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3 **Changes in adipocyte cell size, gene expression of lipid metabolism markers,**
4 **and lipolytic responses induced by dietary fish oil replacement in gilthead sea**
5 **bream (*Sparus aurata* L.)**

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20 **Key words:** Adipocyte cell size, CLA, Glucose uptake, Hormone-sensitive lipase, Lipolysis,
21 Lipoprotein lipase, Liver X receptor, Vegetable oils

22
23 **Abbreviations:** t10,c12-CLA; *trans*-10, *cis*-12 conjugated linoleic acid; 66VO, 66% vegetable
24 oil diet; c9,t11-CLA, *cis*-9, *trans*-11 conjugated linoleic acid; BSA, bovine serum albumin;
25 DPM, desintegrations per minute; FO, fish oil; HSI, hepatosomatic index; HSL, hormone-
26 sensitive lipase; LA, linoleic acid; LPL, lipoprotein lipase; LXR, Liver X receptor; MFI,
27 mesenteric fat index; NEFAs, non-esterified fatty acids; PUFA, polyunsaturated fatty acids; RU,
28 relative units; TNF α , Tumour necrosis factor- α ; VO, vegetable oil

29
30 **ABSTRACT**

31 The effects of fish oil (FO) substitution by 66% vegetable oils in a diet with already 75%
32 vegetable protein (66VO) on adipose tissue lipid metabolism of gilthead sea bream were
33 analysed after a 14-month feeding trial. In the last three months of the experiment, a FO diet was
34 administrated to a 66VO group (group 66VO/FO) as a finishing diet. Hormone-sensitive lipase

35 (HSL) activity was measured in adipose tissue and adipocyte size, and HSL, lipoprotein lipase
36 and liver X receptor gene expression in isolated adipocytes, on which lipolysis and glucose
37 uptake experiments were also performed. Lipolysis was measured after incubation with tumour
38 necrosis factor- α (TNF α), linoleic acid, and two conjugated linoleic acid isomers. Glucose
39 uptake was analysed after TNF α or insulin administration. Our results show that FO replacement
40 increased lipolytic activity and adipocyte cell size. The higher proportion of large cells observed
41 in the 66VO group could be involved in their observed lower response to fatty acid treatments
42 and lower insulin sensitivity. The 66VO/FO group showed a moderate return to the FO
43 conditions. Therefore, FO replacement can affect the morphology and metabolism of gilthead
44 sea bream adipocytes which could potentially affect other organs such as the liver.

45

46 **1. INTRODUCTION**

47

48 World aquaculture production has increased significantly in the last 50 years, with an average
49 annual growth rate of 6.1 percent in volume between 2004 and 2006 (FAO, 2009). The high
50 demand for fish meal to maintain aquaculture production is reaching a critical point (Tacon and
51 Metian, 2008). The study of alternative sources of fish oil (FO) and fish meal for the vegetable
52 components of fish feed has become important for the sustainability of aquaculture (Watanabe,
53 2002). However, vegetable oils (VO) are characterized by a low ratio of omega-3/omega-6 fatty
54 acids, a lack of cholesterol and the presence of phytosterols. Although the VO has a lower ratio
55 of n-3/n-6 fatty acids, different blends of vegetable oils are used trying to obtain a fatty acid
56 composition more similar to a FO diet by means of the use of vegetable oils with a high n-3/n-6
57 ratio like linseed oil (Turchini et al., 2009). Particularly in gilthead sea bream, there is now
58 experimental evidence that the large-scale replacement of marine feedstuffs with ingredients of
59 plant origin alters the tissue-specific profile of fatty acids (Benedito-Palos et al., 2008; 2009,
60 2010) and results in changes in tissue body fat allocation (Saera-Vila et al., 2005) and
61 lipogenic/lipolytic rates (Albalat et al., 2005a; Bouraoui et al., *In Press*). These observations in
62 gilthead sea bream and other fish species (Drew et al., 2007; Menoyo et al., 2005; Todorčević et
63 al., 2009; Torstensen et al., 2008; Turchini et al., 2009) have prompted the study of the lipid
64 metabolism in fish in order to obtain a quality product in farmed fish fed alternative diets.

65 The mesenteric adipose tissue is an organ of fat accumulation in fish which, together with the
66 muscle and liver, controls the lipid homeostasis and energetic balance of the animal (Jobling and
67 Johansen, 2003; Sheridan and Kao, 1998). Adipose tissue stores lipids and provides energy from
68 lipid stores. Triglycerides, which come from the diet, are hydrolyzed by lipoprotein lipase (LPL)
69 and the fatty acids released are taken up by the adipocytes and accumulated in droplet form. In

70 response to energy demands, hormone-sensitive lipase (HSL), after phosphorylation by protein
71 kinase A, can access the lipid droplet and hydrolyze the triglycerides into glycerol and fatty
72 acids (Lafontan and Langin, 2009). In fish as in mammals, the development of adipose tissue is
73 a continuous process which includes the hypertrophy of existing adipocytes and the proliferation
74 of new ones. These processes are known to be affected by diet in mammals, but how dietary
75 changes affect the capacity for enlargement of adipocytes or the differentiation of new ones is
76 poorly understood in fish.

77 Adipose tissue also acts as an endocrine organ secreting adipokines which act as potent
78 messengers to distant organs such as the liver and muscle to maintain the body's energy balance
79 (Gregor and Hotamisligil, 2007). The pro-inflammatory cytokine tumour necrosis factor α
80 (TNF α) is secreted by adipose tissue, among others, and in mammals it is known to act as an
81 adiposity limiting factor and to lead to obesity-induced insulin resistance (Nieto-Vazquez et al.,
82 2008). TNF α also acts by limiting the fat deposits in gilthead sea bream mesenteric adipose
83 tissue (Saera-Vila et al., 2007), it inhibits adipocyte differentiation in rainbow trout (Bouraoui et
84 al., 2008) and it stimulates lipolysis in rainbow trout adipocytes (Albalat et al., 2005b). It has
85 been suggested that excessive lipid accumulation in the liver or steatosis is due in part to the
86 increased hepatic uptake of fatty acids released from adipose tissue with enhanced lipolysis
87 (Benedito-Palos et al., 2008). Therefore, changes in adipose tissue metabolism could affect other
88 organs in fish.

89 The present study is part of multidisciplinary research performed on gilthead sea bream and uses
90 the same diets and animals as in the study of Benedito-Palos et al. (2009). That study reports
91 that FO replacement is reflected in the fatty acid profile of the fish fillet, while a final FO refeed
92 (finishing diet) restores the profile of the FO diet in the fish fillet of animals fed with different
93 degrees of FO substitution (Benedito-Palos et al., 2009). Such results confirm that it is possible
94 to partially substitute the FO in a plant protein based diet without affecting growth, while
95 changing the muscle fatty acid profile. Nevertheless, although this diet did not produce apparent
96 damage to the intestinal and hepatic architecture, the hepatosomatic index (HSI) was increased
97 in animals fed the 66VO diet. This suggests an increase in hepatic lipid content, as previously
98 observed in gilthead sea bream fed vegetable diets (Benedito-Palos et al., 2008). Therefore, the
99 objective of the present study is to analyse the effect of vegetable dietary treatment (66VO diet)
100 and a finishing diet on the lipid metabolism of adipose tissue, which besides being a fat
101 reservoir, also regulates the energy balance in the organism and could be involved in
102 dysregulation of this lipid metabolism. We hypothesize that dietary vegetable oil and subsequent
103 changes in tissue fatty acid composition can affect the metabolism of adipocytes and
104 proliferation of new cells. To this end, we focused on the effects of diet on adipocyte cell size,

105 adipose tissue lipolytic activity and gene expression of lipolytic/accumulation markers in
106 response to hormones and fatty acids in isolated adipocytes.

107

108 **2. MATERIAL AND METHODS**

109

110 *2.1 Animals and diets*

111 Juvenile gilthead sea bream (*Sparus aurata L.*) of Atlantic origin (Ferme Marine de Douhet, Ile
112 d'Oléron, France) were acclimated to laboratory conditions at the Institute of Aquaculture Torre
113 de la Sal (IATS) for 20 days before the study. After this initial acclimation period, fish of 18 g
114 mean initial body weight were distributed into six fibreglass tanks (3000 L each) in groups of
115 150 fish each, where they were kept from July 2006 to September 2007 (as described in
116 Benedito-Palos et al. (2009)). Fish were fed either a FO reference diet or a blend of VO
117 (17:58:25 of rapeseed oil: linseed oil: palm oil) which replaced 66% of the FO (66 VO diet).
118 Table 1 shows the composition of the diets (for more details on diet composition see Benedito-
119 Palos et al., 2009).

120 Twelve weeks before the end of the trial, two tanks with animals fed until then with the 66VO
121 diet were switched to the FO diet (66VO/FO group). The fish in the other 4 tanks continued to
122 be fed with the FO or 66VO diet, as they had been until that time. At sampling, 18 fish per
123 dietary treatment were randomly selected and killed by a blow to the head. Mesenteric adipose
124 tissue was excised, and a piece was frozen for enzymatic activity analysis. The remaining tissue
125 was processed for adipocyte isolation

126

127 *2.2 Adipocyte isolation and lipolysis measurement*

128 Adipocytes were isolated and pooled from six fish for each independent lipolysis experiment
129 performed on each dietary group (n=3), as described elsewhere (Albalat et al., 2005a). Briefly,
130 mesenteric adipose tissue was cut into thin pieces and incubated in a shaking water bath at 18 °C
131 for 60 min with Krebs-Hepes buffer (pH 7.4) pre-gassed with 5% CO₂ in O₂, containing
132 collagenase type II (130 U/mL) and 1% bovine serum albumin (BSA). The cell suspension was
133 filtered through a double layer of nylon cloth and then washed three times by flotation. Cells
134 were carefully resuspended in Krebs-Hepes buffer containing 2% BSA at a density of 7·10⁵
135 cells/mL. Cells were counted using a Fuchs-Rosenthal chamber. Aliquots of 1 mL of this final
136 adipocyte suspension were incubated in polypropylene tubes for up to 3 h at 22 °C in the
137 absence or presence (100 ng/mL) of recombinant human TNF α (Sigma-Aldrich, Madrid, Spain),
138 linoleic acid (LA, 100 μ M; Matreya, USA), *trans*-10, *cis*-12 conjugated linoleic acid (t10,c12-
139 CLA, 100 μ M; Matreya), *cis*-9, *trans*-11 conjugated linoleic acid (c9,t11-CLA; Matreya).

140 At the end of the incubation time, 300 μL of the medium was placed in perchloric acid to give a
141 final concentration of 7% (v/v). The perchloric acid was neutralized for the measurement of
142 glycerol concentration as an index of lipolysis using a spectrophotometric method (Tebar et al.,
143 1996). The remaining medium was removed and a lysis reagent was added for RNA extraction
144 (see below). Control and experimental groups were studied in triplicate for each experiment.

145

146 *2.3 RNA isolation and cDNA synthesis*

147 Total RNA was extracted from the adipocytes by the TriReagent method (Sigma-Aldrich). The
148 quantity and quality of the isolated RNA was determined by spectrophotometry with ND-1000
149 Nanodrop (Labtech Int., UK). For cDNA synthesis, 500 ng of RNA, 3 μL of a blend 2:1 random
150 hexamers (600 μM)/oligo-dT (50 μM), 2 μL dNTP (10 mM), 0.5 μL of reverse transcriptase (20
151 U/ μL), and 0.5 μL of RNase inhibitor (40 U/ μL) were mixed with the kit buffer in a final
152 volume of 20 μL (Transcriptor first strand cDNA synthesis kit, Roche, Germany), and incubated
153 at 50 $^{\circ}\text{C}$ for 60 min, followed by 85 $^{\circ}\text{C}$ for 5 min to inactivate the enzymes.

154

155 *2.4 Real-time PCR assays*

156 PCR measurements were performed applying the primers at 0.5 μM with one fortieth of the
157 cDNA synthesis reaction and SYBR-green PCR mix (BioRad, Spain) in a total volume of 20
158 μL . The RT-PCR primer sequences for target genes (LXR, LPL and HSL) and reference gene
159 (β -actin) are shown in Table 2. Reactions were performed in a MyiQ PCR Detection System
160 (Bio-Rad, Spain) in duplicate, and the fluorescence data acquired during the extension phase
161 were normalized to β -actin by the delta-delta method (Livak and Schmittgen, 2001).

162

163 *2.5 Glucose uptake assay*

164 In the isolated adipocytes, glucose transport was determined as previously described (Capilla et
165 al., 2004). Adipocytes ($2.5 \cdot 10^5$ cells/mL) were incubated in Krebs buffer in the presence or
166 absence of recombinant human insulin (100 nM, Sigma-Aldrich) or recombinant human TNF α
167 (100 ng/mL, Sigma-Aldrich) for 30 min at 22 $^{\circ}\text{C}$ in a shaking water bath. Subsequently,
168 radiolabelled 2-deoxy-D- ^3H glucose (0.8 μCi) was added, and transport was stopped after 2 h
169 with cytochalasin B. The transport assay was terminated by transferring a 200- μL aliquot of the
170 cell suspension into small polyethylene microcentrifuge tubes containing 150 μL of
171 dinonylphthalate. Cells and buffer were separated by centrifugation at 16,000 g for 2 min. The
172 upper phase, which contained the adipocytes, was collected and subjected to liquid scintillation
173 counting. All uptake data were corrected by subtracting the extracellularly and cell-associated
174 radioactivity background by means of measuring the non-specific uptake in cell pre-treated with

175 cytochalasin B.

176

177

178 *2.6 Cell analysis*

179 Adipocytes from mesenteric adipose tissue of animals fed with FO, 66VO and 66VO/FO diets
180 were isolated. Four different pictures from each isolation procedure (n=3 for each experimental
181 group) were taken of the preparations of isolated adipocytes in a Fuchs Rosenthal counting
182 chamber using a digital camera coupled to a microscope (Olympus BX51). All the pictures were
183 acquired using a 10x objective. The cell diameters were measured using image analysis software
184 (analySYS[®], Soft Imaging System).

185

186 *2.7 HSL activity assay*

187 HSL assays are based on the measurement of the release of [³H]oleic acid from tri[³H]olein,
188 which is a measurement of the activated form of HSL influenced by phosphorylation by pKA.
189 The HSL activity assay was performed as previously described (Fredrikson et al., 1981;
190 Østerlund et al., 1996) with minor modifications. Tissue samples were homogenized in three
191 volumes of 0.25 M sucrose buffer containing 1 mM EDTA, 1 mM dithioerythritol, 20 µg/mL
192 antipain and leupeptin, pH 7.4. Infranatants were obtained by centrifugation at 110,000 g for 45
193 min at 4 °C. The protein content of the infranatant was measured by the Bradford method
194 (Bradford, 1976). The tri[³H]olein substrate was emulsified with phospholipids by sonication,
195 and BSA was used as the fatty acid acceptor. Samples of 10 µL of adipose infranatants were
196 incubated in triplicate for 2 h at 18 °C with 100 µL of 5 mM of tri[³H]olein substrate and
197 enzyme dilution buffer (total volume 200 µL). Hydrolysis was stopped by the addition of 3.25
198 mL of methanol/chloroform/heptane (10:9:7), followed by 1.1 mL of 0.1 M potassium
199 carbonate/0.1 M boric acid (pH 10.5). The mixture was centrifuged for 20 min at 1,100 g and 1
200 mL of the upper phase was collected and subjected to liquid scintillation counting. Radioactivity
201 was measured with a TRICARB 2100 β-counter (Packard Bioscience Company, Meriden, CT,
202 USA). The disintegrations per minute (DPM) were referred to protein content to compare the
203 HSL activity between groups.

204

205 *2.8 Plasma parameters*

206 Plasma glucose concentration was determined by a glucose oxidase colorimetric method
207 (Spinreact, Spain). Plasma-free fatty acids and triglycerides were analysed using commercial
208 enzymatic methods (Spinreact, Spain).

209

210 *2.9 Statistical analysis*

211 Statistical analysis was performed using the SigmaStat software. Data are presented as means ±
212 standard error of the mean (S.E.M). Results were analysed by one-way analysis of variance
213 (ANOVA), followed by Tukey's test. A significance of $p < 0.05$ was applied to all statistical tests
214 performed.

215

216 **3. RESULTS**

217

218 *3.1 Effect of diet on biometrics and plasma metabolites*

219 Biometric parameters were measured at the sampling time of the gilthead sea bream fed with
220 FO, 66VO and 66VO/FO diets (Table 3). Liver weight and HSI values were higher in 66VO and
221 66VO/FO groups than in the FO group. However, the 66VO/FO group showed a tendency to
222 recover the liver weight of the FO group. No significant differences were found in adipose tissue
223 weight or mesenteric fat index (MFI) between groups.

224 Plasma glucose and triglycerides did not show any differences between the groups. However,
225 the non-esterified fatty acids (NEFAs) levels were lower in the 66VO/FO group than in 66VO.

226

227 *3.2 Cell size distribution*

228 The cell size of each experimental group was divided into four ranges: 1-20 µm, 20-60 µm, 60-
229 100 µm and over 100 µm (Fig. 1). The adipocytes in the 1-20 µm range were the most abundant
230 in all groups. However, the FO group showed smaller adipocytes, with a considerably higher
231 proportion of cells in the 1-20 µm range than the 66VO group; while the value for the 66VO/FO
232 group was between those of the FO and 66VO groups. The 66VO group had the biggest cells,
233 with a high frequency of cells in the ranges 20-60 µm and 60-100 µm. Conversely, FO group
234 showed smallest cells, and the 66VO/FO had intermediate values in general between the other
235 diet groups.

236

237 *3.3 Effect of diet on hormone-sensitive lipase activity*

238 The HSL activities in mesenteric adipose tissue of the gilthead sea bream fed with FO, 66VO
239 and 66VO/FO diets are shown in Fig. 2. The HSL activity was higher in adipose tissue from the
240 66VO and 66VO/FO groups than in that from the FO group. However, the 66VO/FO group
241 showed a lower induction of HSL activity than the 66VO group did.

242

243 *3.4 Basal lipolysis levels and the effect of incubation with TNF α , LA, c9,t11-CLA and t10,c12-*
244 *CLA on lipolysis.*

245 The measurements of basal lipolysis levels in adipocytes from animals fed FO, 66VO and
246 66VO/FO showed that the animals fed vegetable oils have higher basal lipolysis levels than the
247 animals fed a FO diet (Fig. 3A).
248 The cells incubated with the different treatments showed that the lipolysis level was increased in
249 the FO group with the LA and the t10,c12-CLA (Fig. 3B). The 66VO group adipocyte lipolysis
250 was stimulated with TNF α (Fig. 3C), meanwhile, the 66VO/FO group showed lipolysis
251 induction by LA but not by t10,c12-CLA (Fig. 3D).
252 Stimulation with TNF α tended to increase lipolysis in the 66VO/FO group, but it was not
253 significantly different from the control. Therefore, the 66VO/FO group seems to present an
254 intermediate response to the treatments between FO and 66VO groups (Fig. 3).
255 Comparisons of the effect of the treatments described below between the diet groups showed
256 that the induction of LA and t10,c12-CLA in the FO group is significant in comparison with the
257 66VO group (Fig. 3B and 3C). However, the induction of lipolysis mediated by TNF α in the
258 66VO group is not significantly different from 66VO and 66VO/FO groups.

259
260 *3.5 LPL, HSL and LXR expression profile in isolated adipocytes from animals fed with FO,*
261 *66VO and 66VO/FO diets.*

262 The LPL, HSL and LXR gene expressions were analysed in the adipocytes from the three diet
263 groups (incubated in BSA-Krebs buffer only). The LPL did not show any change between diets,
264 but HSL and LXR expressions were differently regulated in FO, 66VO and 66VO/FO groups
265 (Fig. 4A, B, C). HSL gene transcription showed induction in the 66VO and 66VO/FO groups,
266 with a pattern similar to that of HSL activity (Fig. 2). Conversely, LXR was downregulated in
267 the 66VO and 66VO/FO groups.

268
269 *3.6 Effect of incubation with TNF α , LA, c9,t11-CLA and t10,c12-CLA on LPL, HSL and LXR*
270 *gene expression in isolated adipocytes from animals fed with FO, 66VO and 66VO/FO diets.*

271 The LPL gene expression in the adipocytes from FO group was upregulated by LA and t10,c12-
272 CLA (Fig. 4D). In the 66VO and 66VO/FO groups, the LPL gene expression was not regulated
273 by any treatment (Fig. 4E, F). In contrast, HSL showed a slight upregulation with LA and
274 t10,c12-CLA in the FO group (Fig. 4D), but these tendencies were not significant. However, the
275 66VO/FO group showed upregulation of HSL by LA (Fig. 4F). Neither TNF α nor fatty acids
276 changes HSL mRNA levels in the 66VO adipocytes (Fig. 4E). The LXR gene expression
277 showed downregulation and upregulation by LA in the FO and 66VO/FO groups respectively
278 (Fig. 4D, F).

279 The differences in gene expression levels between the three genes were also observed in each

280 experimental group (Fig. 4D, E, F). Data of gene expression are expressed in relation to the gene
281 with lowest level of expression. The results show that the differences of expression between
282 LPL and HSL are reduced in the 66VO and 66VO/FO because the HSL is expressed at higher
283 levels in these groups (Fig. 4 A) but the LPL expression is not modulated by diet (Fig. 4C).
284 Differences are also observed in the LXR expression profile regarding to the other genes (Fig.
285 4D, E, F).

286 The effect of the treatment in the different diets was also analysed (Fig. 4G). The upregulation
287 of LPL expression by LA observed in the FO group was also maintained when this effect is
288 compared between diets. However the TNF α and c9,t11-CLA did not show significant
289 differences in LPL expression in each experimental group (in comparison with controls). When
290 the effects of these treatments are compared between diets, both showed higher induction of
291 LPL in the FO group than in the 66VO and 66VO/FO groups. The effect of t10,c12-CLA
292 treatment on LPL expression showed differences between FO and 66VO/FO. These results
293 demonstrate that the LPL expression was more affected by the treatments in the control group
294 (FO) than in 66VO and 66VO/FO groups.

295 The HSL expression analyses after the treatments did not show differences between the dietary
296 groups (Fig.4G).

297 The LXR expression analyses showed differences after the LA treatment between the FO and
298 66VO/FO groups as previously observed in comparison with their controls in both groups (Fig.
299 4D, F).

300

301 *3.7 Basal levels of glucose uptake and effects of insulin and TNF α on glucose uptake of isolated*
302 *adipocytes from gilthead sea bream fed with FO, 66VO and 66VO/FO diets.*

303 The basal levels of glucose uptake did not show differences between dietary groups (Fig. 5A).
304 However, after the treatment with insulin only the FO group adipocytes revealed an
305 upregulation of glucose uptake (Fig. 5B, C and D). TNF α did not regulate glucose uptake in
306 adipocytes from any of the three experimental groups.

307

308 **4. DISCUSSION**

309

310 In the present study, although the fish showed good growth performance (Benedito-Palos et al.,
311 2009), the HSI of the animals fed the 66VO diet was greater than that of the FO group, which
312 agrees with the fat accumulation previously observed (Benedito-Palos et al., 2008). This finding
313 indicates that the animals could present a certain degree of lipid metabolism dysregulation and
314 we focus on the morphological, enzymatic activity and gene expression changes of adipose

315 tissue produced by FO replacement in diets with already 75% plant protein together with
316 possible recovery through FO refeeding. Analysing these alterations may make it possible to
317 understand the different lipid metabolism changes produced in other tissues.

318 Gilthead sea bream fed the 66VO diet showed a ~67% increase in HSL activity compared to the
319 FO group. The group with the finishing diet (66VO/FO) also had high levels of HSL activity
320 (~41% increase with regard to the FO group) but slightly lower than the 66VO group. Therefore,
321 the adipose tissue from the 66VO and 66VO/FO groups seems to have more lipolytic activity
322 than that from the FO group. Previous studies of gilthead sea bream have shown that plant
323 components increase basal lipolysis levels in adipocytes (Albalat et al., 2005a) as well as it has
324 been observed in the measurements of the basal lipolysis levels in isolated adipocytes from
325 animals fed vegetable oils (66VO and 66VO/FO groups). This is in agreement with the present
326 results showing enhanced HSL activity. Furthermore, HSI and liver weight values were higher
327 in the 66VO and 66VO/FO groups, suggesting that the fatty acids released from adipose tissue
328 could produce lipid accumulation in the liver, as previously suggested (Benedito-Palos et al.,
329 2008), as has been described in humans (Browning and Horton, 2004). Hepatic triglyceride
330 accumulation is a consequence of human obesity and the derived insulin resistance, which
331 produces a release of fatty acids coming from the increased adipose mass and HSL activity. In
332 turn, this may lead to lipotoxicity in the liver (Browning and Horton, 2004; Lewis et al., 2002).
333 Moreover, liver lipid deposition with vegetable diets has also been related to the increase of
334 lipogenesis caused by the decrease of n-3 polyunsaturated fatty acids (PUFA) (Arzel et al.,
335 1994; Robaina et al., 1998) or alterations of β -oxidation (Izquierdo et al., 2003). However,
336 previous studies of gilthead sea bream showed that the inclusion of VO in the diet did not
337 modify hepatic lipogenic activity (Bouraoui et al., *In Press*). Therefore, these results seem to
338 suggest that the liver lipids come from the adipose tissue.

339 Fat accumulation in the adipose tissue of gilthead sea bream is regulated seasonally, with a
340 tendency for fat deposits to increase during spring, favoured by the upregulation of LPL (Saera-
341 Vila et al., 2005). Subsequent high levels of TNF α expression appear to act as a limiting factor
342 of adiposity. In mammals, it has been reported that TNF α production is linked to adipocyte size
343 which thus limits lipid accumulation (Skurk et al., 2007). An increase in adipokine secretion
344 (e.g., TNF α , IL-6 and IL-8), low cell sensitivity, different patterns of protein expression,
345 accumulation of fatty acids in non-adipose tissue and insulin resistance have all been described
346 as related to adipocyte enlargement in mammals (Blüher et al., 2004; Östman et al., 1975; Skurk
347 et al., 2007). In gilthead sea bream, FO replacement resulted in an increase in adipose cell size,
348 with a partial recovery in the 66VO/FO group. However, the MFI and total mesenteric adipose
349 tissue did not show any differences between groups. Therefore, the adipose tissue undergoes

350 morphological changes through the enlargement of the cells with the VO diet. The increased cell
351 size in the 66VO and 66VO/FO groups could be related to adipocyte metabolic changes. In
352 mammals, a positive correlation between cell size and increased lipolysis rates, and HSL activity
353 and expression has been demonstrated (Berger and Barnard, 1999; Farnier et al., 2003; Gregor
354 and Hotamisligil, 2007). In keeping with this, high HSL activity and mRNA expression was
355 found in the 66VO and 66VO/FO groups, which also had larger cells. However, LPL
356 transcription levels did not show any differences between the groups, in agreement with
357 previous studies of rat adipocytes, in which the increase in adipocyte size induced LPL activity
358 but not its expression (Farnier et al., 2003).

359 The enlargement of adipocytes and increased lipolysis reflect a defective storage capacity in
360 adipose tissue deposits, as occurs in humans during situations of lipodystrophy or obesity,
361 although such deficiency develops differently (Slawik and Vidal-Puig, 2006). Furthermore,
362 hypertrophy of adipocytes is considered a marker of failure in the mechanism of preadipocyte
363 recruitment to develop new (and smaller) adipocytes (Medina-Gomez and Vidal-Puig, 2005).
364 The capacity of adipose tissue to expand through hyperplastic changes is critical for
365 accommodating changes in the availability of energy and type of diet. Studies *in vitro* of salmon
366 adipocytes indicate that incubation with different fatty acids induces different degrees of lipid
367 filling as cellular triglycerides, supporting our conclusion that the composition of dietary fatty
368 acids can affect adipocyte enlargement (Todorčević et al., 2008).

369 However, LXR gene expression showed downregulation with the incorporation of VO into the
370 diet and the FO refeed did not lead to a recovery of LXR gene expression. LXR is a
371 transcription factor recently described in gilthead sea bream and salmonids (Cruz-Garcia et al.,
372 2009a; Cruz-Garcia et al., 2009b) and it is known in mammals to be involved in cholesterol,
373 fatty acid and glucose metabolism (Mitro et al., 2007; Repa et al., 2000; Schultz et al., 2000;
374 Steffensen and Gustafsson, 2004). In salmonids the LXR transcription is downregulated in the
375 liver by VO diets (Cruz-Garcia et al., 2009a) as it is in gilthead sea bream adipocytes from the
376 66VO group. LXR is a cholesterol sensor (Steffensen and Gustafsson, 2004) and can be
377 regulated by fatty acids (Pawar et al., 2002). In this way, the higher proportion of n-6 fatty acids
378 and less n-3 PUFA, the presence of phytosterol or, most probably, the small proportion of
379 cholesterol in the 66VO diet, could be some of the causes of the LXR downregulation.

380 Nevertheless, from our results we cannot elucidate which is the precise cause of the changes in
381 LXR expression. In mammals, the increase in adipocyte size produces an inhibition in
382 cholesterol-sensing (de Ferranti and Mozaffarian, 2008), so this could also be one of the factors
383 involved in the downregulation of LXR in the 66VO and 66VO/FO groups.

384 The enlargement of adipocytes decreases the sensitivity of mammalian cells to insulin action

385 and lipolytic stimulators such as isoproterenol, and makes the cells less dynamic in their
386 responses (Frayn et al., 2007; Jolly et al., 1978; Östman et al., 1975; Smith, 1971). This lack of
387 response associated with the cell morphology seems to be reflected in the incubations with fatty
388 acids of isolated adipocytes from gilthead sea bream. CLAs, omega-6 fatty acids and isomers of
389 linoleic acid, have been widely studied in mammals (Campbell and Kreider, 2008; Churrucá et
390 al., 2009; Evans et al., 2002). The t10,c12-CLA prevents the development of adiposity, reduces
391 the triglyceride content in adipocytes and increases lipolysis (Chung et al., 2005). This last
392 effect of t10,c12-CLA, upregulating lipolysis, is reflected in the gilthead sea bream adipocytes
393 from the FO group. However c9,t11-CLA did not modulate the basal lipolysis in the adipocytes
394 from any of the three diet groups as it has been demonstrated to do in mammals (Chung et al.,
395 2005). The FO group showed an induction of lipolysis with LA and t10,c12-CLA, but the 66VO
396 group did not, while the 66VO/FO group responded to LA, but not to t10,c12-CLA. The lower
397 response to treatments of the 66VO group adipocytes is also observed in the gene expression of
398 LPL, HSL and LXR after fatty acid incubations. This different response to the fatty acids in the
399 66VO adipocytes could be related to the different dietary fatty acid composition. It has been
400 observed in mammals that the variation of the fatty acid composition in the diet produce changes
401 in the fatty acid profile of the tissue affecting the adipocyte metabolism. The changes in the
402 adipocyte metabolism can affect the efficiency of substrate accumulation (Su and Jones, 1993),
403 the permeability of the cell membrane, the ability to recognize extracellular molecules like
404 hormones (Hagve, 1988) and the hyperplasia of the tissue (Launy et al., 1972; Soriguier Escofet
405 et al., 1996). Therefore, this lack of response to the treatments observed in the lipolytic levels
406 and gene expression in adipocytes from animals fed with the VO diet might be associated to the
407 changes in the fatty acid profile of the tissue.

408 The lipolytic response to TNF α in gilthead sea bream has been demonstrated to show
409 differences linked in part to the degree of fish adiposity (Cruz-García et al., 2009b). The relation
410 between TNF α and adipose tissue has been linked to adipocyte size in mammals, and TNF α has
411 been considered a limiting regulator of adipocyte hypertrophy in fish, as in mammals (Saera-
412 Vila et al., 2007; Skurk et al., 2007). Therefore, it is not surprising that the 66VO group
413 adipocytes, with a larger size than those of the other groups, when incubated with TNF α
414 increased their lipolysis level to prevent further hypertrophy, whereas the FO and 66VO/FO
415 group adipocytes did not.

416 At the same time, the hormonal regulation of glucose uptake showed an upregulation by insulin
417 in the FO group but the 66VO and 66VO/FO groups showed no response. In mammals, a lack of
418 response to insulin and consequently a reduction in glucose uptake has been correlated with fat
419 cell size, as observed in the characteristic disorder of insulin resistance defined in mammals (de

420 Ferranti and Mozaffarian, 2008; Maffeis et al., 2007).
421 In conclusion, in the present study we demonstrate that FO replacement changes the lipid
422 metabolism through altering the adipocyte cell size, lipolytic rates and response to fatty acids
423 and hormones. These effects can be moderately reversed by means of a final finishing diet with
424 a FO diet. These metabolic changes can potentially affect other organs such as the liver.
425 However, the total adipose tissue content was not modified by FO replacement. Therefore, the
426 same content of adipose tissue between the different groups jointly with an increase of the cell
427 size in animals fed vegetable oils suggest that the vegetable oils might be affect the hyperplasia
428 of the tissue and consequently producing a hypertrophy of the cells and an adipose tissue
429 metabolism alteration. The lack or reduction in the hyperplasia of the tissue has been associated
430 to changes in the dietary fatty acid profile in humans (Garaulet et al., 2006). Therefore, the
431 different fatty acids profile present in the vegetable oils could be the main factor responsible of
432 the alterations observed in the adipose tissue in the present study. Further study of the effects of
433 dietary FO replacement on adipose tissue could help clarify how this tissue, besides acting as a
434 fat reservoir, contributes to maintaining energy balance and metabolic health.

435

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441

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594

595 **Figure legends**

596

597 **Figure 1. Adipocyte cell size distribution of gilthead sea bream fed with FO, 66VO and**

598 **66VO/FO diets.** Data are shown as mean \pm S.E.M. (n=3). Results were analysed by one-way
599 analysis of variance (ANOVA), followed by Tukey's test. Values that do not share a common
600 letter are significantly different ($P<0.05$).

601
602 **Figure 2. Hormone-sensitive lipase (HSL) activity in adipose tissue of gilthead sea bream**
603 **fed with FO, 66VO and 66VO/FO diets.** Data are shown as mean \pm S.E.M. (n=6). Results
604 were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values
605 that do not share a common letter are significantly different ($P<0.05$).

606
607 **Figure 3. Basal lipolysis levels and effects of incubation with TNF α , linoleic acid (LA), *cis*-9**
608 ***trans*-11 conjugated linoleic acid (c9,t11-CLA) and *trans*-10 *cis*-12 conjugated linoleic acid**
609 **(t10,c12-CLA) on lipolysis of isolated adipocytes of gilthead sea bream fed with FO, 66VO**
610 **and 66VO/FO diets.** Comparison of the basal lipolysis levels from the adipocytes of FO, 66VO
611 and 66VO/FO groups (A). The isolated adipocytes from each experimental group were also
612 incubated with the different treatments and the lipolysis as glycerol released into the medium
613 was measured; FO (B), 66VO (C) and 66VO/FO (D) groups. The treatment vehicle, ethanol
614 (EtOH for fatty acids), was also tested. Data are shown as mean \pm S.E.M. (n=3). Results were
615 analysed by one-way analysis of variance (ANOVA), followed by Tukey's test. Values that do
616 not share a common letter are significantly different ($P<0.05$). Comparisons between each
617 separate treatment were analysed by one-way analysis of variance (ANOVA), followed by
618 Tukey's test. The treatments (TNF α , LA, c9,t11-CLA, t10,c12-CLA) that do not share a
619 common number are significantly different from the other dietary groups ($P<0.05$).

620
621 **Figure 4. LPL, HSL and LXR gene expression profile on isolated adipocytes of gilthead sea**
622 **bream fed with FO, 66VO and 66VO/FO diets and effects of TNF α , linoleic acid (LA), *cis*-9**
623 ***trans*-11 conjugated linoleic acid (c9,t11-CLA) and *trans*-10 *cis*-12 conjugated linoleic acid**
624 **(t10,c12-CLA) on LPL, HSL and LPL expression on isolated adipocytes of gilthead sea**
625 **bream fed with FO, 66VO and 66VO/FO diets.** Gene expression of LPL (A), HSL (B) and
626 LXR (C) of gilthead sea bream fed with FO, 66VO and 66VO/FO diets. Effects of TNF α ; LA;
627 c9,t11-CLA; and t10,c12-CLA on LPL, HSL and LXR of isolated adipocytes of gilthead sea
628 bream fed with FO (D), 66VO (E) and 66VO/FO (F) diets. In the panels D, E and F, the data are
629 compared to the control levels of each gene and also between the different gene expression
630 levels of the three genes analysed. The treatment vehicle, ethanol (EtOH for fatty acids), was
631 also tested. The gene expression data are presented as relative units (RU) using β -actin as a
632 housekeeping gene (delta-delta method). Data are shown as mean \pm S.E.M. (n=3). Results for
633 each gene were analysed by one-way analysis of variance (ANOVA), followed by Tukey's test.
634 Values that do not share a common letter (capital letters for LPL, Greek letters for HSL and
635 lower case letters for LXR) are significantly different ($P<0.05$). The effect of the treatments on
636 LPL, HSL and LXR was also analysed between the three diets (G). Data are shown as mean \pm

637 S.E.M. (n=3). Data are shown as mean \pm S.E.M. (n=3). Results were analysed by one-way
638 analysis of variance (ANOVA), followed by Tukey's test. Values that do not share a common
639 letter are significantly different ($P<0.05$).

640

641 **Figure 5. Basal levels of glucose uptake (2-deoxyglucose, 2-DG) on isolated adipocytes**
642 **from FO, 66VO and 66VO/FO (A) and effect of insulin (Ins) and TNF α on the glucose**
643 **uptake; FO (B), 66VO (C) and 66VO/FO (D) groups. Data are shown as mean \pm S.E.M.**
644 **(n=3). Results were analysed by one-way analysis of variance (ANOVA), followed by Tukey's**
645 **test. Values that do not share a common letter are significantly different ($P<0.05$).**
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Table 1. Ingredients and chemical composition of experimental diets

a	Ingredient (g/Kg)	FO	66VO
	Fish meal (CP 70%)^a	15	15
	CPSP 90^b	5	5
	Corn gluten meal	40	40
	soybean meal	14.3	14.3
	Extruded wheat	4	4
	Fish oil^c	15.1	5.1
	Rapeseed oil	0	1.7
	Linseed oil	0	5.8
	Palm oil	0	2.5
	Soya lecithin	1	1
	Blinder (sodium alginate)	1	1
	Mineral premix^d	1	1
	Vitamin premix^e	1	1
	CaHPO₄.2H₂O (18%P)	2	2
	L-lysine	0.55	0.55
<i>Proximate composition</i>			
	Dry matter (DM, %)	93.4	92.77
	Crude protein (%DM)	53.2	52.62
	Crude fat (%DM)	21.09	20.99
	Ash (%DM)	6.52	6.57

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Fish meal (Scandinavian LT).

^b Fish soluble protein concentrate (Sopropêche, France).

^c Fish oil (Sopropêche, France).

^d Supplied the following (mg/kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.

^e Supplied the following (mg/kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL- α tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B12 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500.

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Table 2

Gilthead sea bream primer sequences used for real-time PCR

Gene	Accession number	Primer sequence	Position
HSL	<u>EU254478</u>	F GCT TTG CTT CAG TTT ACC ACC ATT TC	154-179
		R GAT GTA GCG ACC CTT CTG GAT GAT GTG	275-249
LPL	- <u>AY495672</u>	F GAG CAC GCA GAC AAC CAG AA	500-520
		R GGG GTA GAT GTC GAT GTC GC	672-691
LXR	- <u>FJ502320</u>	F GCA CTT CGC CTC CAG GAC AAG	476-496
		R CAG TCT TCA CAC AGC CAC ATC AGG	582-559
β-actin	- <u>X89920</u>	F TCC TGC GGA ATC CAT GAG A	811-829
		R GAC GTC GCA CTT CAT GAT GCT	861-841

701

702 **Table 3**703
704 Biometric and biochemical plasma parameters of fish sampled at the end of the feeding trial.
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	FO	66VO	66VO/FO	Values are
Body mass (g)	528 ± 14,2 ^a	552 ± 13,35 ^a	563 ± 10,83 ^a	the mean ±
Adipose tissue (g)	6.44 ± 0.32	6.71 ± 0.73	7.04 ± 0.75	S.E.M.
Liver (g)	6.27 ± 0.24 ^a	8.38 ± 0.44 ^b	7.74 ± 0.28 ^b	(n=18).
MFI (%)¹	1.23 ± 0.07	1.14 ± 0.11	1.24 ± 0.12	Differences
HSI (%)²	1.18 ± 0.03 ^a	1.4 ± 0.07 ^b	1.37 ± 0.04 ^b	between
<i>Plasma parameters</i>				groups were
Glucose (mM)	4.23±0.36	4.43±0.40	4.45±0.40	analysed by
NEFAs (mEq/L)³	0.20±0.01 ^{ab}	0.21±0.02 ^a	0.16±0.01 ^b	one-way
Triglycerides (mM)	8.85±0.56	8.86±1.16	7.85±0.50	analysis of
				variance
				(ANOVA),
				followed by
				Tukey's test.

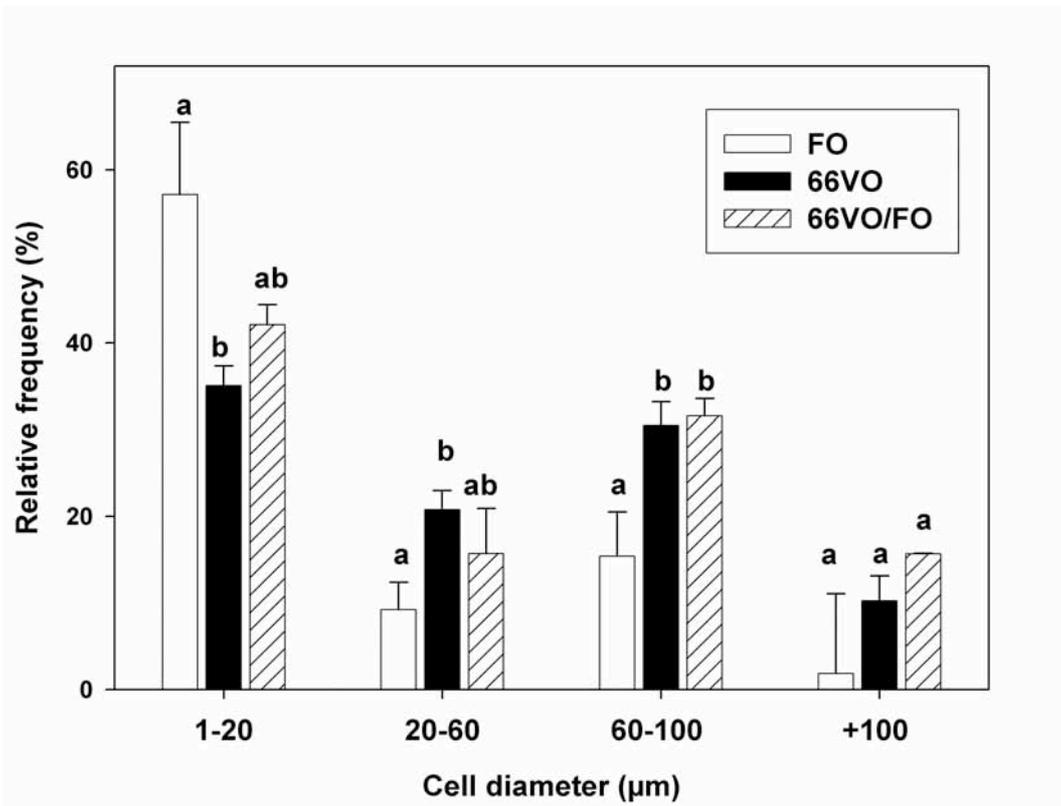
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722 Values that do not share a common letter are significantly different ($P < 0.05$)723 ¹Mesenteric fat index (MFI)=(mesenteric fat mass/fish mass) × 100.724 ²Hepatosomatic index (HSI)=(liver mass/fish mass) × 100.725 ³NEFAs: non-esterified fatty acids

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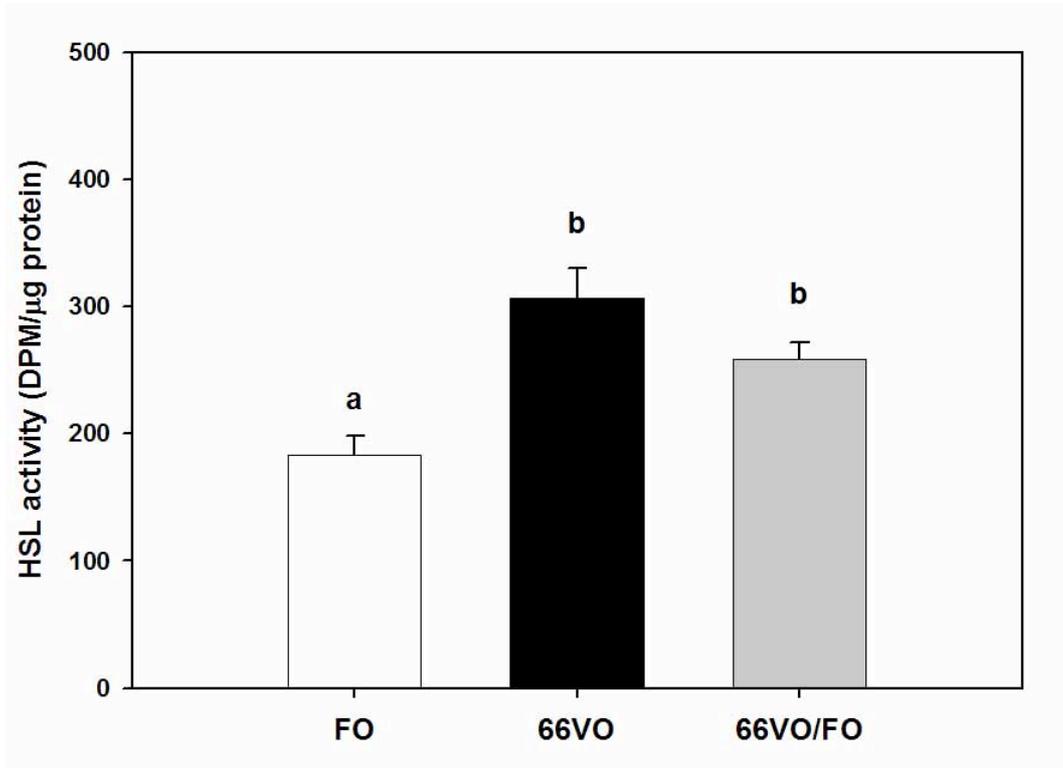
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727 Fig 1
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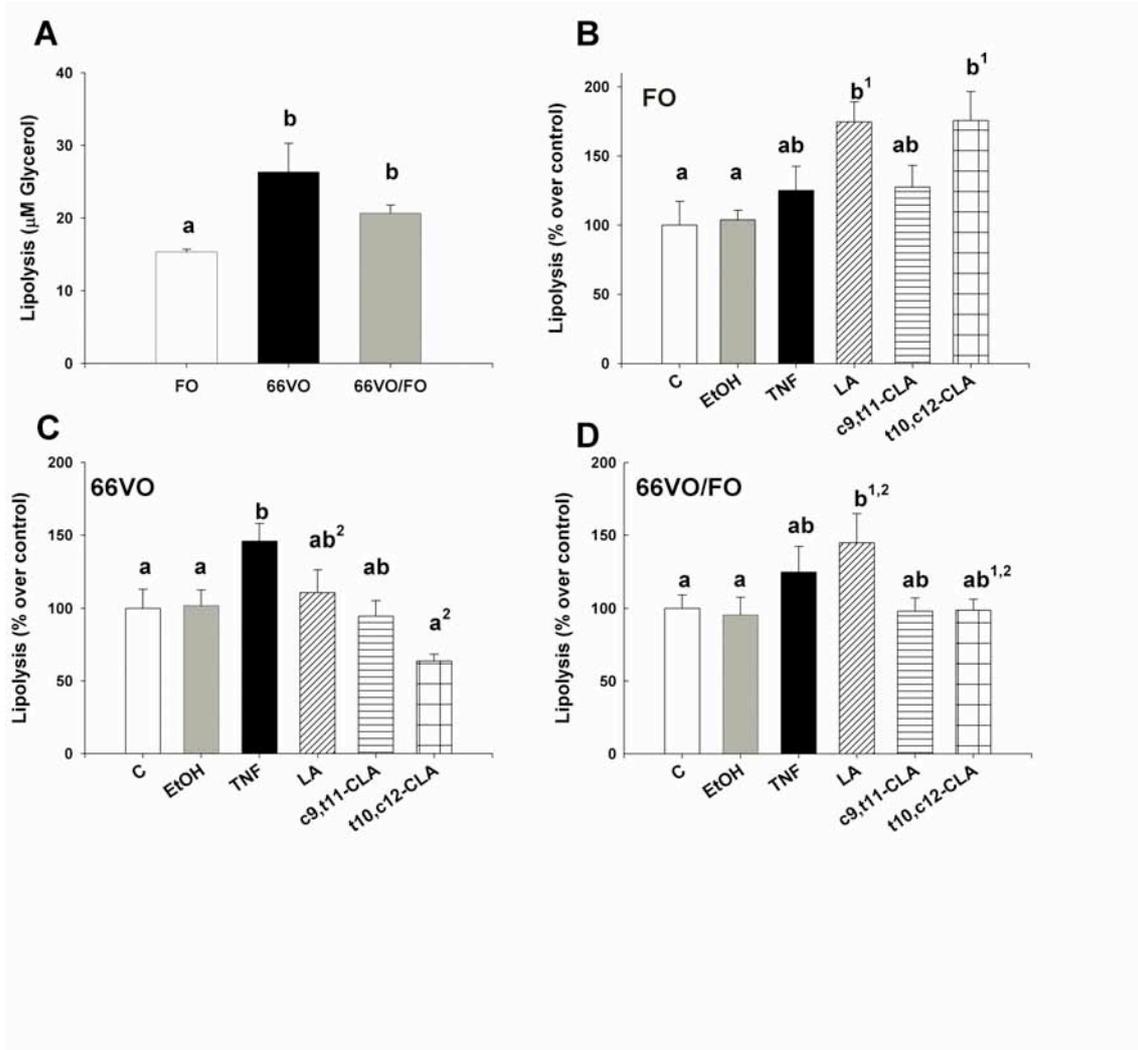


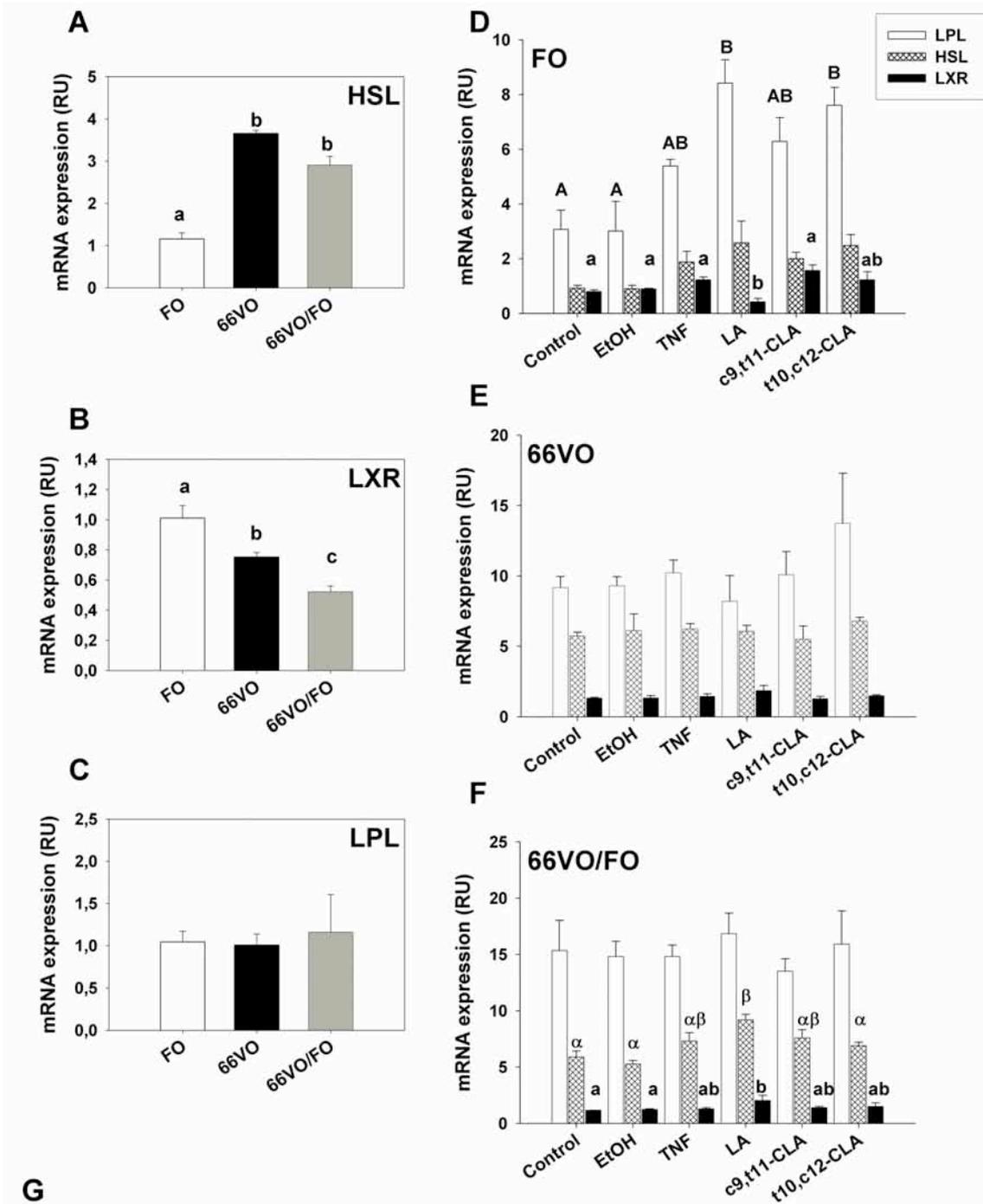
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730 **Fig 2**
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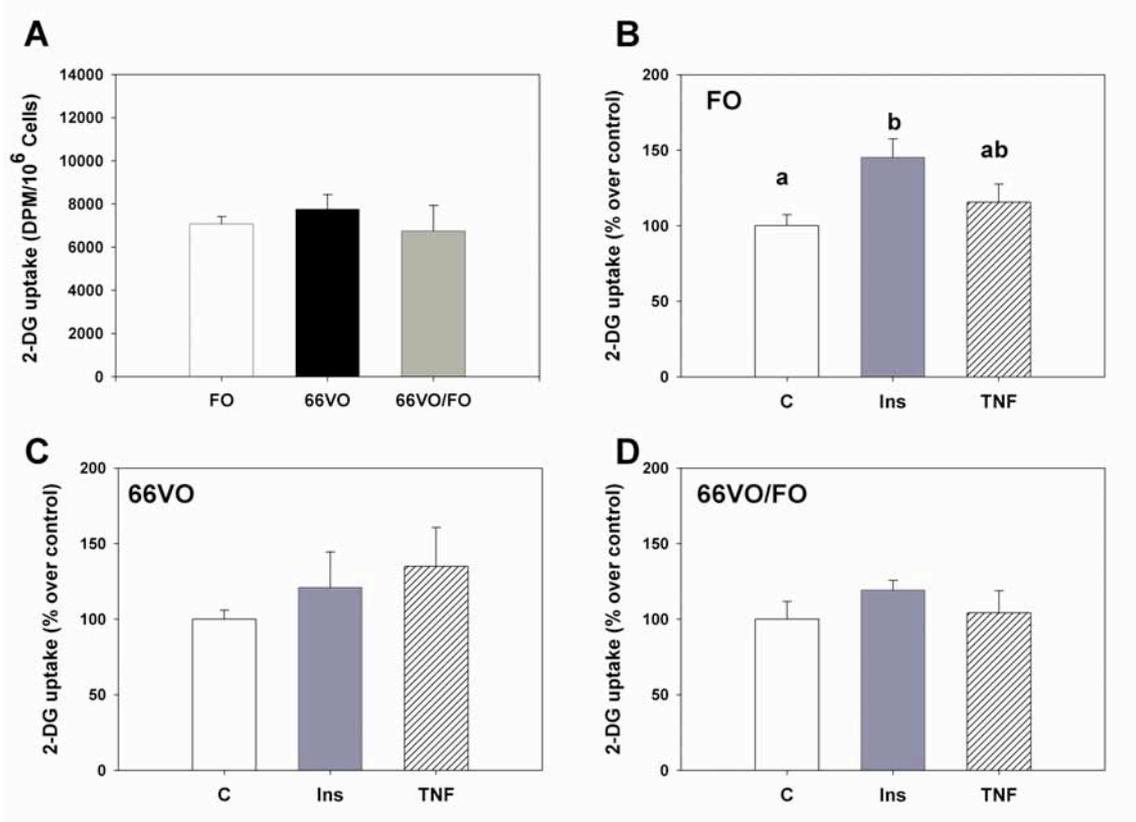




G
Treatments vs diets

	LPL			HSL			LXR		
	FO	66VO	66VO/FO	FO	66VO	66VO/FO	FO	66VO	66VO/FO
TNF α	1.78 \pm 0.29 ^a	1.15 \pm 0.09 ^{ab}	0.94 \pm 0.08 ^b	1.09 \pm 0.31	0.92 \pm 0.06	1.22 \pm 0.12	1.22 \pm 0.10	1.11 \pm 0.15	1.16 \pm 0.11
LA	2.83 \pm 0.28 ^a	0.92 \pm 0.21 ^b	0.97 \pm 0.13 ^b	1.64 \pm 0.51	0.81 \pm 0.10	1.54 \pm 0.08	0.42 \pm 0.13 ^a	1.54 \pm 0.30 ^{ab}	2.05 \pm 0.45 ^b
c9,t11-CLA	2.12 \pm 0.29 ^a	1.13 \pm 0.19 ^b	0.91 \pm 0.07 ^b	0.98 \pm 0.21	0.82 \pm 0.14	1.27 \pm 0.12	1.57 \pm 0.21	1.05 \pm 0.16	1.42 \pm 0.10
t10,c12-CLA	2.56 \pm 0.22 ^a	1.54 \pm 0.40 ^{ab}	0.98 \pm 0.13 ^b	1.58 \pm 0.25	0.93 \pm 0.09	1.15 \pm 0.05	1.23 \pm 0.29	1.23 \pm 0.06	1.51 \pm 0.32

740 Fig 5
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