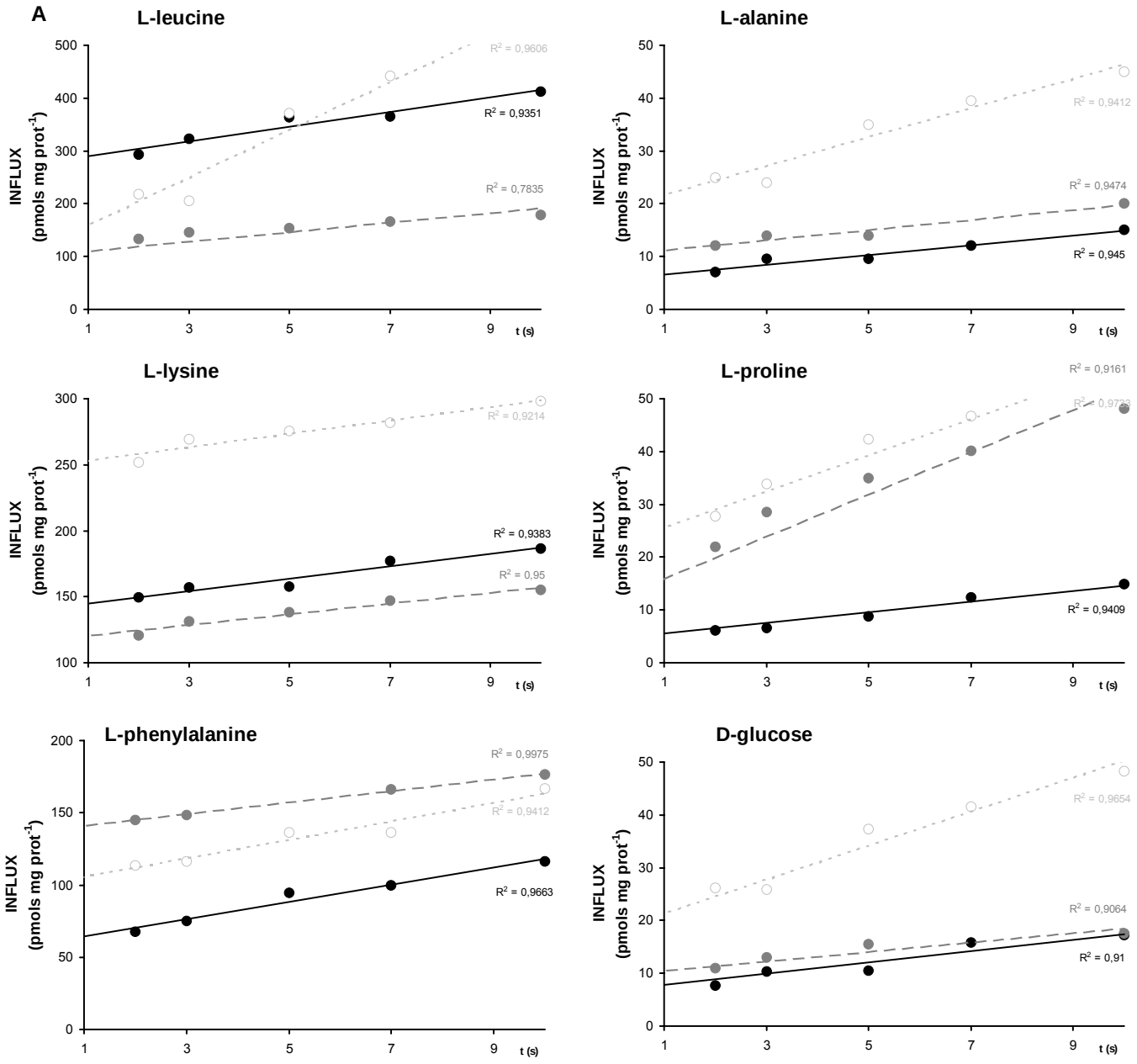
677

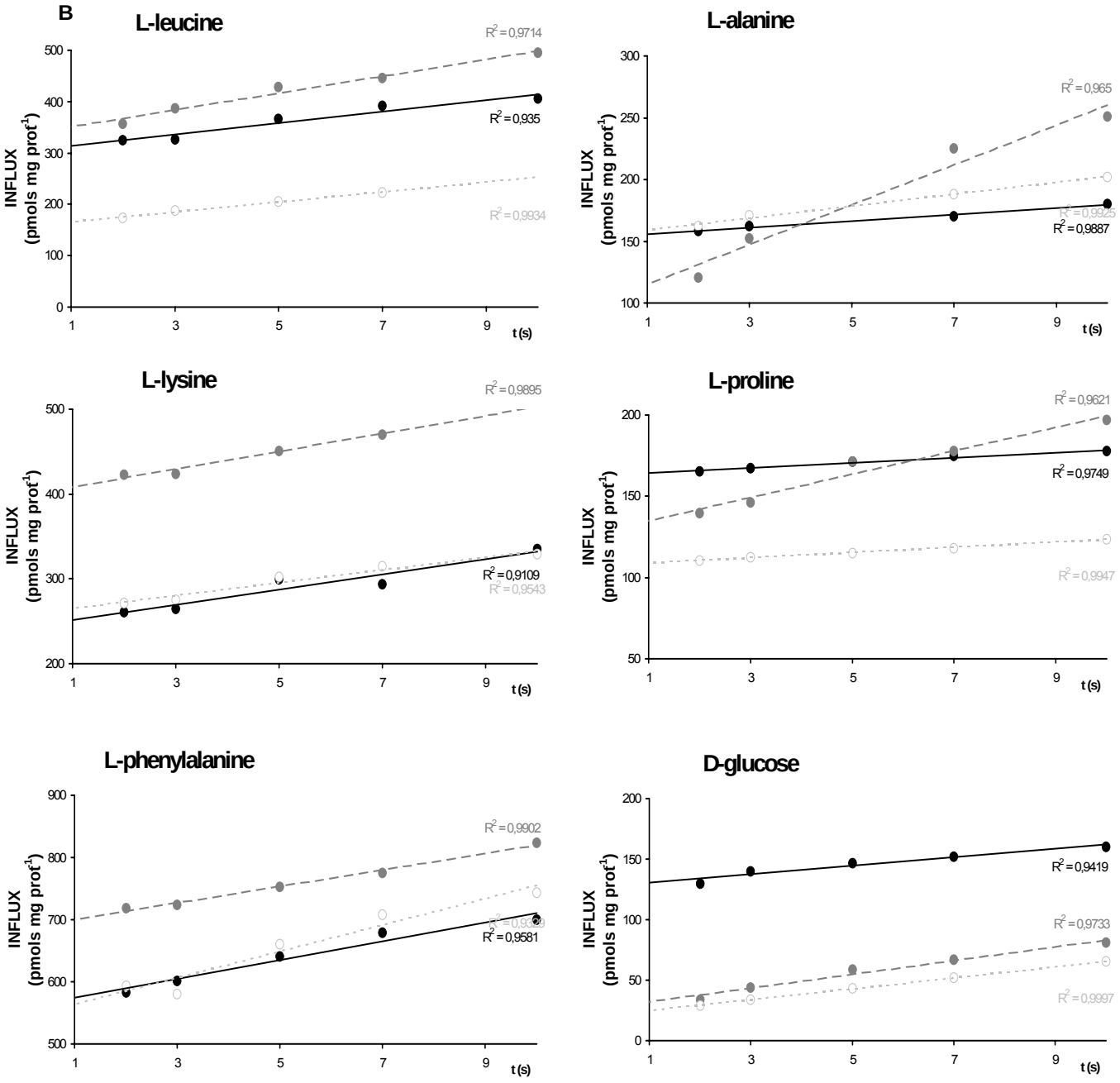
678

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

688 Figure 1

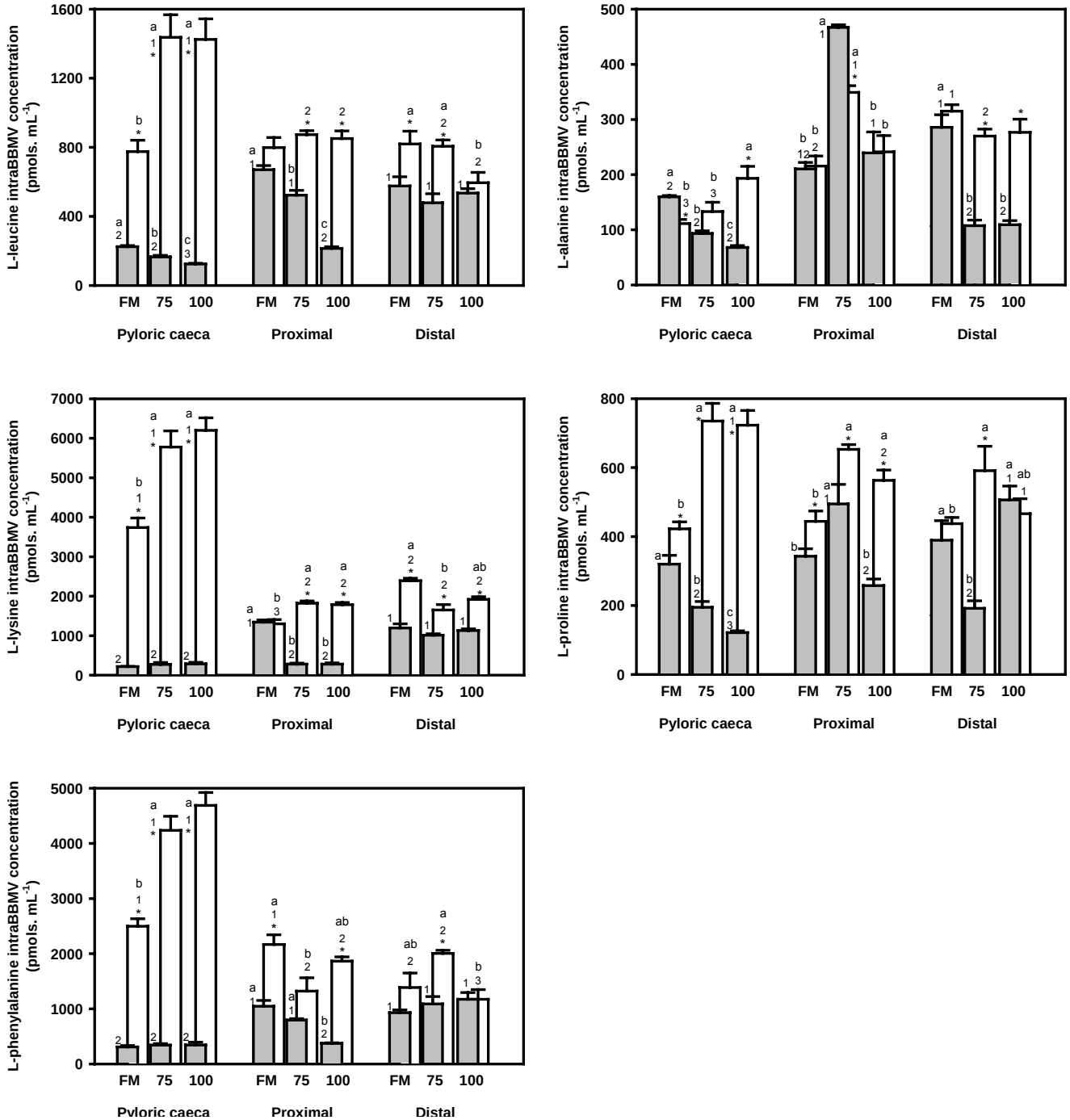
689




B
L-leucine
R² = 0,9714
R² = 0,935
R² = 0,9934
INFLUX (pmols mg prot⁻¹)
t (s)
L-alanine
R² = 0,965
R² = 0,9887
L-lysine
R² = 0,9895
R² = 0,9109
R² = 0,9543
L-proline
R² = 0,9621
R² = 0,9749
R² = 0,9947
L-phenylalanine
R² = 0,9902
R² = 0,9581
D-glucose
R² = 0,9419
R² = 0,9733
R² = 0,9997

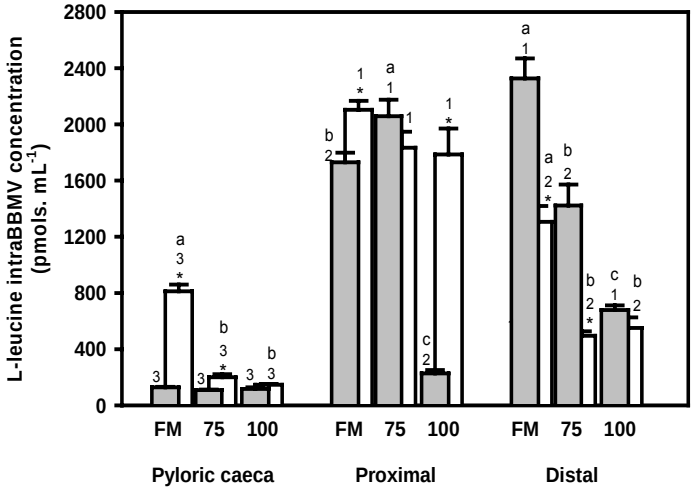
691 Figure 2

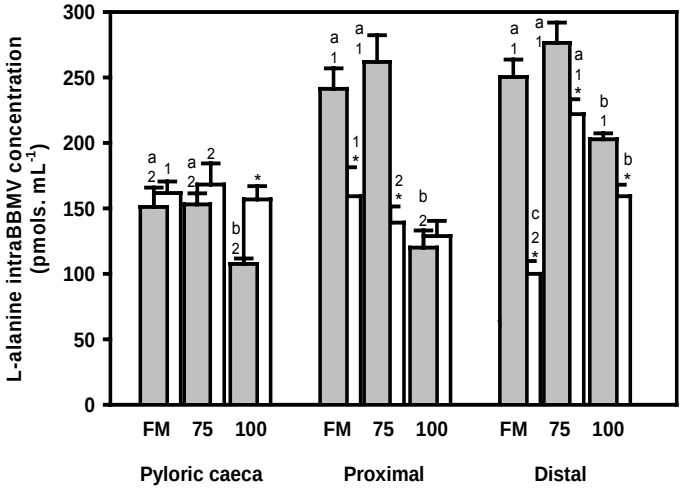
692

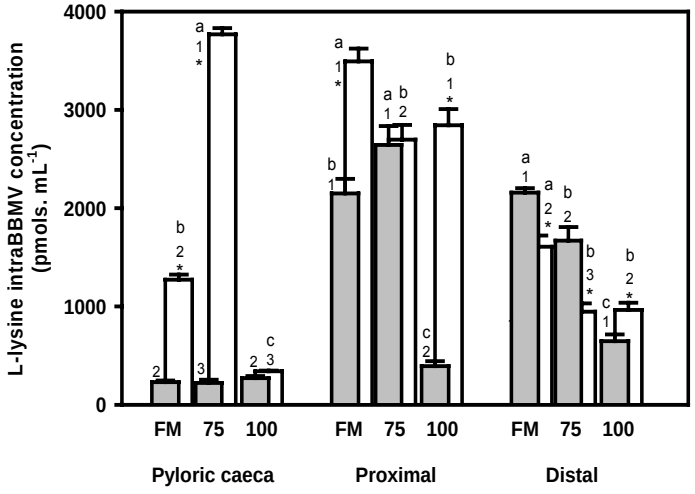


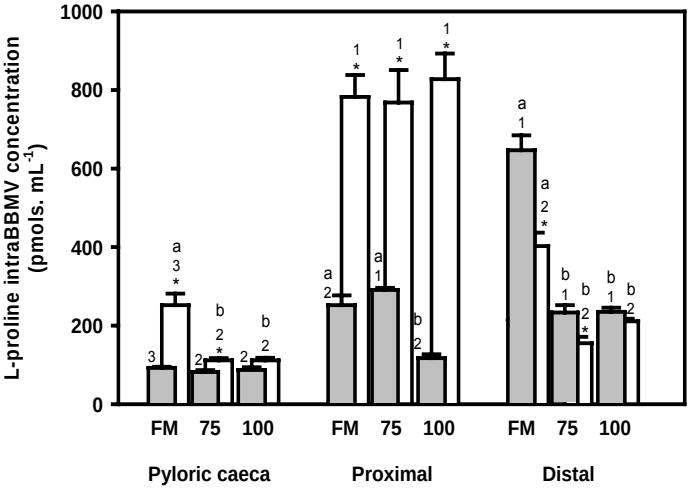
693 Figure 3

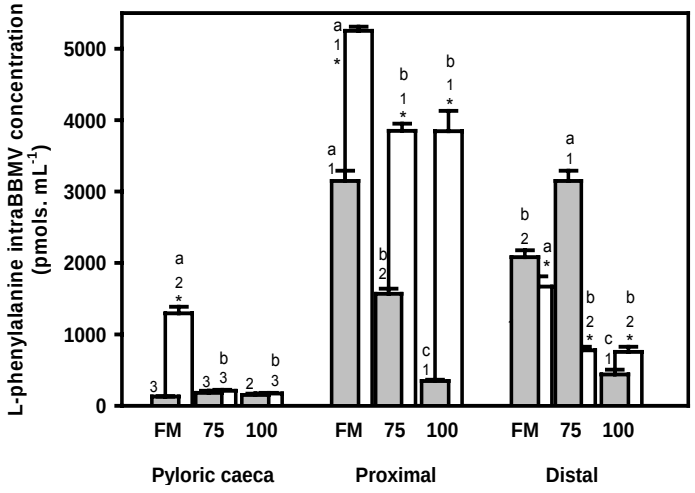
694



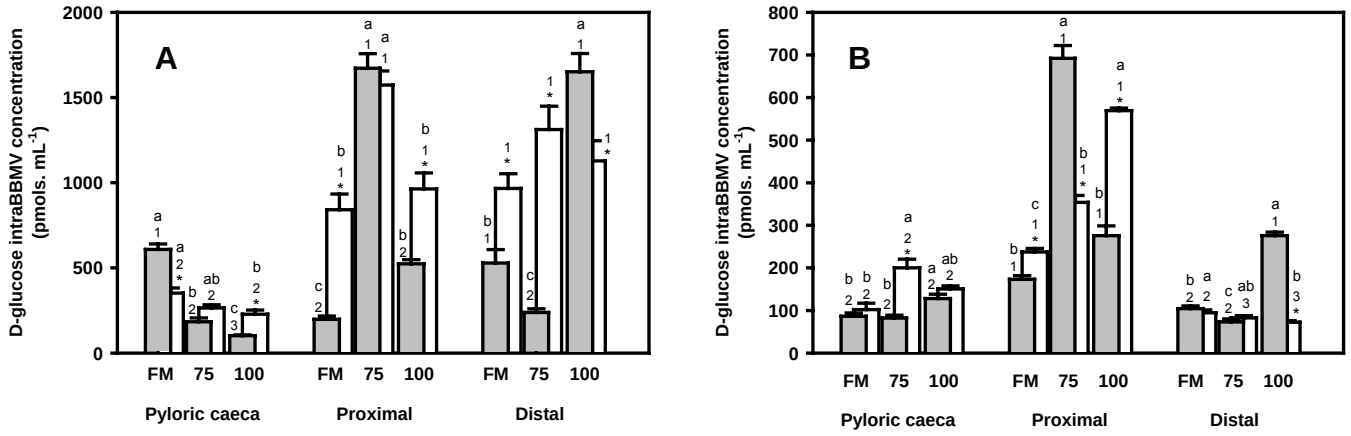








695 Figure 4


A
B
D-glucose intraBBMV concentration (pmols. mL-1)
FM
75
100
Pyloric caeca
Proximal
Distal