

1 **Assessment of the health and antioxidant trade-off in gilthead sea bream (*Sparus aurata***
2 **L.) fed alternative diets with low levels of contaminants**

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24 **ABSTRACT**

25 The aim of the present work was to analyze the effect of partial and total replacement of fish
26 oil (FO) by a blend of vegetable oils on the health and antioxidant status of gilthead sea
27 bream (*Sparus aurata* L.) fed primarily plant-protein based diets. The study included
28 measurements of feed-borne contaminants, gene expression analyses of detoxifying and
29 antioxidant pathways and measures of antioxidant and innate immune descriptors.
30 Polybrominated diphenyl ethers (PBDEs) were almost undetectable in all diets, and the
31 loading-charges of polychlorinated biphenyls (PCBs), dioxin-like PCBs, organochlorine
32 pesticides (OCs), and polycyclic aromatic hydrocarbons (PAHs) were at trace levels
33 decreasing their concentrations according to the level of FO replacement with vegetable oils
34 (0%, 33%, 66%, 100%). Hepatic detoxifying pathways were down regulated by FO
35 replacement, and the hepatic transcription of cytochrome P450 1A1 and aryl hydrocarbon
36 receptor 1 was significantly reduced in fish fed the 100% vegetal oil diet. Dietary
37 intervention did not alter the hepatic expression of the recycling glutathione reductase,
38 whereas glutathione peroxidase-1 and phospholipid glutathione peroxidase were either down
39 or up-regulated by the total FO replacement. This suggests that vegetable oils prime the *in*
40 *situ* repair of peroxidized phospholipids rather than the increased turnover of membrane
41 phospholipids from the undamaged pool of cytosolic free fatty acids. The hepatic expression
42 of non-enzymatic antioxidants (metallothionein, glucose regulated protein 75) was down
43 regulated in fish fed 66% and 100% vegetable oil diets. Hepatic glutathione levels and total
44 plasma antioxidant capacity were also lowest in fish fed high levels of vegetable oils, but the
45 concurrent increase in the GSH/GSSG ratio was interpreted as an index of reduced oxidative
46 stress. This redox balance agrees with the enhanced respiratory burst of blood leucocytes
47 after PMA stimulation in fish feed the 100% vegetable oil. Total plasma peroxidases and
48 plasma alternative complement pathway were not affected by dietary treatment, whereas
49 plasma lysozyme was significantly decreased in fish fed the 66% vegetable oil diet. Taken
50 together, the results suggest that the health and the antioxidant status of gilthead sea bream
51 was not damaged by high levels of FO replacement in eco-friendly diets, but both the
52 scavenging and production of reactive oxygen species were modulated in concert by complex
53 and nutritionally-mediated readjustments.

54 **Key words:** fish oil, vegetal oil, plant proteins, aryl hydrocarbon receptors, cytochrome
55 P450 1A1, glucose regulated protein 75, metallothionein, glutathione, alternative
56 complement pathway, lysozyme, peroxidases, ROS production.

57

58 1. Introduction

59

60 The increasing demand of fish oil (FO) to meet the expanding aquaculture industry,
61 together with the opposing trend of fisheries and the increasing use of FO in nutraceutical
62 and agricultural industries, has lead to the search for alternative sources of dietary lipids in
63 fish feeds (Miller et al., 2008). Different vegetable oils at different levels of inclusion have
64 been tested with variable results in freshwater and marine fish (Bell and Waagbo, 2008;
65 Webster et al., 2007). Indeed, vegetable oils are rich in C₁₈ polyunsaturated fatty acids
66 (PUFA), but they are lacking in n-3 long-chain polyunsaturated fatty acids (LC-PUFAs).
67 This means that fish feeding on vegetable oils would have to desaturate and elongate C₁₈
68 PUFAs to their LC-PUFA derivates. However, all marine fish so far studied, including
69 gilthead sea bream, appear to have lost the ability to make such conversion (Mourente and
70 Tocher, 1994; Seiliez et al., 2003; Zheng et al., 2004), and therefore they have absolute
71 dietary requirement for C₂₀ and C₂₂ PUFAs. On the other hand, marine derived products are
72 also the main source of environmental pollutants, even in human dietary supplements
73 (Storelli et al., 2004). Furthermore, the high levels found in some farmed fish have led to
74 reconsider the possible beneficial properties of fish consumption in some population groups
75 (Foran et al., 2005; Hamilton et al., 2005). Thus, efforts to reduce this contaminant load have
76 also been directed towards the use of alternative vegetable oils in fish feeds (Bethune et al.,
77 2006) and even to engineering oil seeds to produce n-3 LC-PUFA (Damude and Kinney,
78 2008).

79 The anti-inflammatory effect of n-3 LC-PUFAs has been extensively documented,
80 and supplementation of domestic foods with marine FO is becoming an accepted practice to
81 improve the nutritional quality of most animal products (e.g., meat, milk, eggs). However,
82 LC-PUFAs are extremely vulnerable to oxidation and dietary antioxidants (e.g., vitamin C,
83 vitamin E, polyphenols, carotenoids, biologically active peptides) help to counteract the

84 negative effects of lipid peroxidation, having beneficial effects on growth, fertility,
85 immunocompetence, ageing and pollutant susceptibility (Catoni et al., 2008; Erdmann et al.,
86 2008; Fang et al., 2002). In fish, most studies dealing with antioxidant systems have focused
87 on vitamin E, carotenoids and some minerals (Martínez-Alvarez et al., 2005; Mourente et al.,
88 2007a), but now there is also evidence for the antioxidant properties of plant protein
89 ingredients in practical diets for gilthead sea bream (Sitjà-Bobadilla et al., 2005), a highly
90 valued fish for the Mediterranean aquaculture. Also, we have earlier shown that both fish
91 meal and FO can be replaced up to 65-75% without growth retardation and signs of histo-
92 pathological damage (Benedito-Palos et al., 2007; 2008). The goal of the present study is to
93 gain more understanding about the risk and benefits of these eco-friendly diets, in terms of
94 the health and anti-oxidant status of the fish. For this issue, plant protein-based diets with a
95 partial or total replacement of FO with vegetable oils were formulated, and the loading-
96 charges of the most common persistent organic pollutants (POPs) were firstly monitored. The
97 transcriptional and nutritionally-mediated effects on detoxifying and antioxidant defence
98 systems were assessed by hepatic mRNA measurements of aryl hydrocarbon receptors
99 (AhR1 and AhR2), cytochrome P450 1A1 (CYP1A; EC 1.14.14.1), metallothionein (MT),
100 glucose regulated protein 75 (GRP75), glutathione reductase (GR; EC 1.8.1.7), glutathione
101 peroxidase (GPx-1; EC 1.11.1.9) and phospholipid glutathione peroxidase (PHGPx; EC
102 1.11.1.12). Hepatic glutathione levels and total plasma antioxidant capacity were monitored
103 as antioxidant indexes. Lastly, immunological and pro-inflammatory status was assessed
104 through the alternative complement pathway, leucocyte production of reactive oxygen
105 species (ROS), and plasma measures of lysozyme and total peroxidase activities.
106

107 2. Materials and methods

108

109 2.1 Experimental setup

110

111 Animals and samples were the same as those described in a previous study (Benedito-
112 Palos et al., 2008). Briefly, juvenile gilthead sea bream (*Sparus aurata* L.) of 16 g initial
113 mean body weight were distributed into 12 fibreglass tanks (500 l) in groups of 60 fish per
114 tank at the research experimental facilities of IATS (Castellón, Spain). Each triplicate group
115 received from May 23rd to September 19th one of the four experimental diets nominally
116 CTRL, 33VO, 66VO and VO (Table 1). Added oil was either Scandinavian FO (CTRL diet)
117 or a blend of vegetable oils, replacing the 33% (33VO diet), 66% (66VO diet) and 100% (VO
118 diet) of FO. All diets were manufactured using a twin-screw extruder at the INRA
119 experimental research station of Donzaq (Landes, France), dried under hot air, sealed and
120 kept in air-tight bags until use.

121 Fish were reared under natural day-length and water temperature following the
122 natural changes at IATS latitude (40° 5'N; 0° 10'E). Water flow was 20 l/min and feed was
123 offered by hand to apparent visual satiety twice a day (9.00 h-14.00 h). Each 3-4 weeks, fish
124 were counted and group-weighed under moderate anaesthesia (3-aminobenzoic acid ethyl
125 ester, MS 222; 100 µg/ml). There was no reduction in growth or feed efficiency (wet weight
126 gain/dry feed intake = 1.06-1.02) with the partial replacement of FO (183-186 g final mean
127 body weight for fish fed CTRL 33VO, 66VO diets). A decrease in feed intake and weight
128 gain of about 10% was found with the total FO replacement (VO diet).

129 At the end of the feeding trial, randomly selected fish (4 fish per tank; 12 fish per
130 treatment) were killed by a blow on the head prior to blood and tissue sampling. Blood was
131 taken with heparinised syringes from caudal vessels, and kept on ice. One aliquot was

132 immediately used to measure respiratory burst activity of circulating leucocytes. The
133 remaining blood was centrifuged at 3000 g for 20 min at 4 °C, and plasma aliquots were
134 stored at -80 °C until use. Liver was extracted and rapidly excised, frozen in liquid nitrogen,
135 and stored at -80 °C until analyses.

136

137 *2.2 Contaminant analyses*

138

139 Organochlorine pesticides (OCPs), polychlorinated biphenyls (PCBs), dioxin-like
140 PCBs (DL-PCBs) and polybrominated diphenyl ethers (PBDEs) were analyzed in fish feeds
141 as described elsewhere (Serrano et al., 2003a). Briefly, feed-borne contaminants were
142 extracted by refluxing ca. 8 g during 4 h. Clean-up was performed by means of sulphuric acid
143 digestion prior to normal phase liquid chromatography (NPLC). Identification and
144 quantification of PCBs, DL-PCBs and selected OCPs were performed using a gas
145 chromatograph (GC, Varian CP-3800) coupled to a Varian Saturn 4000 ion trap mass
146 spectrometry detector (system operated in MS/MS mode). Instrumental determination of
147 PBDEs was carried out by means of a GC system (Agilent 6890N, Palo Alto, USA),
148 equipped with an autosampler (Agilent 7683) coupled to a triple quadrupole (QqQ) mass
149 spectrometer (Quattro Micro GC; Micromass, Boston, USA) operating in CI mode.

150 For polycyclic aromatic hydrocarbons (PAHs) analysis, a first saponification step was
151 carried out. PAH analytes were then extracted twice with 8 ml of n-hexane and concentrated
152 under gentle nitrogen stream at 40 °C to 1 ml. The resultant extract was purified in Florisil
153 SPE cartridge. The final extract free of interference compounds was analyzed by means of
154 the Quattro Micro GC system working in EI (MS/MS). The analytical method offered
155 satisfactory results in linearity (0.5-90 µg/ml), accuracy (recoveries between 70-120 %, n=6,

156 at 1, 10 and 20 ng/g levels), precision (RSD < 30%) and selectivity (using two transitions
157 from tandem mass spectrometry).

158 In all assays, isotopically labelled standards were added before extraction as
159 surrogates for quality control. Quantification was performed using the internal standard
160 method with external calibration curves.

161

162 *2.3 RNA extraction and RT procedure*

163

164 Total RNA extraction was performed with the ABI PRISM™ 6100 Nucleic Acid
165 PrepStation (Applied Biosystems, Foster City, CA, USA). Briefly, liver tissue was
166 homogenized at a ratio of 25 mg/ml with a guanidine-detergent lysis reagent. The reaction
167 mixture was treated with protease K, and RNA purification was achieved by passing the
168 tissue lysate (0.5 ml) through a purification tray containing an application-specific
169 membrane. Wash solutions containing DNase were applied, and total RNA was eluted into a
170 96-well PCR plate. The RNA yield was 40-50 µg with absorbance measures ($A_{260/280}$) of 1.9-
171 2.1.

172 Reverse transcription (RT) with random decamers was performed with the High-
173 Capacity cDNA Archive Kit (Applied Biosystems). For this purpose, 500 ng total RNA were
174 reverse transcribed in a final volume of 100 µl. RT reactions were incubated 10 min at 25 °C
175 and 2 h at 37 °C. Control reactions were run without reverse transcriptase and were used as
176 negative real-time PCR controls.

177

178

179 *2.4 Gene expression profile*

180

181 The abundance of hepatic transcript levels was analyzed by way of real-time PCR
182 assays, using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA) as
183 previously described (Calduch-Giner et al., 2003). Briefly, diluted RT reactions were
184 conveniently used for PCR reactions in 25- μ l volume. Each PCR-well contained a SYBR
185 Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.3-0.9 μ M to
186 obtain amplicons of 51-150 bp in length (Table 2). β -actin was used as housekeeping gene,
187 and the efficiency of PCR reactions for target and the reference gene varied between 95%
188 and 98%, respectively. The dynamic range of standard curves (serial dilutions of RT-PCR
189 reactions) spanned five orders of magnitude, and the amount of product in a particular
190 sample was determined by interpolation of the cycle threshold (Ct) value. The specificity of
191 reaction was verified by analysis of melting curves and by electrophoresis and sequencing of
192 PCR amplified products. Reactions were performed in triplicate and the fluorescence data
193 acquired during the extension phase were normalized to β -actin by the delta-delta method
194 (Livak and Schmittgen, 2001). No changes in β -actin expression were found in response to
195 dietary intervention.

196

197 *2.5 Glutathione determinations*

198

199 Frozen liver samples were homogenised with 5 volumes of ice-cold buffer [200 mM
200 2-(N-morpholino) ethanesulphonic acid, 50 mM phosphate, 1 mM EDTA, pH 6], and
201 centrifuged for 15 min at 20 000 g and 4°C. Supernatants were deproteinized with
202 metaphosphoric acid, and oxidized (GSSG) and total (tGSx) levels of glutathione were
203 determined enzymatically with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA)

204 based on the recycling reaction of reduced glutathione (GSH) with DNTB (5,5'-dithio-2-
205 nitrobenzoic acid) in the presence of an excess of GR. Measurements were made in a
206 microplate reader, and the GSH/GSSG ratio was calculated as the quotient of reduced GSH
207 equivalents.

208

209 *2.6 Antioxidant capacity*

210

211 Total antioxidant capacity in plasma samples was measured with a commercial kit
212 (Cayman Chemical, Ann Arbor, MI, USA) adapted to 96-well microplates. The assay relies
213 on the ability of antioxidants in the sample to inhibit the oxidation of ABTS [2,2'-azino-di-(3-
214 ethylbenzthiazoline sulphonate)] to ABTS radical cation by metamyoglobin, a derivatized
215 form of myoglobin. The capacity of the sample to prevent ABTS oxidation is compared with
216 that of Trolox (water-soluble tocopherol analogue), and is quantified as millimolar Trolox
217 equivalents.

218

219 *2.7 Leucocyte ROS production*

220

221 Induction of the respiratory burst (RB) activity in blood leucocytes was measured
222 directly from heparinised blood, following the method described by Nikoskelainen et al.
223 (2005). Briefly, 100 µl of diluted blood (1:25) in HBSS (Hanks balanced salt solution, pH
224 7.4) were dispensed in white flat-bottomed 96-wells, and incubated with 100 µl of a freshly
225 prepared luminol suspension (2mM luminol in 0.2 M borate buffer pH 9.0, with 2 µg/ml
226 phorbol myristate acetate (PMA)) for 1 h at 24-25 °C. Luminol-amplified chemiluminescence
227 was measured every 3 min with a plate luminescence reader for generation of kinetic curves.

228 Each sample was run by duplicate and read against a blank in which no blood was added.
229 The integral luminescence in relative light units (RLU) was calculated.

230 Total plasma peroxidases (PO), which include myeloperoxidase, were chosen as a
231 measure of the oxidizing capacity of the plasma, because of their involvement in the
232 production of ROS (Spickett et al., 2000). They were measured following the procedure
233 described in Sitjà-Bobadilla et al. (2005). Briefly, 15 μ l of plasma were mixed in flat-
234 bottomed well plates with 135 μ l of HBSS-plus (HBSS, without Ca^{2+} and Mg^{2+} , 0.1% NaCl
235 and antimycotic/antibiotic mixture) and 50 μ l of 3,3',5,5'-tetramethylbenzidine
236 hydrochloride (TMB). After 2 min of incubation, the reaction was stopped with 25 μ l of 1N
237 H_2SO_4 , and the optical density was read at 450 nm. Wells in which no plasma was added
238 were run as blanks.

239

240 *2.8 Lysozyme and alternative complement pathway*

241

242 The lysis by the alternative complement pathway (ACP) was determined as in Sitjà-
243 Bobadilla et al. (2005), with some modifications. Briefly, sheep red blood cells (SRBC)
244 obtained from sheep defibrinated blood (Durviz, Valencia, Spain) were used as targets at a
245 final concentration of 2.85×10^8 cells/ml. Triplicates of tested plasma (100 μ l), diluted in
246 HBSS-EGTA (Hank's Balanced Salt Solution, plus 10 mM Mg^{2+} and 10 mM ethylene
247 glycolbistetra-acetate, pH 7.6), were mixed with 25 μ l of SRBC in 96-well plates.
248 Microplates were then incubated for 100 min at 20°C with constant shaking and centrifuged
249 to spin down the remaining SRBC. The absorbance of the supernatant was read at 415 nm.
250 The dilution corresponding to 50% haemolysis was expressed as ACH_{50} .

251 Plasma lysozyme was measured by a turbidimetric assay adapted to 96-well
252 microplates, as previously described (Sitjà-Bobadilla et al., 2005). Briefly, lyophilized

253 *Micrococcus lysodeikticus* (0.3 mg/ml) (Sigma) in 50 mM sodium phosphate buffer at pH 6.2
254 was used as a substrate for the plasma lysozyme. Triplicates of test plasma (diluted 1:2, 10
255 μ l) were added to 200 μ l of the bacterial suspension, and the reduction in absorbance at 450
256 nm was measured after 0.5 and 4.5 min. A unit of lysozyme activity was defined as the
257 amount of enzyme that caused a decrease in absorbance of 0.001 per min.

258

259 *2.9 Statistical analysis*

260

261 One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test
262 was used to compare means of the four different groups. The significance level was set at $P <$
263 0.05. All statistical analyses were performed using Sigma Stat software (SPSS Inc., IL).

264

265

266 **3. Results**

267

268 *3.1 Feed-borne contaminants*

269

270 Although the charge of POPs in all experimental feeds was in the range of trace
271 levels, FO replacement with vegetable oils resulted in a significant and progressive reduction
272 of feed-borne contaminants (Table 3). The total PCB/DL-PCB concentration on a wet weight
273 basis ranged between 9.6 and 2.2 ng/g for the two extreme diets (CTRL and VO). Detectable
274 amounts of HCB, DDT and DDT-derivates were found in all diets, varying the sum of these
275 selected OCPs from 9.8 in the CTRL diet to 1.6 ng/g in the VO diet. Regardless of diet, most
276 PBDE congeners were below the limit of detection (< 0.1 ng/g). Most PAHs were also below
277 the limit of detection (< 0.1 - 0.5 ng/g), decreasing the sum of detectable PAH congeners

278 (phenanthrene + anthracene, pyrene, chrysene, benzo [a] anthracene, benzo [b] fluoranthene,
279 benzo [k] fluoranthene) from 9.5 ng/g in the CTRL diet to 4.1 ng/g in the VO diet.

280

281 *3.2 Hepatic transcripts*

282

283 The expression pattern of AhRs and CYP 1A is shown in Fig. 1. Hepatic
284 transcription of AhR1 was down-regulated by FO replacement, and a significant 30%
285 reduction in AhR1 mRNA levels was found in fish fed the VO diet (Fig. 1A). The relative
286 expression of AhR2 was not significantly altered by dietary treatment (Fig. 1B). The trend
287 for CYP1A was similar to that of AhR1, and a two-fold reduction in CYP1A mRNA levels
288 was found in fish fed the VO diet (Fig. 1C).

289 The expression pattern of antioxidant enzymes of the hepatic glutathione pathway is
290 shown in Fig. 2. The amount of GR mRNA transcripts was not affected by dietary treatment
291 (Fig. 2A). The expression of GPx-1 was down-regulated by FO replacement, and a slight but
292 significant 20% reduction in GPx-1 mRNA levels was found in fish fed the VO diet when
293 comparisons were made with fish fed the CTRL diet (Fig. 2B). The trend for PHGPx was
294 opposite to that of GPx-1, and the expression of PHGPx mRNA was enhanced by a 30% in
295 fish fed the VO diet (Fig. 2C).

296 The hepatic expression of MT was reduced by FO replacement, and the abundance
297 of MT mRNA levels in fish fed 66VO and VO diets was two-fold lower than in fish fed
298 CTRL and 33VO diets (Fig. 3A). The relative expression of GRP75 was significantly
299 reduced by a 40% in fish fed the VO diet in comparison to data found in fish fed the CTRL
300 diet (Fig. 3B).

301

302

303 3.3 *Glutathione and antioxidant defence system*

304

305 The hepatic synthesis of glutathione was inhibited by FO replacement, and tGSx
306 levels (oxidized plus reduced forms) were significantly and progressively decreased in fish
307 fed 66VO and VO diets (Fig. 4A). The oxidized form (GSSG) also decreased with FO
308 replacement (Fig. 4B), but the GSH/GSSG ratio increased in concert and the highest quotient
309 was found in fish fed the VO diet (Fig. 4C). Dietary intervention also altered the total plasma
310 antioxidant capacity, and the values registered in fish fed the VO diet were significantly
311 lower than in CTRL fish (Fig. 5).

312

313 3.4 *Immune status*

314

315 The RB of circulating leucocytes was triggered by the graded replacement of FO.
316 Thus, ROS production after PMA stimulation was lowest in fish fed the CTRL diet and two-
317 three folds higher in fish fed the VO diet (Fig. 6A). Non significant differences were found in
318 plasma PO (Fig. 6B). Likewise, dietary intervention did not modify the ACP, although the
319 maximum ACH₅₀ values (no statistically significant) were found in fish fed the 66VO diet
320 (Fig. 6C). Conversely, plasma lysozyme levels were reduced in this group of fish, and the
321 measured values in fish fed the 66VO diet were significantly lower than in the CTRL group
322 (Fig. 6D).

323

324

325 4. Discussion

326

327 The cardioprotective and anti-inflammatory properties (Calder, 2008) of n-3 LC-
328 PUFAs are behind the recommendation of increased fish consumption in contemporary
329 western diet. However, n-3 LC-PUFAs have a higher susceptibility to oxidation (Jobling and
330 Bendiksen, 2003), and FOs and seafood in general are considered the most important source
331 of dietary pollutants in the human diet (Abalos et al., 2008a; Borga et al., 2001; Kidd et al.,
332 2001; Serrano et al., 2003a). In the current study, the total replacement of FO by vegetable
333 oils reduced the total charge of POPs in fish feeds by a 45-85%. Moreover, PBDEs were
334 below the limit of detection, and the load-charge of PCBs, DL-PCBs and OCPs was lower
335 than or in the same range as previously reported in salmon feeds (Bell et al., 2005; Berntssen
336 et al., 2005, Easton et al., 2002; Hites et al., 2004) and other marine fish feeds (Serrano et al.,
337 2003b; 2008a; 2008b). In consequence, the analyzed feed-borne contaminants were at low or
338 trace levels regardless of the diet, even in the FO diet. However, the detoxifying
339 cytochrome/AhR pathway of gilthead sea bream appears to be sensitive enough to detect the
340 progressive reduction of contaminants when FO was progressively reduced in fish fed 66VO
341 and VO diets.

342 Cytochrome P450s constitutes a major family of drug metabolizing enzymes that
343 transform xenobiotics to non-toxic or procarcinogenic metabolites. The transcriptional or
344 post-transcriptional induction of CYP1A and related enzymes is mediated in gilthead sea
345 bream (Ortiz-Delgado et al., 2002; Ortiz-Delgado and Sarasquete, 2004) and other teleosts
346 (Barron et al., 2004; Billiard et al., 2004; Jönsson et al., 2004; Panserat et al., 2008; Yuan et
347 al., 2006) by planar halogenated compounds (PAHs, PCBs, TCDD, etc.) that bind to
348 cytosolic AhRs, leading to the dissociation of heat-shock protein-90 from the activated
349 ligand-receptor complex. This specific binding ultimately results in the up-regulation of the

350 CYP1A gene through the activation of xenobiotic-responsive elements in the promoter
351 region of the CYP1A gene (Hankinson, 2005). Flavonoids are thought to promote optimal
352 health, partly via their antioxidant effects in protecting cellular components against free
353 radicals. However, recent *in vitro* studies in mammals have also shown that some food
354 flavonoids down-regulate CYP1A at the functional level of AhR, inhibiting the toxic effects
355 of PCBs (Ramadass et al., 2003; Van der Heiden et al., 2009). Thus, in the current work, the
356 significant decrease of CYP1A and AhR1 found in fish fed vegetable oils could be due not
357 only to the reduction of contaminants, but also to the possible presence of some compounds
358 of plant origin, such as flavonoids.

359 Fish differ from mammals and birds in having not one, but at least two AhR genes
360 designated as AhR1 and AhR2. Phylogenetic analyses and gene mapping indicate that AhR1
361 of bony and cartilaginous fish is the ortholog of the mammalian AhR (Hahn, 2002).
362 However, there are different roles and tissue-specific profiles for each AhR between and
363 within fish species. Thus, AhR1 paralogs are transcribed at very low levels in Atlantic
364 salmon, and Hansson et al. (2004) suggested that if any AhR loci become dysfunctional
365 (pseudogenes) these should be related to AhR1. By contrast, red sea bream, which belongs to
366 the order Perciformes (Sparidae family), shows a wide and prominent tissue expression of
367 AhR1 (Yamauchi et al., 2005). Similarly, in the present study, the expression of AhR1 was
368 modulated in gilthead sea bream by dietary exposure to trace levels of environmental
369 pollutants, whereas transcript levels of AhR2 remained almost unchanged. This suggests that
370 AhR2 is more constitutively expressed than AhR1 in sparids. However, long-term dietary
371 exposure to PCDD/Fs, and in particular to the most toxic congener 2,3,7,8-
372 tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), clearly induced in gilthead sea bream the
373 expression of AhR2 (Abalos et al., 2008b). Unfortunately, no data are available in this last
374 study for AhR1 and the dual response of fish AhRs needs to be addressed in a specific and

375 dose-dependent manner not only in gilthead sea bream, but also in a wide range of sentinel
376 fish species.

377 The primary enzymatic antioxidant defence system is the glutathione redox system
378 that reduces hydrogen peroxide and lipid hydroperoxides at the expense of oxidizing GSH to
379 its disulfide form (GSSG). GR returns the oxidized glutathione form to GSH using NADPH
380 as reducing equivalents. In the present study, the hepatic expression of GR was not
381 nutritionally regulated. However, selenium-dependent glutathione peroxidases were inhibited
382 (GPx-1) or induced (PHGPx) by FO replacement according to their different substrate
383 specificity and cellular distribution, as it has been observed in other fish models (Imai and
384 Nakagawa, 2003). Thus, the cytosolic GPx-1, which acts alone or in combination with
385 phospholipase A₂ on H₂O₂ and free fatty acid hydroperoxides, was down regulated in fish fed
386 the VO diet. Conversely, mitochondrial PHGPx, which acts primarily on peroxidized fatty
387 acids of membrane phospholipids, was significantly overexpressed in the same group of fish.
388 Since these two enzymes probably operate in concert, it can be postulated that fish fed
389 vegetable oils prime the *in situ* repair of peroxidized phospholipids rather than the turnover
390 of membrane phospholipids from the cytosolic pool of undamaged free fatty acids. This
391 strategy should be especially advantageous in marine fish because their low elongase and
392 desaturase rates for bioconversion of C₁₈ vegetable oils into C₂₂ PUFA reduce the pool of
393 LC-PUFA in fat depots (Benedito-Palos et al., 2007; 2008).

394 Dietary intervention also modified the transcriptional regulation of non-enzymatic
395 antioxidant markers, and the VO group showed the lowest hepatic expression of GRP75 and
396 MT. The GRP75, also named mortalin/HSPA9B/PBP74/mtHSP70, is a mitochondrial-type
397 stress-protein of the heat shock protein 70 (HSP 70) family that performs a broad spectrum of
398 cellular functions, making this protein and its yeast homologue (SSC1) life-essential (Craven
399 et al., 2005; Kaul et al., 2007). Likewise, MT is a ubiquitous low molecular cystein rich-

400 protein that protects lower and higher vertebrates against heavy metals and oxidant stressors
401 (Gornati et al., 2004; Scudiero et al., 2005). Previous studies in common dentex and gilthead
402 sea bream indicate that both GRP75 and MT transcripts are regulated in these two sparid fish
403 by stressful behaviour and stress confinement, protecting fish against oxidative insults
404 (Bermejo-Nogales et al., 2007; 2008). In this way, the down regulation of these two oxidative
405 markers with FO replacement, together with the lowered plasma antioxidant capacity, can be
406 interpreted as a reduced oxidative stress. This is supported by the increased hepatic
407 GSH/GSSG ratio in VO fish despite of the reduction in absolute glutathione levels. This
408 result agrees with the recent concept that low antioxidant levels in farmed animals and long-
409 live mammals and birds predict low rates of ROS generation (Pamplona, 2008; Lykkesfeldt
410 and Svendsen, 2007).

411 Dietary fatty acids are capable of modulating the immune system in mammals (De
412 Pablo and De Cienfuegos, 2000) and n-3 LC-PUFA present in FO, and more specifically
413 eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids, have well recognized anti-
414 inflammatory properties (Calder, 2007). Conversely, many environmental toxicants
415 frequently cause inflammation by damaging tissues and inducing signalling pathways that are
416 oxidative stress-sensitive (Bols et al., 2001). Thus, FO replacement by vegetable oils could
417 provoke a double-edged effect in fish. However, in the current study, the anti-inflammatory
418 action of FO clearly over passed the possible inflammatory action of FO-borne contaminants,
419 since its replacement by vegetable oils progressively increased the PMA-induced RB of
420 blood leucocytes. This could be due to the fact that toxicant-induced inflammation usually
421 occurs at high toxicant concentrations, but not at the trace dietary levels found in the current
422 study (Pimpao et al., 2008). Similarly, the *in vitro* addition of EPA and DHA provoked a
423 marked reduction of superoxide anion generation in human polymorphonuclear leucocytes
424 (Chen et al., 1994), and dietary EPA caused a dose-dependent decrease in neutrophil RB in

425 elderly men (Rees et al., 2006). The immunosuppressive effect of n-3 LC-PUFA is thought to
426 be caused by changes in eicosanoid production, but recent studies have shown that the effects
427 of FO occur by eicosanoid-independent mechanisms, including actions upon receptor,
428 intracellular signalling pathways, transcription factor activity and gene expression (Gorjão et
429 al., 2006). However, we cannot disregard the possible inflammatory effect of vegetable
430 components. For example, the addition of soy phosphatidylcholine in human diet induced a
431 significant increase in neutrophil superoxide generation (Guarini et al., 1998), and even
432 undefined “herbal mixtures” added to fish diet appear to enhance some immune parameters,
433 including the RB (Yuan et al., 2007).

434 In fish, changing the dietary n-3 LC-PUFA levels can have both beneficial and, in
435 some instances, detrimental effects on disease resistance and immune status. Thus, the intake
436 of high levels of dietary n-3 LC-PUFA suppressed some immune functions and reduced
437 survival after pathogen challenge (Erdal et al., 1991; Fracalossi and Lovell, 1994; Kiron et
438 al., 1995; Misra et al., 2006). Conversely, other studies found significantly higher mortality
439 rates in fish fed some vegetable oils compared to those fed with FO when subsequently
440 challenged with bacteria (Brandsen et al., 2003; Thompson et al., 1996). In grouper
441 (*Epinephelus malabaricus*), a high dietary DHA/EPA ratio significantly enhanced phagocytic
442 and RB activities (Wu et al., 2003), whereas 100% replacement of FO with corn oil for 8
443 weeks significantly reduced the RB of circulating leucocytes (Lin and Shiau, 2007).
444 Similarly, 40% substitution of FO by a mixture of vegetable oils, or individual vegetable oils
445 in the diet of European sea bass (*Dicentrarchus labrax*), significantly reduced the RB of head
446 kidney leucocytes (Mourente et al., 2005; 2007b). In gilthead sea bream, a 204 days-trial
447 with 60 and 80% FO replacement by single or mixed vegetable oils did not affect the RB of
448 circulating neutrophils (Montero et al., 2003). The same happened when FO was 100%
449 replaced by different vegetable oils in Atlantic salmon (*Salmo salar*) (Bell et al., 1996).

450 These inconsistent results concerning the effect of vegetable oils on RB may, to some extent,
451 be due to the type of cells and the method of assessment. In our case, RB was assayed on
452 whole blood, which avoids the mechanic impact of the isolation procedures on the cells.
453 Furthermore, luminol-enhanced chemiluminescent is thought to measure intracellular as well as
454 extracellular ROS, whereas other methods measure only one type of ROS production.

455 In the current study, total peroxidases and ACH₅₀ were not significantly affected by
456 the dietary treatment. The even levels of PO could indicate that the release of these enzymes
457 from leucocytes was not elevated when FO was replaced. This should be regarded as
458 potentially beneficial for fish fed vegetable oils, since high plasma MPO levels in humans are
459 considered as a specific index of leucocytic activation in inflammatory diseases and also
460 correlate with heart diseases and atherosclerosis (Meuwese et al., 2007; Vita et al., 2004). In
461 accordance with the present results, ACH₅₀ was not affected by 60% replacement by soybean
462 oil (SO), rapeseed oil (RO) or linseed oil (LO) or a mixture of them when fed for a short-
463 term to gilthead sea bream. However, when fed for a longer time (204 days), SO fed fish had
464 significantly lower values (Montero et al., 2003). When the level of replacement was
465 increased to 100% with SO or LO for 6 months, ACH₅₀ was again decreased (Montero et al.,
466 2008), but these effects were not seen when a mixture of LO and SO was used. By contrast,
467 partial (but not total) replacement of FO with corn oil increased ACH₅₀ in grouper (Lin and
468 Shiao, 2007), and feeding with canola oil or with LO or safflower oil had no significant
469 effect in the ACH₅₀ of largemouth bass (*Micropterus salmoides*) (Subhadra et al., 2006), and
470 rainbow trout (*Oncorhynchus mykiss*) (Kiron et al., 2004), respectively.

471 The only humoral innate factor modified by the current dietary treatment was
472 lysozyme, which was significantly decreased in fish fed the 66VO diet. Similarly, serum
473 lysozyme of hens fed maize oil was significantly lower than in those fed with FO (Guo et al.,
474 2004). By contrast, serum lysozyme appears to be unaffected in most studies with fish fed

475 diets rich in vegetable oils (Bell et al., 1996; 2006; Kiron et al., 2004; Montero et al., 2003;
476 Mourente et al., 2005; Subhadra et al., 2006). These apparent contradictory effects on
477 immune factors are probably due to different experimental conditions (fish meal inclusion,
478 type of vegetable oil, PUFAs ratios, feeding time, etc.), type of immunocytes involved and
479 species model. One of the differences of our dietary trial is that the basal diet has also a high
480 level of substitution of fish meal by plant proteins, and most fish trials replace either fish
481 meal or FO.

482 In conclusion, the present results show that the concurrent decrease of feed-borne
483 contaminants and inclusion of vegetable oils produced in gilthead sea bream the readjustment
484 of detoxifying pathways and ROS production and scavenging (enzymatic and non-enzymatic
485 antioxidants) processes. The lowering of the plasma antioxidant capacity seems not to
486 endanger fish health, since the hepatic GSH/GSSG ratio was increased, evidencing a lower
487 risk of lipid peroxidation and oxidative damage. Anyway, the biological significance of the
488 lowered lysozyme or other induced changes in the defence mechanism of the fish must be
489 further explored with experimental infections for the complete development and validation of
490 eco-friendly aquafeeds based on the concurrent FO and fish meal replacement.

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492

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501 **References**

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798
799
- 800

801 **Figure Legends**

802

803 Figure 1. Effects of dietary treatment on hepatic transcript levels of aryl hydrocarbon
804 receptor 1 (A), aryl hydrocarbon receptor 2 (B) and cytochrome P450 1A1 (C). Data in fish
805 fed the control diet were used as arbitrary reference values in the normalization procedure
806 (values > 1 or < 1 indicate increase or decrease respect to reference values). Different letters
807 indicate significant differences ($P < 0.05$) among dietary treatments (Student-Newman-Keuls
808 test).

809

810 Figure 2. Effects of dietary treatment on hepatic transcript levels of glutathione reductase
811 (A), glutathione peroxidase (B) and phospholipid glutathione peroxidase (C). Data in fish fed
812 the control diet were used as arbitrary reference values in the normalization procedure
813 (values > 1 or < 1 indicate increase or decrease respect to reference values). Different letters
814 indicate significant differences ($P < 0.05$) among dietary treatments (Student-Newman-Keuls
815 test).

816

817 Figure 3. Effects of dietary treatment on hepatic transcript levels of metallothionein (A) and
818 glucose regulated protein 75 (A). Data in fish fed the control diet were used as arbitrary
819 reference values in the normalization procedure (values > 1 or < 1 indicate increase or
820 decrease respect to reference values). Different letters indicate significant differences ($P <$
821 0.05) among dietary treatments (Student-Newman-Keuls test).

822

823 Figure 4. Effects of dietary treatment on hepatic glutathione levels. Total glutathione (A),
824 oxidized glutathione (B) and reduced oxidized glutathione ratio (C). Different letters

825 indicate significant differences ($P < 0.05$) among dietary treatments (Student-Newman-Keuls
826 test).

827

828 Figure 5. Effects of dietary treatment on the total plasma antioxidant capacity. Different
829 letters indicate significant differences ($P < 0.05$) among dietary treatments (Student-Newman-
830 Keuls test).

831

832 Figure 6. Effects of dietary treatment on the respiratory burst of circulating leucocytes (A),
833 plasma peroxidases (B), serum alternative complement pathway (C) and plasma lysozyme
834 levels (D). Different letters indicate significant differences ($P < 0.05$) among dietary
835 treatments (Student-Newman-Keuls test).

836

837 **Table 1.** Ingredients and chemical composition of experimental diets. For details in amino
 838 acid and fatty acid composition see Benedito-Palos et al. (2007).
 839

Ingredient (g/kg)	CTRL	33VO	66VO	VO
Fish meal (CP 70%) ¹	150	150	150	150
CPSP 90 ²	50	50	50	50
Corn gluten	400	400	400	400
Soybean meal	143	143	143	143
Extruded wheat	40	40	40	40
Fish oil ³	151.5	101.5	51.5	0
Rapeseed oil	0	8.5	17	25.8
Linseed oil	0	29	58	87.9
Palm oil	0	12.5	25	37.9
Soya lecithin	10	10	10	10
Binder	10	10	10	10
Mineral premix ⁴	10	10	10	10
Vitamin premix ⁵	10	10	10	10
CaHPO ₄ ·2H ₂ O (18%P)	20	20	20	20
L-Lys	5.5	5.5	5.5	5.5
<i>Proximate composition</i>				
Dry matter (DM, %)	93.42	94.16	94.79	95.38
Protein (% DM)	48.98	48.74	49.03	48.65
Fat (% DM)	22.19	22.26	22.11	22.31
Ash (% DM)	6.54	6.57	6.62	6.41

840

841 ¹Fish meal (Scandinavian LT)842 ²Fish soluble protein concentrate (Sopropêche, France)843 ³Fish oil (Sopropêche, France)

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

848 ⁵Supplied the following (mg / kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL- α
 849 tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5,
 850 nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B₁₂ 0.025, ascorbic acid 250,
 851 inositol 500, biotin 1.25 and choline chloride 500.

852

853 **Table 2.** Forward and reverse primers for hepatic real-time PCR assays. Aryl hydrocarbon receptors (AhR1, AhR2),
 854 cytochrome P450 1A1 (CYP1A), metallothionein (MT), glucose regulated protein 75 (GRP75), glutathione reductase
 855 (GR), glutathione peroxidase (GPx-1), phospholipid glutathione peroxidase (PHGPx) and β -actin.
 856

Gene	GenBank accession	Primer sequence	Position
AhR1	EU254480	F CCT GGG ACT GAA CGC CGA AG	1027-1046
		R GCT AAG TGT TGG GAT GTG GTT GG	1120-1098
AhR2	AY129956	F TCA GAG GGA TTG GTG TTT TAT GTC	358-381
		R TGG GTT TAG AGC AAA GTG AAG C	507-486
CYP1A	AFO11223	F GCA TCA ACG ACC GCT TCA ACG C	903-924
		R CCT ACA ACC TTC TCA TCC GAC ATC TGG	1071-1047
GR	AJ937873	F TGT TCA GCC ACC CAC CCA TCG G	927-948
		R GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG	1041-1029
GPx-1	DQ524992	F GAA GGT GGA TGT GAA TGG AAA AGA TG	34-59
		R CTG ACG GGA CTC CAA ATG ATG G	162-141
PHGPx	AM977818	F TGC GTC TGA TAG GGT CCA CTG TC	237-259
		R GTC TGC CAG TCC TCT GTC GG	312-293
MT	U93206	F CTC TAA GAC TGG AAC CTG	75-92
		R GGG CAG CAT GAG CAG CAG	167-150
GRP75	DQ524993	F TCC GGT GTG GAT CTG ACC AAA GAC	358-381
		R TGT TTA GGC CCA GAA GCA TCC ATG	500-477
β -actin	X89920	F TCC TGC GGA ATC CAT GAG A	811-829
		R GAC GTC GCA CTT CAT GAT GCT	861-841

883

Table 3. Concentration of PCBs, DL-PCBs (*), OCPs, PBDEs and PAHs in experimental diets. Each value is the mean of three separate determinations. Coefficient of variation is under parentheses.

Compound (ng/g wet wt)	CTRL	33VO	66VO	VO
PCB 28+31	0.2(3)	0.1(3)	0.1(3)	< 0.1
PCB 52	0.5(5)	0.3(6)	0.2(4)	0.1(9)
PCB 101	1(13)	0.7(5)	0.4(10)	0.2(10)
PCB 77*	< 0.2	< 0.2	< 0.2	< 0.2
PCB 118*	1(5)	0.8(11)	0.5(12)	0.2(21)
PCB 153	2.5(5)	2(7)	1.5(5)	0.6(4)
PCB 105*	0.4(2)	0.3(21)	0.2(8)	< 0.1
PCB 138	1.4(2)	1.3(3)	0.8(15)	0.4(5)
PCB 126*	< 0.2	< 0.2	< 0.2	< 0.2
PCB 128	0.3(30)	0.2(4)	0.2(7)	< 0.1
PCB 156 *	0.3(28)	0.2(16)	< 0.1	0.1(25)
PCB 180	0.7(6)	0.8(21)	0.5(3)	0.3(15)
PCB 169*	0.2(10)	0.1(18)	< 0.1	< 0.1
PCB 170	0.3(5)	0.3(11)	0.2(12)	< 0.2
HCB	0.6(6)	0.4(10)	0.4(10)	0.2(11)
p,p'-DDT	5.8(8)	4.1(4)	2.6(3)	0.9(12)
p,p'-DDE	2.2(7)	1.7(5)	1.1(11)	0.3(27)
p,p'-DDD	1.2(6)	1.1(12)	0.7(7)	0.3(24)
PBDE 28	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 71	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 47	0.13(11)	0.12(12)	< 0.1	< 0.1
PBDE 66	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 100	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 99	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 85	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 154	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 153	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 138	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 183	< 0.1	< 0.1	< 0.1	< 0.1
PBDE 209	< 0.1	< 0.1	< 0.1	< 0.1
Naphthalene	< 0.5	< 0.5	< 0.5	< 0.5
Acenaphthylene	< 0.1	< 0.2	< 0.2	< 0.2
Acenaphthene	< 0.2	< 0.2	< 0.2	< 0.2
Fluorene	< 0.3	< 0.3	< 0.3	< 0.3
Phenanthrene +Anthracene	0.62(15)	1.50(5)	0.38(11)	< 0.2
Fluoranthene	< 0.2	< 0.2	< 0.2	< 0.2
Pyrene	1.33(7)	0.83(15)	< 0.2	< 0.2
Benzo [a] anthracene	1.12(5)	< 0.2	< 0.2	< 0.2
Chrysene	1.41(5)	< 0.2	< 0.2	< 0.2
Benzo [b] fluoranthene	2.68(4)	2.01(19)	2.20(5)	2.06(8)
Benzo [k] fluoranthene	2.31(8)	2.19(7)	2.16(7)	2.07(6)
Benzo [a] pyrene	< 0.2	< 0.2	< 0.2	< 0.2
Indeno [1,2,3-cd] pyrene	< 0.2	< 0.2	< 0.2	< 0.2
Dibenzo [a,h] anthracene	< 0.2	< 0.2	< 0.2	< 0.2
Benzo [g,h,i] perylene	< 0.2	< 0.2	< 0.2	< 0.2
$\sum PCBs + DL-PCBs$	8.66	7.03	4.71	2.22
$\sum OCPs$	9.8	7.3	4.8	1.6
$\sum PAHs$	9.5	6.5	4.7	4.1
DL-PCBs TEQ, ng/g wet wt to humans	0.00231	0.00123	0.00114	0.0011
DL-PCBs TEQ, ng/g wet wt to fish	0.0000385	0.0000315	0.000029	0.000027

Figure 1

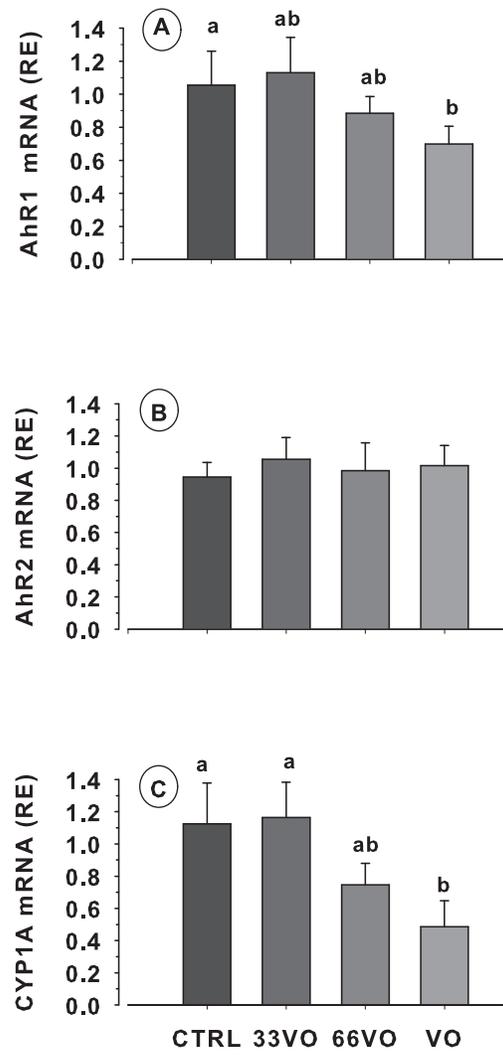


FIG. 1

Figure 2

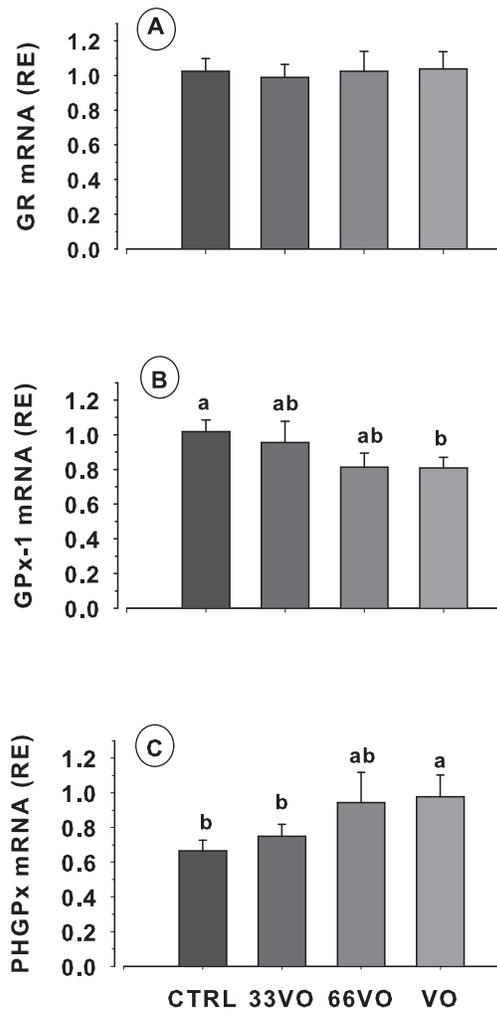


FIG. 2

Figure 3

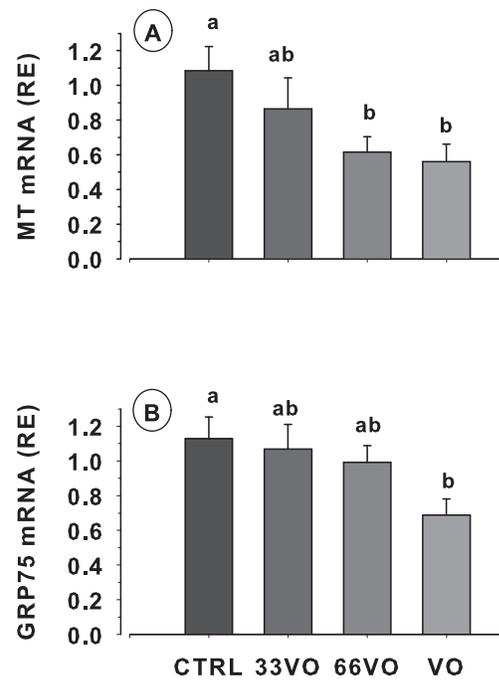


FIG. 3

Figure 4

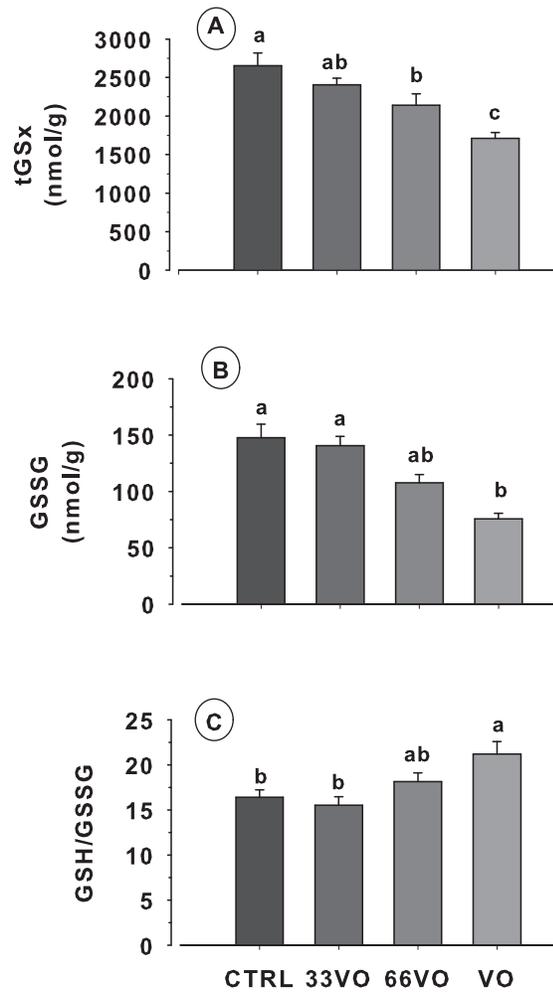


FIG. 4

Figure 5

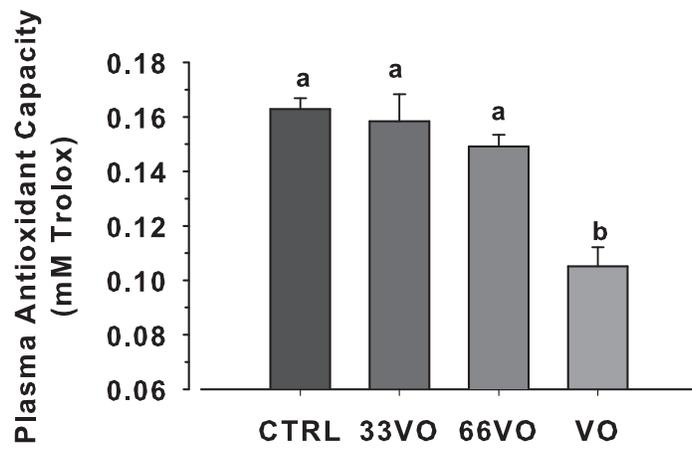


FIG. 5

Figure 6

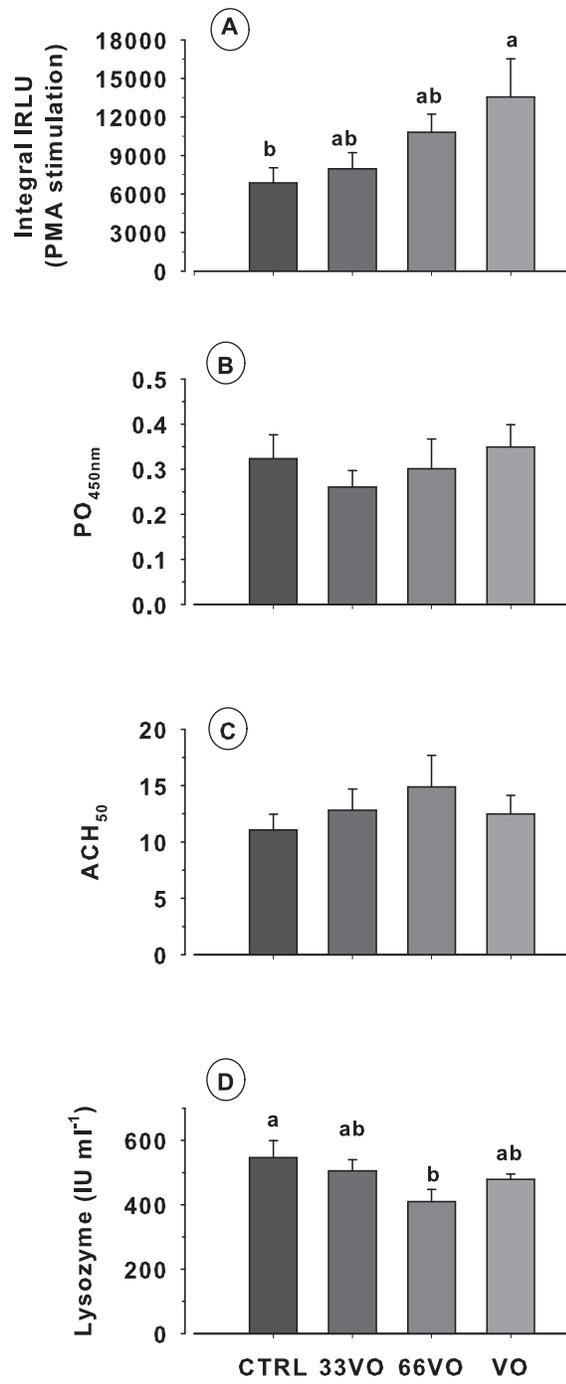


FIG. 6