Plant oils inclusion in high fish meal substituted diets. Effect on intestinal digestion and nutrient absorption in sea bream (*Sparus aurata*)

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Running title: Digestive response of sea bream to FM- and FO-replaced diets

1. ABSTRACT

Here we performed an 11-week growth trial in juvenile sea bream to study the effects of four experimental diets on digestion, nutrient absorption and histological intestinal integrity. For this purpose, all diets formulated had a low FM content (25%) and were rich (75%) in plant protein sources (corn gluten meal, soybean meal and extruded wheat). FO was replaced at 0, 33, 66 and 100% (referred to as FO, 33VO, 66VO and 100VO) by graded levels of a blend of vegetable oils (VOs; rapeseed, linseed and palm oils).

Protease activity was increased in pyloric caeca and decreased in the proximal intestine of the 66VO and 100VO groups in response to FO replacement by VO, while lipase and α-amylase activites were not modified. The capacity of brush border membrane vesicles obtained from pyloric caeca, proximal intestine and distal intestine to absorb amino acids was not modified; however, D-glucose and linoleic acid absorption diminished when VO was included in the diets.

These results indicate a progressive delay in transit rate when up to 66% of FO is replaced by VO. Only the 100VO group showed impaired digestion, with reduced total protease activity and the accumulation of lipid droplets in the enterocytes of the proximal intestine.

33 Keywords: protease; amylase; lipase; brush border membrane vesicles;
34 vegetable oil.
35

36 2. INTRODUCTION

37 The limited availability of fish oil (FO) and fish meal (FM), together with the
38 increase in aquaculture (SOFIA, 2008), calls for the identification of alternative
39 lipid and protein sources for the development of sustainable fish farming
40 activities. Plant ingredients are among the alternatives (Bell & Waagbø, 2008;
41 Turchini et al., 2009).

42 The inclusion of vegetable oils (VOs) in the diets of carnivorous fish yields
43 growth rates similar to those obtained under FO diets (Glencross et al., 2003;
44 Mourente et al., 2005). However, digestive and absorptive processes can be
45 affected. Modifications in the composition of enterocyte membranes have been
46 described in fish fed VOs (Caballero et al., 2003; Sitjà-Bobadilla et al., 2005).
47 These alterations can compromise intestinal function by reducing lipid (Francis
48 et al., 2007; Geruden et al., 2009) and protein (Francis et al:, 2007) digestibility.
49 Similarly, changes in brush-border enzyme activities have been reported in
50 response to diet fatty acid (FA) composition (Cahu et al., 2000), and it has also
51 been hypothesized that the digestibility of a given FA can be altered by the
52 presence of another FA (Torstensen et al. 2000). Moreover, in rat jejunum,
53 dietary fat modulates disaccharidase activities (Goda and Takase, 1994).
54 Furthermore, the modification of membrane fluidity may alter nutrient transport
55 (Drozdowski & Thomson, 2006). In this regardHigher uptake rates for amino
56 acids and free FAs have been found in fish fed VO than in those fed FO (Jutfelt
57 et al. 2007). However, in spite of these drawbacks and given the decrease in
58 FO availability, alternative sustainable fat sources that allow satisfactory growth
59 rates and an acceptable quality of the final product are required (Rosenlund et
60 al., 2001).

61 Concerning FM replacement, it has been demonstrated that in sea bream
62 (*Sparus aurata*) up to 75% of FM can be replaced by plant protein sources
63 when diets are balanced to match fish amino acid requirements. FM
64 replacement initially leads to a slight decrease in growth rate (Gomez-Requeni
65 et al., 2004), which is overcome at the end of a complete culture cycle (De
66 Francesco et al., 2007). However, previous studies on this species have
67 reported that digestion processes (Santigosa et al., 2008) and protein retention

68 (Kissil et al., 2000) are affected by plant protein diets because of the presence
69 of anti-nutritional factors (ANFs) in meal (Francis et al., 2001; Gatlin et al.,
70 2007).
71 Less information is available on the effects of simultaneous FO and FM
72 replacement on sea bream (Bouraoui et al., 2006; Benedito-Palos et al., 2007;
73 Benedito-Palos et al., 2008) or other species (Subhadra et al., 2006; Drew et
74 al., 2007; Torstensen et al., 2008; Panserat et al., 2009; Salze et al., 2010). To
75 our knowledge, no study has addressed the modifications of digestive enzymes
76 and intestinal nutrient absorption caused by diets in which FM and FO have
77 been replaced by proteins and lipids derived from plants, respectively.
78 In vertebrates, the gastrointestinal tract is the main site of food digestion and
79 nutrient uptake. Digestion depends on the signalling pathway of the peptide
80 hormone cholecystokinin (CCK). This hormone is synthesized by I-cells in the
81 mucosal epithelium of the small intestine and secreted in the proximal intestine,
82 where it stimulates the digestion of fat and protein by triggering the release of
83 pancreatic enzymes that are active in the lumen. Serine proteases,
84 glucosidades and lipases then break down the ingested proteins, carbohydrates
85 and fats, and produce metabolites, which are then absorbed by enterocytes.
86 Absorption occurs by diffusion, facilitated transport or active transport. In
87 mammals, the apical translocation of lipolytic products into the enterocyte
88 seems to occur by passive or active diffusion at high and low FA
89 concentrations, respectively, by means of specialized carrier proteins, such as
90 FATP (Stremmel et al., 1985). The uptake of amino acids, oligopeptides and
91 glucose, which is mediated by both facilitated and active transport, follows
92 asymptotic kinetics and depends on the presence of protein transporters
93 immersed in the lipid bilayer (Casirola et al., 1995). Fish absorption
94 mechanisms have been widely studied (Ferraris & Ahearn, 1984; Collie &
95 Ferraris, 1995) and are similar to those of mammals, although fish show lower
96 rates (Reshkin & Ahearn, 1987). These rates are programmed to match diet
97 composition, and transport proteins are modulated to fit lumen nutrient
98 concentration (Ferraris & Ahearn, 1984; Buddington et al., 1987).

Here we performed an 11-week growth trial in sea bream to study the effects of four experimental diets, in which FM and FO were simultaneously replaced by plant proteins and VO respectively, on the following parameters: 1) luminal protease, α-amylase and lipase activities; 2) intestinal absorption capacities and 3) intestinal absorption regionalization pattern. The experimental diets contained 75% plant protein and graded (0, 33, 66 and 100%) levels of a blend of VOs.

3. MATERIAL AND METHODS

3.1. Diets

Four experimental diets with a low level (25%) of FM and rich (75%) in plant protein sources sources (corn gluten meal, soybean meal and extruded wheat) were formulated. FO was replaced at 0, 33, 66 and 100% (FO, 33VO, 66VO and 100VO) by graded levels of a blend of vegetable oils (VOs; rapeseed, linseed and palm oils). Diets were supplemented with L-lysine and the DHA/EPA ratio (1.1-1.2) was kept constant (Table 1). For a more detailed description (including the FA profile) of the diets see Benedito-Palos *et al*., 2007.

3.2. Fish and sampling

Nine hundred and sixty gilthead sea bream (*Sparus aurata*) obtained from Ferme Marine de Douhet (Ile d'Olérn, France) were acclimatized at the *Instituto de Acuicultura de Torre de la Sal –CSIC* (Castellón, Spain) for 20 days. At the start of the experiment, fish weighing 16.2 ± 0.05 g were randomly distributed in 12 fibreglass tanks (500-L). The water (37.5‰ salinity) flow was 20 L·min^{-1} and the oxygen content of outlet water was higher than 85% saturation. During the 11-week trial, triplicate groups of animals were manually fed the corresponding diet twice a day (9:00 and 14:00 h) until visual satiety. Day length and water temperature followed natural changes over the course of the trial (May-August). Growth coefficients and feed efficiency at the end of the growth trial were similar in the FO, 33VO and 66VO groups. The 100VO group showed reduced feed intake and growth (Benedito-Palos et al., 2007). At the end of the experiment, four animals per tank (12 fish per condition) were anesthetized (MS-222 0.1g L^{-}

131 ^{1}), weighed and sacrificed by severing the spinal cord 5 h after the morning
132 manual feeding. Digestive tracts were isolated on ice and adherent tissue was
133 removed. A portion of pyloric caeca (PC) and the first proximal segment (0.5
134 cm), including the intestinal content, were rapidly frozen in liquid nitrogen and
135 maintained at –80ºC until enzymatic studies. A second segment of the proximal
136 intestine (PI) (0.5 cm) was pre-conserved in 4% buffered formol for histological
137 observation The remaining PC and PI, and the whole distal intestine (DI) were
138 cut lengthwise, washed in isosmotic saline solution containing 0.1M protease
139 inhibitor (phenyl-methyl-sulphonyl-fluoride, PMSF), and frozen in liquid nitrogen
140 until nutrient absorption experiments.

141 3.3. Digestive enzyme activity

142 *Preparation of enzyme extracts*: Samples were individually homogenized in 50
143 mM TrisHCl; pH 7.5 (Politron 2000, Sorvall TC; 4ºC) to a final concentration of
144 250 mg tissue $\cdot mL^{-1}$ for lipase activity analyses and to a final concentration of
145 50 mg tissue$\cdot mL^{-1}$ for protease and α-amylase activity measurement.
146 Homogenates were then centrifuged (1100x*g*, 15 min, 4ºC. Jouan CR 411) and
147 supernatants were recovered and stored at –20ºC.

148 *Total protease activity measurement*: Acid protease activity in stomach samples
149 was measured following Alarcón et al. (1998) using 0.5% haemoglobin in 0.1M
150 glycine-HCL pH 2.0 (30 min, 20ºC). Alkaline protease activity in PC and PI
151 extracts was measured by the casein method described by Kunitz (1947), as
152 modified by Walter (1984), using 1% casein in TrisHCl 50 mM pH 9.0 (30 min,
153 20ºC). Both reactions were stopped by adding 12% trichloracetic acid (TCA).
154 Samples were then centrifuged (7500*g*, 5 min, 4ºC. Sigma 2-15) and
155 supernatant absorbance at 280 nm was recorded (UV-1603, Shimadzu). All
156 measurements were analyzed in triplicate and the blank for each sample was
157 measured separately. Activity was referred to the soluble protein concentration
158 of the extract, which was determined following Bradford (1976) using bovine
159 serum albumin as standard. Alkaline protease activity was measured as BAEE
160 units.

161 *Modifications of individual protease activities:* By means of zymography
162 (García-Carreño *et al*. 1993), we identified changes in individual protease

163 activity in PC and PI. This method allowed the viewing of bands with protease
164 activity in polyacrylamide (12%) gels (10 x 10.5 x 0.1 cm). Electrophoresis was
165 performed at a constant current of 15 mA per gel (100 min, 4ºC. EPS 301
166 Power Supply) and gels were incubated in a TrisHCl buffer pH 8.2 containing
167 2% casein (30 min, 4ºC and 90 min 25ºC). They were then washed and stained
168 in a methanol:acetic:water solution (40:10:40) with 0.1% of Coomassie brilliant
169 blue R-250. Distaining was done using the same solution without colorant.
170 Molecular weights were established using a molecular weight marker (RPN800,
171 Amersham Pharmacia Biotech, Spain). The combination of the extracts in
172 polyacrylamide gels with specific inhibitors allowed identification of the specific
173 activities of the bands (Santigosa et al., 2008). Individual PC and PI fish
174 extracts were analyzed by substrate-SDS-PAGE.
175 *α-Amylase activity*: We analyzed α-amylase activity in PC and PI extracts
176 following the method described by Dold et al. (1995), as modified by Santigosa
177 et al. (2008). This method involves the use of the tracer material amylose azure,
178 a potato starch covalently labelled with Ramazol Brillant Blue R dye. Briefly,
179 100 μL of homogenate was combined with 400 μL of 0.5% tracer solution.
180 Soybean trypsin inhibitor (SBTI, 0.04 mg mL^{-1}) was added to prevent
181 proteolysis. The reaction was stopped after 30 min by adding 6% acetic acid.
182 After 30 min at 4°C, the tubes were centrifuged (13,000*g*, 10 min, 4 °C).
183 Supernatant absorbance was recorded at 595 nm. Each sample was analyzed
184 in triplicate and individual blanks were established. α-Amylase was used as
185 standard. One unit of enzyme activity was defined as 1.0 mg of maltose
186 released from starch in 3 min at pH 6.9 at 20 °C.
187 *Lipase activity*: Lipase activity was analysed in PI extracts using a modification
188 of the method described by Panteghini (2001). Briefly, 50 μL of homogenate
189 was combined with 950 μL of a buffer containing (in mM) 20.5 Tris, 3.6
190 taurodeoxicolate, 0.9 deoxicolate, 0.8 tartrate, 0.12 DGGR (1,2-o-dilauryl-rac-
191 glycero-3-glutaric acid-(6'-methylresorufin) ester), 0.05 CaCl2, 30 mannitol, and
192 1 mg L^{-1} colipase (pH 8.3), and the increase in absorbance was recorded at
193 580 nm between 10 and 20 min. Each sample was analyzed in triplicate and
194 individual blanks were established. Lipase was used as standard. One unit

195 hydrolyzes 1.0 microequivalent of fatty acid from triacetin in 1 h at pH 7.4 at
196 37°C.
197 3.4. Nutrient uptake analysis
198 Brush border membrane vesicles (BBMVs) from PC, PI and DI were obtained
199 as described by Sala-Rabanal et al. (2004). Briefly, samples were thawed in a
200 hypoosmotic buffer (in mM: 100 mannitol, 2 HEPES, pH 7.4) and placed in a
201 Waring blender at low speed to separate mucose from the muscular layers. The
202 resulting eluate was filtered through a Büchner funnel and homogenized at high
203 speed. The homogenate was subsequently filtered through nylon stocking
204 material, and $MgCl_2$ was added to the eluent to a final concentration of 10 mM,
205 for selective precipitation of most cellular membranes except for the brush
206 border. Subsequent selective centrifugations allowed the purification and
207 concentration of apical enterocyte membranes, which were vesiculated using an
208 insulin syringe.
209 *Validation of the BBMV suspensions*: To ensure the enrichment in brush border
210 membrane, as well as the depletion in other cellular fractions, the activity of
211 membrane enzymes considered suitable cellular markers (Scalera *et al*., 1980)
212 was measured (20ºC) in the initial homogenate and in the final BBMV
213 preparations. Thus, alkaline phosphatase activity was measured following
214 Weiser (1973) to ensure an increase in brush border membrane during
215 vesiculation. The decrease in Na^+/ K^+-ATPase activity (Sala-Rabanal *et al*.
216 2004) and in citrate synthase activity (Srere, 1969) ruled out possible
217 contaminations with baso-lateral and mitochondrial membranes, respectively.
218 The orientation of BBMVs was studied using a modification of the approach
219 described by Del Castillo & Robinson (1982). Thus, vesicle preparations were
220 incubated in the presence and absence of a solution of 2 mM sodium
221 deoxycholate containing EDTA 15 mM, which disrupts cellular membranes. The
222 total saccharase activity of the solution was then measured by adding 100 μM
223 sacarose to the preparations. An increase in saccharase activity in disrupted
224 *versus* non-disrupted BBMVs indicates the presence of vesicles in a non-
225 physiological orientation.

226 *BBMV uptake of amino acids, glucose and linoleic acid:* The capacity of
227 BBMVs to absorb 3 essential amino acids (L-leucine, L-lysine and L-
228 phenylalanine) and 3 non-essential amino acids (L-alanine, L-glutamic and L-
229 proline) was studied. Glucose and linoleic influxes were also determined. In all
230 cases, we used the technique described by Sala-Rabanal et al. (2004).
231 For this measurement, 10μL of BBMVs was mixed with 40μL of incubation
232 buffer (in mM: 250 NaSCN, 100 mannitol, 40 HEPES, 0.1 $MgSO_4 \cdot 7H_2O$, 8.16
233 LiN_3, 0.15 unlabelled nutrient, 0.01 ^{3}H-nutrient. Osm 320; pH 7.4). After 5 s
234 (lineal uptake zone previously determined. See Fig. 1) the reaction was stopped
235 by adding 1 mL of cold stop buffer (in mM: 300 mannitol, 20 HEPES, 0.1
236 $MgSO_4 \cdot 7H_2O$, 4.08 LiN_3. Osm 320; pH 7.4). We then rapidly passed 990 μL of
237 the resulting mix under negative pressure through 0.22 μm cellulose nitrate
238 filters (Millipore, Bedford MA) previously wet in cold stop buffer. Filters were
239 washed with 10 mL of stop solution and dissolved in Filtron-X scintillation liquid
240 (ITISA S.A, Spain). Samples were counted in a scintillation counter (Packard
241 TRI-CARB 2100 TR). All measurements were done at 20ºC. Because of the
242 hydrophobic properties of linoleic acid, it was complexed with albumin (following
243 Tocher *et al*., 2002) before addition to the incubation buffer. Thus, an aliquot of
244 ^{14}C FA (0.75 μCi) was incubated for 10 min with a solution containing 0.428
245 mmols KOH · μCi^{-1} and then for 45 min in the incubation buffer previously
246 described containing 600 μg · μCi^{-1} FA-free bovine serum albumin. We then
247 mixed 40 μL of the resultant solution with 10 μL of vesicles and performed the
248 analysis as previously described.
249 *Determination of vesicular volume using L-alanine*: The BBMV volume varies
250 depending on the intestinal segment studied (Sala-Rabanal et al., 2004). Diet
251 composition may also modify this parameter. Thus, we measured the vesicular
252 volume of BBMV preparations following Sala-Rabanal et al. (2004). Briefly, L-
253 alanine retained inside the vesicles in the steady state was measured by
254 incubating 10 μL of BBMV preparation with 40 μL of the incubation buffer on
255 ice. After 90 min the reaction was stopped and L-alanine was measured
256 following the procedure described above.

257 The protein concentration of the individual BBMV preparations was measured
258 following Bradford (1976) using the BIORAD[R] protein assay reagent.

259 3.5. Histological observation

260 Samples conserved in buffered formol were embedded in paraffin, cut (5 μm),
261 and stained with haematoxylin-eosin for observation under a light microscope.

262 3.6. Statistical analysis

263 All data presented a normal distribution ($p>0.05$ in Kolmogorov-Smirnov Test).
264 The *t*-Student test was used to establish significant differences ($p<0.05$)
265 between two groups. Differences between intestinal regions or dietary
266 treatments were determined using one way ANOVA and Tukey test. The
267 software used was SPSS 12.0 (SPSS Inc., US).

268 3.7. Chemicals

269 Radiolabelled nutrients were obtained from Amersham Pharmacia Biotech
270 (Spain). The remaining reagents were purchased from Sigma-Aldrich (Spain),
271 unless stated otherwise.

272

273 4. RESULTS

274 Total protease activity (TPA) was measured in PC and PI 5 h post-feeding (Fig.
275 1). The FO group showed maximal TPA activity in PI, with a TPA PI/PC ratio of
276 2.57. This pattern was maintained in the 33VO group, although the ratio
277 decreased to 1.77. Fish in the 66VO and 100VO treatments showed higher TPA
278 activity in PC than in PI (PI/PC ratio of 0.78 and 0.90, respectively). Moreover,
279 the 100VO group showed a decrease in total alkaline TPA activity compared to
280 the rest of the groups.

281 In the zymograms of PC and PI extracts, five bands (90, 60, 50, 30 and 25KDa)
282 with proteolytic activity were detected (Fig. 2). The combination of the same
283 extracts in polyacrylamide gels with specific inhibition solutions allowed
284 identification of the specific activity of each band (Santigosa et al., 2008). Thus,
285 the two highest molecular weight bands presented trypsin-like activity, while the
286 three lower bands (50, 30 and 25KDa) showed chymotrypsin-like activity. The
287 60 KDa trypsin-like and 25 KDa chymotrypsin-like bands showed the greatest

288 activity, while a minor contribution was related to the highest molecular weight
289 band (90KDa).
290 In PC zymograms (Fig. 2A), the increase in dietary VO resulted in a higher
291 intensity and amplitude of trypsin- and chymotrypsin-like proteolytic bands. The
292 progressive increase in the magnitude of all four PC proteolytic bands supports
293 the results obtained using the spectrophotometric quantitative approach.
294 In PI extracts (Fig. 2B), although the 90 and 50 KDa bands were not modified in
295 response to the replacement of the lipid source, a decrease in the intensity of
296 30 and 25-KDa bands was detected in the 66VO group and a decrease in the
297 intensity of both 60 and 25 KDa bands and especially the 30 KDa band in the
298 100VO group.
299 α-Amylase activity in PC and PI samples 5h after manual feeding (Fig. 3) was
300 not significantly modified by the replacement of FO by VOs. Neither was
301 significant the difference found between the two intestinal regions of any of the
302 VO-fed groups.
303 Lypase activity in PC samples (Fig. 4) was not significantly modified when FO
304 was replaced by VOs (6.88 ± 2.80 and 4.81 ± 1.51 mU^{-1} protein · min^{-1} for FO
305 and VO animals, respectively).
306 In order to study the effect of the diet on the capacity to absorb amino acids, D-
307 glucose and linoleic acid, as well as the regionalization of this capacity along
308 the intestinal tract, we also examined BBMVs from PC, PI and DI from fish on
309 the 4 experimental diets. Vesicular preparations were checked in order to
310 determine their purity (Table 2) and physiological orientation. The increase in
311 alkaline phosphatase activity together with the decrease in Na^{+}/K^{+} ATPase and
312 citrate synthase activities in the final preparations when compared to the initial
313 homogenates indicated that final suspensions were enriched in BBMVs and
314 ruled out contamination with other cellular fractions. In all cases, vesicles
315 presented a physiologic orientation (99.42 ± 0.25%; n=12). For all the nutrients
316 examined, uptake was linear for almost 7 s for PC, PI and DI (Fig. 5). Vesicular
317 volume was determined in BBMV preparations from the three vesicular types
318 (Table 3). This parameter differed among intestinal regions and dietary
319 treatments, being significantly higher in vesicular suspensions obtained from DI

320 than in PC and PI. Moreover, the volume of PI BBMVs was higher in the 66VO
321 group. As a result of these changes we decided to normalize influx values,
322 which are presented as intravesicular concentration after 5 s of incubation.
323 We detected a regionalization of amino acid uptake capacity along the
324 digestive tract, with a decrease in absorption capacity from PC to DI (Tables 4
325 and 5). In general, the replacement of FO by VO had no effect on amino-acid
326 uptake. Similar values were observed for each intestinal segment in the 4
327 experimental groups. D-glucose uptake (Fig. 6A) was significantly higher in
328 BBMVs from PI of the FO group. In VO groups, uptake decreased, whilst PC
329 increased in response to 100% FO replacement. Linoleic acid uptake (Fig. 6B)
330 was maximal in PC BBMVs obtained from the FO group. The uptake of this FA
331 was lower in BBMVs obtained from the three intestinal segments in the VO
332 groups.
333 Histological preparations of PI from fish on the FO (Fig. 7A) and 100VO (Fig.
334 7B) diets showed no differences in fold length or intestinal layer integrity at the
335 end of treatment. However, an increase in lipid droplet content, together with a
336 decrease in the number of goblet cells, was detected in the 100VO group.
337 These changes were progressive as the percentage of VO in the diet increased
338 (images of fish on 33VO and 66 VO diets not shown).
339 5. DISCUSSION
340 The FO and FM traditionally used in aquafeeds are limited primary resources
341 (SOFIA, 2008). Plant protein may provide a suitable substitute for the
342 formulation of carnivorous fish diets.
343 Digestive enzymatic activity is compromised when diets contain mid or high
344 levels ofplant protein sources (Santigosa et al., 2008). Here we fed gilthead sea
345 bream with diets containing 75% of plant protein and graded levels (0, 33, 66
346 and 100%) of a blend of vegetable oils. Fish showed 5 proteolytic bands in PC
347 and PI 5 h after feeding. These results correspond to the alkaline proteolytic
348 bands reported by Alarcón et al. (1999) for this species. A 55KDa band with
349 proteolytic activity detected in a previous trial (Santigosa et al., 2008) was not
350 detected in the present study. This absence may be attributable to the distinct
351 blends of VO used to replace FM in the studies. Furthermore, each plant

352 protein source has specific ANFs (Francis et al., 2001; Gatlin et al., 2007).
353 Moreover, the modification of the dietary lipid sources may be the responsible of
354 the quantitative modification of enzymatic digestive activities 5 h post-feeding in
355 PC and PI of the 66VO and 100VO groups. In this regard, a significant
356 decrease in apparent protein digestibility in Murray cod fed VO diets has been
357 described (Francis et al., 2007). Similarly, lower trypsin activities were detected
358 in Senegalese sole fed on *Artemia* enriched with soybean oil emulsion than
359 those fed *Artemia* enriched with FO emulsion (Morais et al. 2006). Our data
360 show a progressive variation of the TPA PI/PC ratio, which could be related to
361 the inhibition of luminal proteases as a result of the presence of ANFs in the
362 VOs, as also reported when plant protein sources are used in carnivorous fish
363 aquafeed (Moyano et al., 1999; Santigosa et al., 2008). However, the
364 progressive increase in PC TPA respective to VO inclusion seems to contradict
365 this hypothesis, and points to a decrease in transit rate as the most plausible
366 explication. In this regard, the modification of dietary lipid sources could be
367 responsible for modified emulsification of the dietary bolus and a differential
368 micellar incorporation, which could lead to slower passage along the intestine,
369 with a concomitant slower release of proteases in the lumen. Since proteases
370 exert distinct functions in digestion (Guillaume and Choubert, 2001), the
371 modifications described in the trypsin/chymotrypsin ratio may imply differential
372 availability of luminal oligopeptides and amino acids, which may lead to an
373 amino acid imbalance under these feeding conditions.
374 This prompted us to study possible modifications in absorptive phenomena
375 along the intestinal tract. Our data show a regionalization of intestinal amino
376 acid transport, with absorption being greater in PC and PI segments, which is
377 consistent with the findings of other studies on nutrient uptake in carnivorous
378 fish (Sala-Rabanal et al., 2004; Bakke-McKellep et al., 2000; Jutfelt et al.,
379 2007). We detected no differences in the capacity of intestinal BBMVs to absorb
380 the 3 essential amino acids (L-leucine, L-lysine and L-phenylalanine) and the 3
381 non-essential amino acids (L-alanine, L-glutamic and L-proline) in response to
382 dietary lipid source. This observation is in accordance with the satisfactory
383 growth rates of marine species fed VO diets (Glencross et al., 2003; Mourente

384 et al., 2005). However, diet lipid composition modified the volume of the BBMVs
385 in PI. This modification may be attributable to a variation in the lipid bilayer
386 composition. In this regard, changes in the FA profile of intestinal mucosa polar
387 lipids have been described in sea bream (Caballero et al., 2003; Sitjà-Bobadilla
388 et al., 2005) in response to dietary plant lipids. The consideration of the
389 vesicular volume in this experience may explain the divergence of our data from
390 other existing works (Jutfelt et al., 2007; Geurden et al., 2009) in which VO
391 inclusion modified intestinal uptake. Moreover, in mammals the modification of
392 intestinal mucosa polar lipids alters passive properties across the membrane
393 (Spector and Yorek, 1985). This observation could explain the decrease in
394 linoleic acid uptake in the three intestinal segments observed in sea bream on
395 33VO, 66VO and 100VO diets, although no differences in intestinal lipase
396 activity were found in this study. However, since enterocytes incorporate this FA
397 at high concentrations by diffusion (Stremmel et al., 1985) as shown in this
398 experiment, the increase in luminal linoleic availability in response to the
399 progressive replacement of FO by VO (from 12.1% to 21.3% of total FAME in
400 FO vs. 100VO groups, respectively; Benedito-Palos et al., 2007) should also be
401 taken into account.
402 Our study indicates that the inclusion of VO in feed for juvenile sea bream does
403 not alter amino acid absorption capacity. This finding contrasts to what occurs
404 when FM is replaced by plant protein (data not shown). Our findings indicate
405 good amino acid digestion and absorption efficiency of fish on FO, 33VO and
406 66VO diets and reinforce the previously stated hypothesis of decreased transit
407 rate *versus* protease inactivation. This hypothesis is supported by the high SGR
408 values obtained after 11 weeks of dietary treatment (Benedito-Palos et al.,
409 2007). The lower growth rates recorded in the 100VO group after the 11-week
410 trial could be associated with reduced feed intake (Benedito-Palos et al., 2007),
411 or to the accumulation of lipid droplets in PI enterocytes, which has been
412 previously related to impaired transport rate from intestinal mucosa to the blood
413 (Salhi et al., 1999). Although VO inclusion in finfish diets has been associated
414 with intestinal lipid accumulation (Caballero et al., 2003, Olsen et al., 2003), the
415 histological study of the posterior intestine in sea bream on the same diets

416 showed no pathological lipid accumulation in the enterocytes (Benedito-Palos et
417 al., 2008). These discrepancies may be due to sample origin (anterior *versus*
418 posterior intestine), but may also be related to the supplementation of diets with
419 phospholipids in the form of soya lecithin, as previously proposed by Benedito-
420 Palos et al. (2008).
421 Our data showed that in fish fed the FO diet, D-glucose is absorbed mainly in
422 the PI. This result is in agreement with those of other studies performed in sea
423 bream (Sala-Rabanal et al., 2004). The three groups fed VO diets showed a
424 decrease in monosaccharide uptake in this intestinal segment. This observation
425 may indicate modifications in the density of specific transporters, as described
426 in channel catfish (Houpe et al., 1997), or changes in the conformation of the
427 binding sites of transport proteins, as described for rabbit SGLT1 and GLUT5
428 (Meddings et al., 1990). As expected, since carbohydratases are genetically
429 programmed to be low (Cahu & Infante, 1995), fishon diets in which FM and FO
430 were simultaneously replaced by plant protein and VO respectively did not have
431 the capacity to modulate the activity of α-amylase in response to a decrease in
432 glucose uptake.
433 In summary, the replacement of up to 66% of FO by a blend of VOs when 75%
434 of FM is replaced by plant protein sources and slowed down protease digestion,
435 increasing the trypsin/chymotrypsin ratio at 5 h post-feeding respective to VO-
436 inclusion. No differences in the capacity of amino acid absorption were found.
437 Total FO replacement resulted in decreased TPA and increased the
438 accumulation of lipid droplets in the PI enterocytes. All these modifications
439 could explain the lower growth performance in the fish on the diet with maximal
440 simultaneous FO replacement (Benedito-Palos et al., 2007).

441 6. ACKNOWLEDGMENTS

442 This research was funded by a Spanish project (AGL2004-06319-CO2). E.
443 Santigosa and I. García-Meilán received a fellowship from the *Universitat de*
444 *Barcelona*.
445
446 Table 1. Composition of the experimental diets.

	FO	33VO	66VO	100V0

Ingredient (g Kg^{-1})				
Fish meal	150			
CPSP 90	50			
Corn gluten meal	400			
Soybean	143			
Extruded wheat	40			
L-Lys	5.5			
Fish oil	155.0	101.5	51.5	0
Rapeseed oil	0	8.5	17.0	25.8
Linseed oil	0	29.0	58.0	88.0
Palm oil	0	12.5	25.0	38.8
Proximate composition				
Protein (%)	49.0	48.8	49.0	48.6
Lipids (%)	22.2	22.3	22.1	22.3
Energy (MJ·Kg^{-1} DM)	24.7	24.7	24.7	24.5

447

448 Diets were supplemented with minerals and vitamins. For more details on the
449 composition see Benedito-Palos et al. (2007).

450

451 Table 2. BBMV marker enzyme enrichment factor.

Intestinal segment	Phosphatase alkaline	Na^+/K^+ATPase	Citrate synthase
PC	5.21 ± 0.45	0.42 ± 0.02	0.03 ± 0.01
PI	3.39 ± 0.19	0.66 ± 0.02	0.12 ± 0.04
DI	5.15 ± 0.46	0.80 ± 0.01	0.09 ± 0.03

452

453 Enrichment factor is the ratio between the specific activity measured in the final
454 BBMV preparations and the initial homogenate. Results are presented as the
455 mean ± S.E.M. of the different treatments for each marker enzyme (n=16). No
456 differences among treatment were found. Pyloric caeca (PC), proximal intestine
457 (PI) and distal intestine (DI).
458
459 Table 3. Vesicular volume referred to protein content (μL · mg^{-1} protein) of
460 BBMVs obtained from intestinal segments of sea bream.

Intestinal segment	**FO**	**33VO**	**66VO**	**100VO**
PC	1.29 ± 0.13 ^{2}	1.65 ± 0.18 ^{2}	1.79 ± 0.40 ^{2}	2.10 ±0.12 ^{2}
PI	1.42 ± 0.04 $^{b, 2}$	1.81 ± 0.50 $^{b, 2}$	3.85 ± 0.63 $^{a, 12}$	2.81 ± 0.29 $^{ab, 2}$
DI	4.97 ± 0.34 ^{1}	6.48 ± 0.66 ^{1}	5.64 ± 0.94 ^{1}	4.70 ± 0.17 ^{1}

461
462 Results are presented as the mean ± S.E.M. of 8 measurements for each
463 condition. Significant differences ($p<0.05$) between diets are shown by different
464 letters; significant differences between intestinal segments are shown by
465 different numbers. Pyloric caeca (PC), proximal intestine (PI) and distal intestine
466 (DI).

Table 4. Intravesicular essential amino acid concentration (pmols mL^{-1}) in BBMVs obtained 5 h post-feeding.

	Intestinal segment	FO	33VO	66VO	100VO
L-Leu	PC	1084.9 ±127.4 ^{1}	1568.4 ± 124.9 ^{1}	1449.1 ± 250.5 ^{1}	1166.20 ± 29.74 ^{1}
	PI	1090.0 ± 56.2 ^{ab,1}	1236.9 ± 97.6 ^{a,1}	662.6 ± 120.5 ^{b,2}	1092.30 ± 104.01 ^{ab,1}
	DI	298.4 ± 17. 4 ^{b,2}	473.2 ± 80.0 ^{a,2}	486. 8 ± 39.9 ^{a,2}	455.92 ± 23.21 ^{ab,2}
L-Lys	PC	2553.5 ± 314.8 ^{1}	2619.8 ± 234.0 ^{1}	2015.5 ± 359.1 ^{1}	2401.8 ± 24.1 ^{1}
	PI	1811.8 ± 283.7 ^{ab,1}	2140.4 ± 437.2 ^{a,1}	1530.6 ± 465.3 ^{b,12}	1856.5 ± 98.1 ^{ab,2}
	DI	457.7 ± 12.5 ^{b,2}	737.0 ± 69.0 ^{a,2}	594.1± 42.8 ^{ab,2}	736.6 ± 59.5 ^{a,3}
L-Phe	PC	4059.2 ± 690.92 ^{1}	4965.2 ± 368.6 ^{1}	4003.7 ± 414.2 ^{1}	4272.34 ± 236.02 ^{1}
	PI	3565.8 ± 272.19 ^{a,1}	3356.7 ± 138.4 ^{a,2}	1000.0 ± 49.1 ^{b,2}	3009.74 ± 192.87 ^{a,2}
	DI	936.2 ± 55.8 ^{b,2}	1626.6 ± 255.5 ^{a,3}	1214.9 ± 232.3 ^{ab,2}	1002.22 ± 159.13 ^{ab,3}

Results are presented as the mean ± S.E.M of 8 measurements. Significant differences ($p<0.05$) between diets are shown by different letters; significant differences between intestinal segments are shown by numbers. Pyloric caeca (PC), proximal intestine (PI) and distal intestine (DI).

1 Table 5. Intravesicular non-essential amino acid concentration (pmols mL^{-1}) in BBMVs obtained 5 h post-feeding.

	Intestinal segment	FO	33VO	66VO	100VO
L-Ala	PC	147.3 ± 13.1 [b,2]	608.2 ± 268.0 [a]	734.6 ± 127.7 [ab,1]	684.42 ± 71.89 [ab]
	PI	438.8 ± 53.1 [12]	1029.4 ± 245.0	826.9 ± 83.9 [1]	701.59 ± 152.02
	DI	672.8 ± 113.6 [1]	424.8 ± 144.1	393.1± 37.2 [2]	487.33 ± 80.40
L-Glu	PC	233.5 ± 38.8 [1]	197.0 ± 7.5 [1]	218.6 ± 54.5 [1]	174.3 ± 5.6 [1]
	PI	222.9 ± 5.4 [a,1]	189.6 ± 13.4 [ab,1]	164.4 ± 9.4 [b,12]	161.0 ± 4.2 [b,1]
	DI	119.1 ± 8.6 [a,2]	110.0 ± 11.6 [ab,2]	90.4 ± 5.6 [ab,2]	80.3 ± 5.5 [b,2]
L-Pro	PC	436.954 ± 45.0 [1]	570.8 ± 34.6 [1]	451.4 ± 75.9 [1]	571.73 ± 48.43 [1]
	PI	499.551 ± 8.5 [a,1]	491.8 ± 13.5 [a,2]	301.74 ± 32.5 [b,12]	421.98 ± 34.17 [a,2]
	DI	147.851 ± 3.0 [2]	186.3 ± 8.0 [3]	185.9 ± 19.6 [2]	174.74 ± 11.59 [3]

2

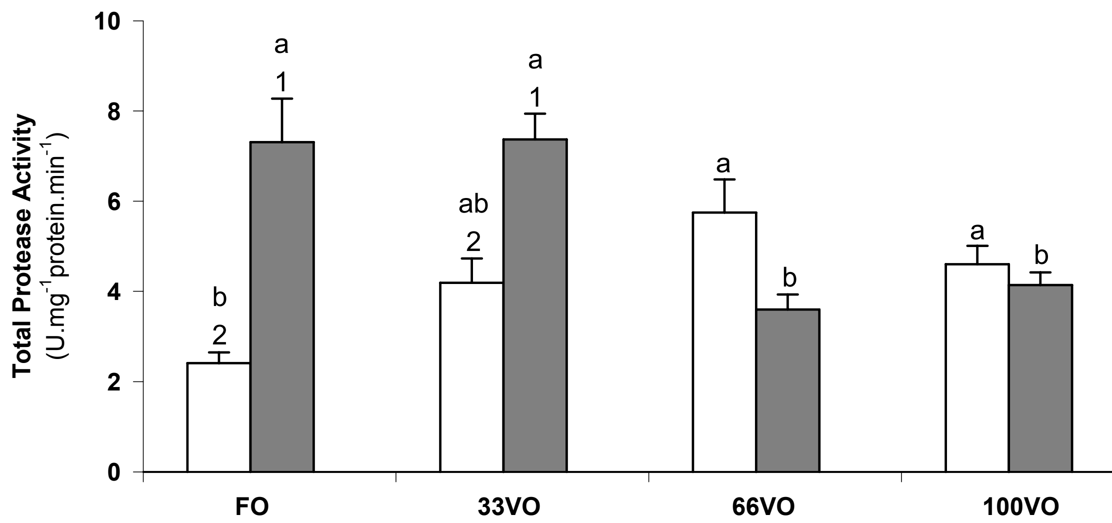
3 Results are presented as the mean ± S.E.M of 8 measurements. Significant differences ($p<0.05$) between diets are shown by
4 different letters; significant differences between intestinal segments are shown by numbers. Pyloric caeca (PC), proximal
5 intestine (PI) and distal intestine (DI)

1 LEGEND OF FIGURES
2 Figure 1. Total protease activity at 5h post-feeding. Values are represented as
3 the mean ± S.E.M of 12 fish. Significant differences ($p<0.05$) between diets are
4 shown by different letters; significant differences ($p<0.05$) between intestinal
5 segments are shown by different numbers. Pyloric caeca (white bars) and
6 proximal intestine (grey bars). The FO diet contained fish oil as the only source
7 of lipids; in the 33VO, 66VO and 100VO diets, 33, 66 and 100% of the fish oil
8 was replaced by a blend of vegetable oils respectively.
9 Figure 2. Model zymograms of the alkaline proteolytic activity in pyloric caeca
10 (A) and proximal intestine (B) extracts. Figure shows the molecular weight.
11 Figure 3. α-amylase activity at 5h post-feeding. Values are represented as the
12 mean ± S.E.M of 12 fish. No significant differences ($p<0.05$) were found
13 between diets or intestinal segments. Pyloric caeca (white bars) and proximal
14 intestine (grey bars).
15 Figure 4. Lipase activity in pyloric caeca at 5h post-feeding. Values are
16 represented as the mean ± S.E.M of 9 fish. No significant differences ($p<0.05$)
17 were found between diets.
18 Figure 5. Time course (0 to 10 s) of nutrient uptake of BBMV obtained from
19 pyloric caeca (black circles), proximal intestine (grey circles) and distal intestine
20 (open circles) of sea bream: Values are represented as the mean ± S.E.M of 4
21 measurements. r^2 is shown for all the conditions.
22 Figure 6. Intravesicular D-glucose and linoleic acid concentration in BBMVs
23 obtained from pyloric caeca (PC, white bars), proximal intestine (PI, grey bars)
24 and distal intestine (DI, black bars) of sea bream 5h post-feeding. Results are
25 presented as the mean ± S.E.M of 8 measurements. Significant differences
26 ($p<0.05$) between diets are shown by different letters; significant differences
27 between intestinal segments are shown by numbers.
28 Figure 7. Histological appearance (400X) of the proximal intestine obtained from
29 sea bream after 11 weeks of dietary treatment. (A) Fish on the FO diet. (B) Fish
30 on the 100VO diet. Lipid droplets (black arrows) and goblet cells (dotted
31 arrows).
32

1 **Figure 1**

2

3



1 **Figure 3**

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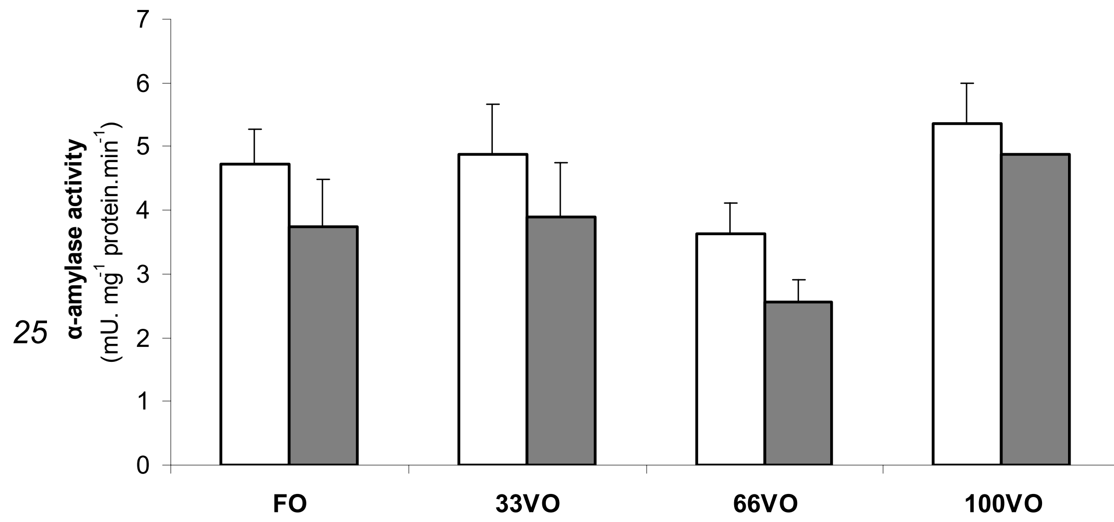

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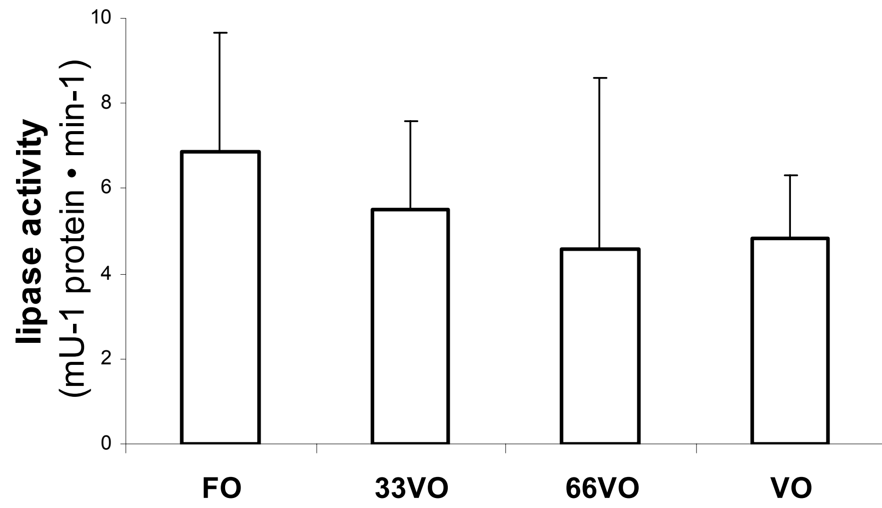

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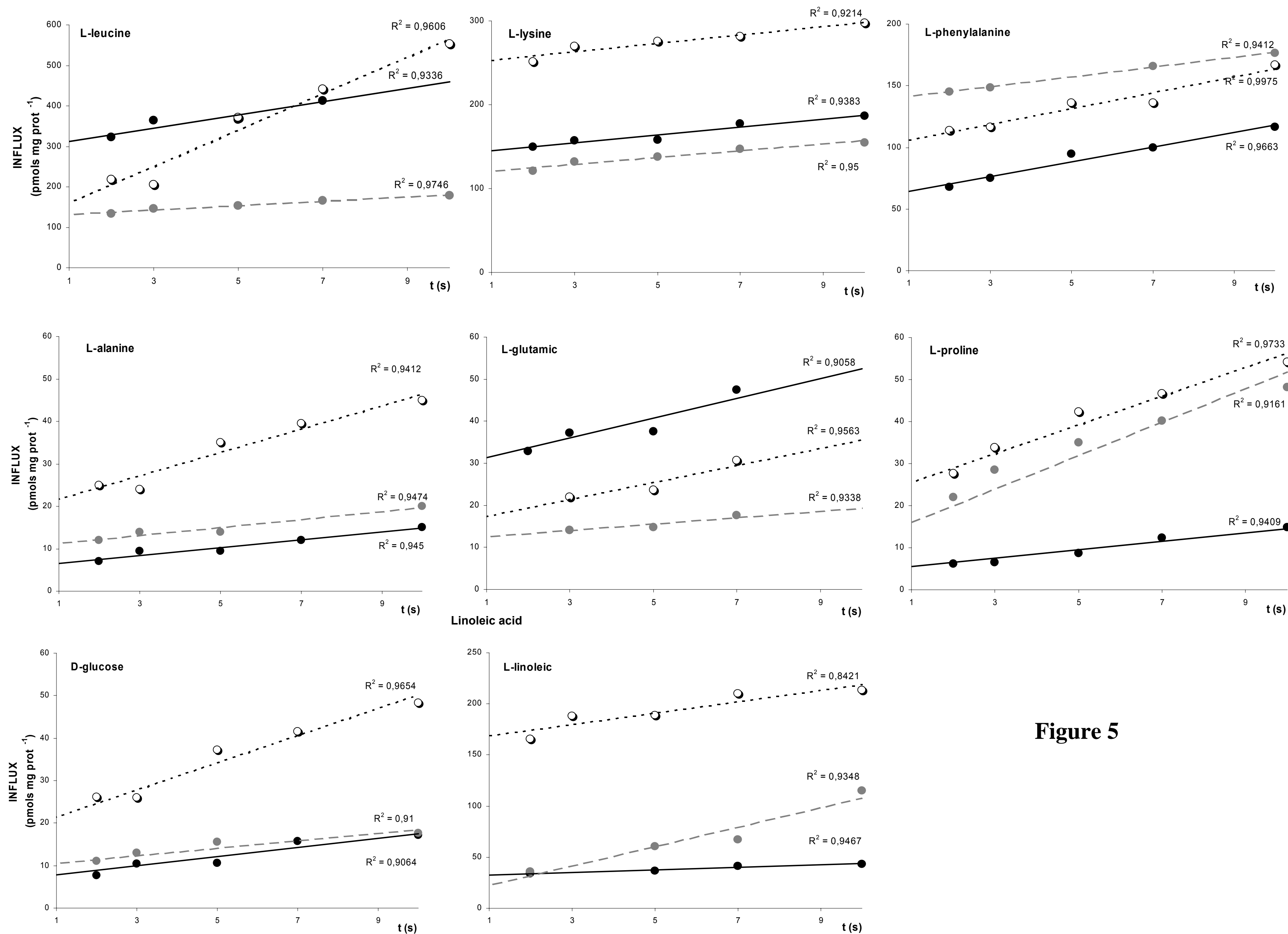

Figure 5

1 **Figure 6**

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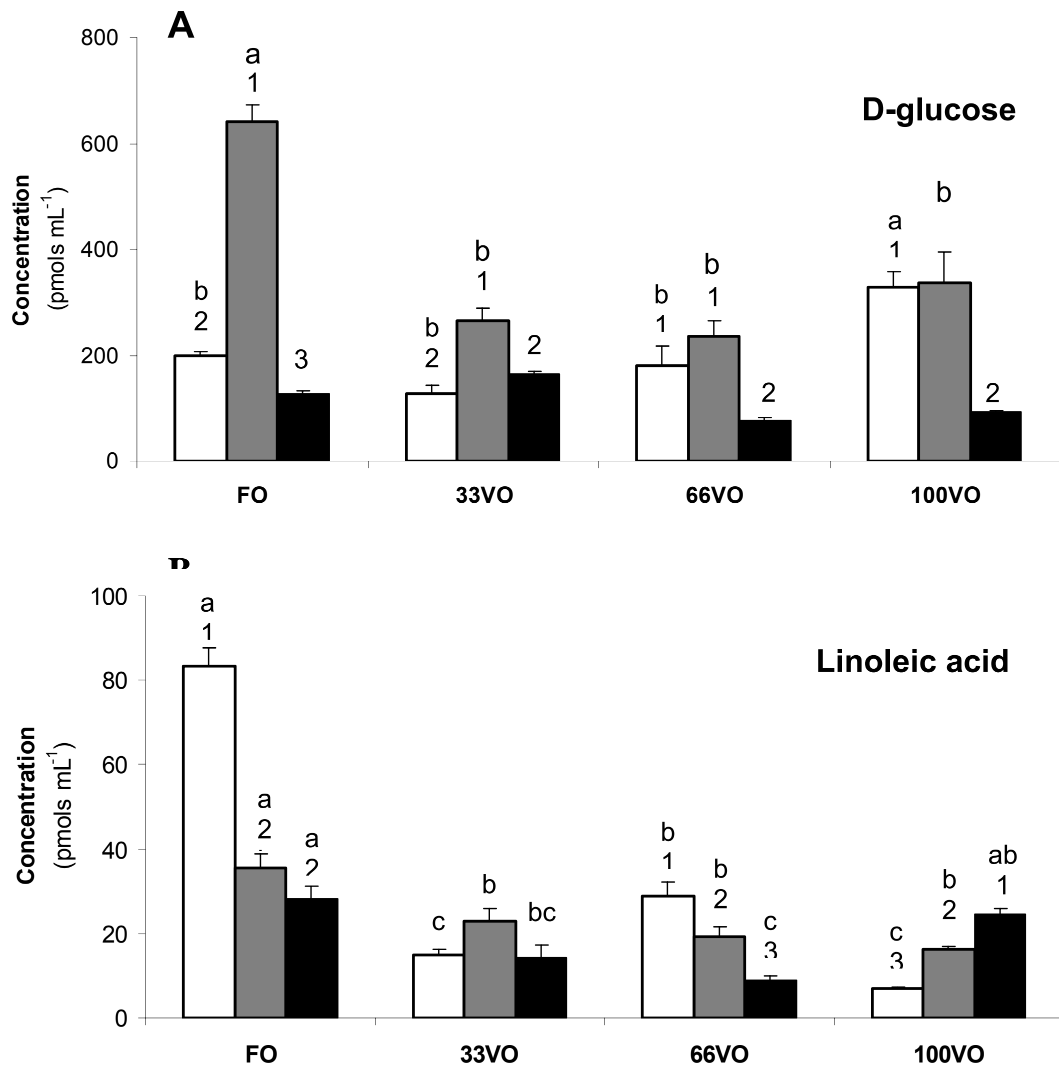

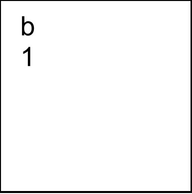

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