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**EFFECT OF IVERMECTIN ON THE LIVER OF GILTHEAD SEABREAM *Sparus aurata*: A PROTEOMIC APPROACH**

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33 **ABSTRACT**

34 Gilthead sea bream *Sparus aurata* is the most commercialized Mediterranean aquacultured  
35 fish species. Ivermectin has recently (experimentally) started to be used to control  
36 ectoparasitic infestations in Mediterranean cultured marine fish. The potential hepatotoxicity  
37 of ivermectin was investigated in gilthead sea bream juveniles (35 g) following oral  
38 administration at the recommended dose of 0.2 mg kg<sup>-1</sup> fish for 10 days. Difference Gel  
39 Electrophoresis Technology (DIGE) was used to study the effect of this treatment in gilthead  
40 sea bream liver protein profile under routine culture conditions. The 2D-DIGE protein maps  
41 obtained were analyzed using the DeCyder 6.5 software. The results obtained showed  
42 significant changes in the expression of 36 proteins respect to the control group. Among  
43 these proteins, six increased in abundance, and 30 decreased. Spot showing differential  
44 expression respect to the control were analysed by mass spectrometry and database search,  
45 which resulted in three positive identifications corresponding to hepatic proteins involved in  
46 lipid metabolism (apoA-I), oxidative stress responses and energy generation (beta-globin,  
47 ATP synthase subunit beta). These proteins have not been previously associated to  
48 ivermectin effect.

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50 **Keywords:** Proteomics; 2D-DIGE; ivermectin; gilthead seabream; new biomarkers; mass  
51 spectrometry (MS)

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70 **Introduction**

71 Gilthead sea bream, (*Sparus aurata*) is the most important Mediterranean  
72 aquacultured fish species. As with other intensive animal farming practices, aquaculture  
73 activities involve the cultivation of large numbers of fish confined in a reduced space and  
74 therefore, disease outbreaks are common regardless of the improvement of vaccine  
75 development and the quality of the hygiene practised. Pathogens such as bacteria, parasites,  
76 viruses and fungi may cause infection in cultured fishes. Disease outbreaks in aquaculture  
77 are normally confronted with mass therapy, usually orally administered via incorporation of  
78 drugs into the feed. It is generally accepted that nowadays exoparasitic outbreaks remain the  
79 most significant source of mortalities in Mediterranean fish cage farming (Rigos and  
80 Katharios, 2009).

81 Ivermectin, is a wide spectrum antiparasitic drug, belonging to the family of  
82 avermectins. Avermectins are macrocyclic lactones isolated from the fermentation products  
83 of the actinomycetes *Streptomyces avermitilis* (Campell et al., 1983). The general mode of  
84 action of these compounds is to interrupt the transmission of signals in the nervous system of  
85 invertebrates. The high-affinity binding of avermectins to glutamate-gated chloride ion  
86 channels in invertebrate excitable cells leads to paralysis and death (Arena et al., 1995).  
87 These neurotoxins are highly effective when used against arthropod and nematode infections  
88 in a number of terrestrial farm animals (Campell, 1989), and also in the prevention of some  
89 parasitic infections in humans, like river blindness (onchocerciasis) (Laffont et al., 2002).

90 In Atlantic salmon (*Salmo salar*) sea lice infestations produce general stress and  
91 osmorregulatory problems due to disruption of the skin by the feeding behavior of the  
92 parasites, as well, as changes in the feeding behavior of fish (Dawson et al., 1999). Although  
93 according to Sanderson et al. (2007) ivermectin has not officially been used for ectoparasite  
94 treatment in aquaculture, it has been used “de facto” for the control of sea lice in salmon  
95 farms in Chile, Ireland, Canada and UK (Roth, 2000). Besides, this drug has also started to  
96 be used in Mediterranean aquaculture to control ectoparasitic copepod (*Lernathropus*  
97 *kroyeri*) and isopod (*Ceratothoa oestroides*) infestations (Athanasopoulou et al., 2001).

98 Several authors have reported the efficacy and toxicity of ivermectin against  
99 ectoparasitic infestations of several farmed fish (Palmer et al., 1987; Spencer, 1992; Davies  
100 and Rodger, 2000; Athanasopoulou et al., 2001, 2002; Katharios et al., 2002a, 2004;  
101 Mladineo et al., 2006). The most commonly used oral doses in farmed fish range between  
102 0.05 and 0.2 mg kg<sup>-1</sup> fish, with different schemes of administration (i.e. single dose, once or  
103 twice a week), without apparent side effects to the fish (Palmer, et al., 1987; Johnson et al.,  
104 1993; Davies and Rodger, 2000; Athanasopoulou et al., 2001).

105 Regarding pharmacokinetics, ivermectin is accumulated in fatty tissues including liver,  
106 and exhibits an enterohepatic circulation in vertebrates (Davies and Roger, 2000). Katharios

107 et al. (2002a) obtained a rapid uptake, high bioavailability and fast elimination in gilthead sea  
108 bream after intraperitoneal injection of ivermectin. However, when the drug was orally  
109 administered, a slow absorption and elimination profile was evident, with bile being the major  
110 route of excretion mainly as parent drug (Hoy et al., 1990). Furthermore, the high brain  
111 concentrations of ivermectin found in Atlantic salmon, *Salmon salar*, and gilthead sea bream  
112 after oral and intraperitoneal administration, respectively (Hoy et al., 1990; Katharios et al.,  
113 2002a, 2004) point towards a less selective barrier in fish as compared to mammals.  
114 Katharios et al. (2002b) investigated the toxic side effects of ivermectin administered  
115 intraperitoneally to gilthead sea bream at single doses of 0.1, 0.2, 0.4 and 0.8 mg Kg<sup>-1</sup> fish.  
116 Their study concluded that the most important side effect of ivermectin was a significant  
117 reduction of the hematocrit value in fish treated with the highest dose (0.8 mg kg<sup>-1</sup> fish). Also,  
118 signs of neurotoxicity like lethargy, loss of appetite and dark color were observed at this  
119 dose. However, neither mortality nor histopathological alterations in the different tissues  
120 examined including liver were observed.

121 Up to date, all previous works on the toxicity effect of ivermectin in marine fish,  
122 including gilthead sea bream, have been carried out using conventional approaches, and no  
123 studies have been published about the capacity of the drug to produce liver injury at the  
124 molecular level after a standard treatment. Recently, the introduction of new and improved  
125 proteomic technology, has allowed investigating changes in biological events at molecular  
126 level. Proteomics-based approaches have a great potential since they massively assess  
127 protein alterations without any previous knowledge of toxicity mechanisms (López-Barea and  
128 Gómez-Ariza, 2006), and may be useful to find new biomarkers of hepatotoxicity in animals,  
129 including marine fish.

130 The aim of this study was to evaluate the possible hepatotoxicity effects of ivermectin  
131 in gilthead sea bream juveniles after oral administration at the recommended dose of 0.2 mg  
132 kg<sup>-1</sup> fish. The liver was examined as the principal target of toxicity due to the role of this  
133 organ in energetic and xenobiotic metabolism. To evaluate the effect of the treatment, a  
134 proteomic approach was carried out using 2-D differential gel electrophoresis (2D-DIGE) and  
135 proteins of interest were identified by mass spectrometry analysis and database search.

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## 137 **Materials and Methods**

### 138 *Animals and treatment*

139 Gilthead sea bream weighting 35 ± 9 g were purchased from a local commercial  
140 aquaculture farm (Acuícola Marina SL, Burriana, Castellón, Spain), and kept in the facilities  
141 of the aquarium plant of the University of Valencia. Fish were distributed in several 2000 L  
142 fibreglass tanks (120 fish per tank) filled with seawater (salinity: 32‰) supplied with

143 continuous aeration under room temperature ( $19 \pm 0.5$  °C) in a closed circuit, and acclimated  
144 for 1 week before starting the experiment.

145 The test fish were fed pelleted dry food medicated with ivermectin at a concentration  
146 of  $0.2 \text{ mg kg}^{-1}$  fish, for 10 days (1% body weight daily feeding ratio with fish delivered feed  
147 once a day). The control fish were administered unmedicated pelleted feed at the same  
148 feeding schedule. The experimental feeds were made at the Fish Nutrition and Pathology  
149 Laboratory, Institute of Aquaculture, of the Hellenic Center for Marine Research in Athens  
150 (Greece). Commercial feed (Biomar) with the following composition: fish meal (35%), soya  
151 bean meal (20%), wheat meal (15%), rapeseed meal (2.7%), wheat gluten (5%), corn gluten  
152 (10%), fish oil (13%), premix (0,3%). Ingredients were grounded, mixed with the antiparasitic  
153 drug, and prepared as dry pellets suitable for the size of the fish. The same procedure was  
154 followed for the preparation of the unmedicated diet.

155 After treatment, 8 fish from each experimental group were anaesthetized with clove  
156 oil ( $20 \text{ mg L}^{-1}$ ) and sacrificed before livers being quickly dissected. The samples were  
157 immediately frozen in liquid nitrogen and stored at  $-80$  °C until analyzed.

158

## 159 *2-D difference gel electrophoresis (2D-DIGE)*

### 160 *Liver homogenization and protein labelling*

161 Individual livers were homogenized with the aid of a grinding kit system (General  
162 Electric Healthcare) in 9 volumes of DIGE buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30  
163 mM Tris) in which antiproteolytic agent (Complete Mini Roche) was added. The solubilized  
164 proteins were separated from non-solubilized cellular components by centrifugation ( $20,000$   
165  $\text{g} \times 20$  min). Proteins present in the supernatants were precipitated using the 2D Clean-up kit  
166 (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions and  
167 resolubilized in DIGE buffer. The pH of protein extract was adjusted to 8.5 by adding 50 mM  
168 NaOH, and protein concentration was determined using the Bradford Biorad Protein Assay  
169 (RcDc kit) with  $\gamma$ -globulin as standard.

170 Liver proteins extracted from control ( $n=8$ ) and treated ( $n=8$ ) fishes were randomly  
171 labelled with Cy3 or Cy5. For DIGE minimal labelling,  $50 \mu\text{g}$  of protein sample was mixed  
172 with  $400 \text{ pmol}$  CyDye (GE healthcare) by vortexing and incubated on ice in the dark for 30  
173 min. The labelling reaction was stopped by the addition of  $1 \mu\text{L}$  10 mM lysine followed by  
174 incubation on ice for a further 10 min. The internal standard sample was prepared by pooling  
175  $25 \mu\text{g}$  of protein from each liver sample studied and by labelling by Cy2 as above described.  
176 Combinations of a Cy3 and a Cy5 labelled sample were then mixed with Cy2-labeled internal  
177 standard, and DTT (65 mM final concentration) and ampholytes (1% final concentration, pH  
178 = 3-10) were added to the mixture before running the first dimension.

179

180 *Gel electrophoresis (2D-DIGE gel) and image capture*

181 The 16 liver protein samples were analysed on a total of 8 analytical 2-D gels. IPG  
182 strips (24 cm, pH = 3-11NL) were rehydrated in 8 M urea, 4% CHAPS, DeStreak (12  $\mu$ L mL<sup>-1</sup>),  
183 and ampholytes (1% final concentration, pH = 3-10) overnight at room temperature. Cy-  
184 labelled samples were applied onto IPG rehydrated strips via anodic cup loading, and IEF  
185 was performed on a Ettan IPGphor II horizontal electrophoresis system (Amersham  
186 Biosciences) at 20 °C using the following program: step 1:300 V 4 h, gradient to 1000 V 6h,  
187 gradient to 8000 V 3 h; step 2: 8000 V until reached 32000 V h.

188 After IEF, the strips were reduced in equilibration buffer (Tris 50 mM, urea 6 M and  
189 glycerol 30% (v/v), 2% SDS (w/v)) containing 2% DTT, for 15 min at room temperature;  
190 followed by alkylation in equilibration buffer containing 2.5% iodoacetamide, for 15 min at  
191 room temperature.

192 Then, the strips were transferred to the second dimension 12.5% acrylamide SDS-  
193 PAGE gels (25 cm x 21 cm x 1 mm) made between low fluorescence glass plates, and  
194 overlaid with 0.5% low melting agarose. The gels were run in Ettan Dalt Six Unit (GE  
195 Healthcare) electrophoresis system at 2 W per gel for 1 h and 15 W per gel for 6 h.

196 After electrophoresis, the 2-D gels were scanned directly in a Typhoon™ 9400  
197 Variable Mode Imager to visualize the labelled proteins. Excitation and emission wavelengths  
198 were chosen specifically for each of the dyes according to manufacturer's recommendations.  
199 (GE Healthcare).

200

201 *Data analysis*

202 Intra-gel and Inter-gel matching and statistical analysis were performed using  
203 DeCyder™ V. 6.5 and DeCyder™ EDA software V.1.0.

204 Gel images were processed by the DeCyder-DIA (Differential In-gel Analyses)  
205 software module to co-detect and differentially quantify the protein spots in the images,  
206 taking the internal standard sample as a reference to normalize the data. DIA software uses  
207 a normal distribution model to determine the differentially expressed spots. The threshold  
208 was set to 2 standard deviations based on an assumption that 95% of the protein spots were  
209 not expected to be differentially expressed. Each protein in the individual sample is  
210 represented in the pooled internal standard, thus a comparison between the test samples  
211 and the identical protein in the internal standard can be used to generate a ratio of relative  
212 expression. Then, the DeCyder-BVA (Biological Variation Analysis) was applied. BVA does a  
213 gel-to-gel matching of the internal standard spots maps from each gel. The differences in  
214 average ratios of protein expression were analysed by the Student's t- test ( $p \leq 0.02$ ).

215 EDA module from DeCyder™ software was used for multivariate statistical analysis of  
216 data. Principal Components Analysis (PCA) was carried out following the nonlinear iterative

217 partial least squared method, including only proteins present in at least 80% of the spot maps  
218 and applying a t-test filter ( $p \leq 0.02$ ). A hierarchical cluster analyses was performed using the  
219 same protein selection criteria.

220

#### 221 *Protein identification by mass spectrometry (MALDI, MS/MS) analysis*

222 Proteins of interest were manually excised from analytical gels and digested with  
223 sequencing grade trypsin (Promega) as described elsewhere (Shevchenko *et al.*1996), and  
224 subject to PMF (MALDI) and/or LC/MS/MS analyses.

225 The digestion mixture was dried in a vacuum centrifuge, resuspended in 7  $\mu\text{L}$  of 0.1%  
226 TFA (trifluoroacetic acid, Sigma), and 1  $\mu\text{L}$  was spotted onto the MALDI target plate. After  
227 the droplets were air-dried at room temperature, 0.5  $\mu\text{L}$  of matrix (5  $\text{mg mL}^{-1}$  CHCA ( $\alpha$ -  
228 cyano-4-hydroxycinnamic acid, Sigma) in 0.1% TFA-ACN/H<sub>2</sub>O (1:1, v/v) was added and  
229 allowed to air-dry at room temperature. The resulting 576 fractions were analyzed in a 4700  
230 Proteomics Analyzer (Applied Biosystems, Foster City, USA) in positive reflection mode  
231 (2000 shots every position). Five of the most intense precursors (according to the threshold  
232 criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap:  
233 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis.  
234 And, MS/MS data corresponding to the control was acquired using the default 1 kV MS/MS  
235 method.

236 The MS and MS/MS information was sent to MASCOT via the GPS software (Applied  
237 Biosystems). Database search on Swiss-Prot and NCBI databases was performed using  
238 MASCOT search engine (Matrix-Science). Searches were done with tryptic specificity  
239 allowing one missed cleavage and a tolerance on the mass measurement of 100 ppm in MS  
240 mode and 0.8 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed  
241 modification and oxidation of Met and deamidation of Asn and Gln as variable modifications.

242 The samples without a positive identification were analyzed by LC/MS/MS. Peptide  
243 separation by LC-MS/MS was performed using an Ultimate nano-LC system (LC Packings)  
244 and a QSTAR XL Q-TOF hybrid mass spectrometer (MDS Sciex-Applied Biosystems).  
245 Samples (5  $\mu\text{L}$ ) were delivered to the system using a FAMOS autosampler (LC Packings) at  
246 40  $\mu\text{L min}^{-1}$ , and the peptides were trapped onto a PepMap C18 pre-column (5 mm  $\times$  300 m  
247 i.d.; LC Packings). Peptides were then eluted onto the PepMap C18 analytical column (15  
248 cm  $\times$  75 m i.d.; LC Packings) at 200  $\text{nL min}^{-1}$  and separated using a 55 min gradient of 15–  
249 50% CAN. The QSTAR XL was operated in information-dependent acquisition mode, in  
250 which a 1-s TOF MS scan from 400–2000  $\text{m/z}$ , was performed, followed by 3-s product ion  
251 scans from 65–2000  $\text{m/z}$  on the three most intense doubly or triply charged ions.

252 The MS/MS information was sent to MASCOT via the MASCOT DAEMON software  
253 (MATRIX SCIENCE). The search parameters were defined as for MS-MS/MS analysis.

254

#### 255 *Quantitative RT-PCR of selected proteins*

256 Total RNA was extracted from the same liver used for the proteomic analysis with the  
257 RNAspin MiniRNA isolation kit (GE HealthCare), according to the manufacturer's  
258 instructions, and stored at -80 °C. The purity and quantity of extracted RNA were measured  
259 with the Experion System (Bio-Rad). Two hundred ng of total RNA was reverse transcribed  
260 into cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems), with  
261 a final reaction volume of 20 µL. The RT conditions were: 10 min at 25 °C, 120 min at 37 °C  
262 and 5 sec at 85 °C. The cDNA samples were stored at -20 °C until use.

263 Transcript measurements were made by real-time PCR using an iCycler IQ Real-time  
264 Detection System (Bio-Rad, Hercules, CA, USA) as previously described (Calduch-Giner et  
265 al., 2003). RT reactions were conveniently diluted and 7.5 µL were used for PCR reactions in  
266 a 25 µL volume. Each PCR-well contained a SYBR Green Master Mix (Bio-Rad) with specific  
267 primers at a final concentration of 0.9 µM (see Table 1). Alpha-tubulin was used as  
268 housekeeping gene and the efficiency of PCR reactions for target and reference genes  
269 varied between 88% and 95%, respectively. The dynamic range of standard curves spanned  
270 five orders of magnitude, and the amount of product in a particular sample was determined  
271 by interpolation of the cycle threshold (Ct) value. The specificity of reaction was verified by  
272 analysis of melting curves. Fluorescence data acquired during the extension phase were  
273 normalized to α-tubulin by the delta-delta method (Livak and Schmittgen, 2001), using data  
274 in control fish as reference values. No changes in α-tubulin expression were found in  
275 response to treatment.

276 The difference in gene expression levels between control and treated fish was  
277 calculated by two-tailed independent Student t-test using SPSS statistics software v 17.0. A  
278 p value ≤ 0.05 was considered statistically significant.

279

#### 280 *Histology*

281 For histological analyses subsamples from the same liver used for the proteomic study were  
282 immediately fixed in buffered formalin at 4°C for 24 h. Samples were transferred to 70%  
283 alcohol and kept there until processing. Finally, samples were dehydrated in alcohol, wax-  
284 embedded, cut into 6 µm sections and stained with the hematoxylin and eosin technique.

285

#### 286 **Results**

287 A representative 2D-DIGE-gel image of total liver proteins extracts from treated  
288 *versus* control fish is shown in Figure 1. Proteins over the range of pH applied in this

289 experiment (pH = 3-11NL) and with a molecular weight from approximately 10 to 250 kDa  
290 were resolved, and 3993 spots in the master gel were detected using the DeCyder BVA  
291 software. Each protein spot was assigned an average ratio (i.e. change in expression level  
292 due to ivermectin treatment) and *p*-value (Student's t-test) to indicate the level of  
293 significance. A total of 36 protein spots showed significant changes in the expression  
294 compared to the control group (standardized average volume ratio  $\geq 1.3$ , t-student  $\leq 0.02$ ).  
295 This represented about 1% of the total protein spots analysed. Among these protein spots, 6  
296 were up regulated and 30 down regulated by ivermectin. The positions of those differentially  
297 expressed protein spots in the 2D-DIGE are shown in Figure 1. The PCA and hierarchical  
298 cluster analyses of data are shown in Figure 2. The PCA results obtained indicated that two  
299 components are enough to cluster the different experimental groups (control vs treated), with  
300 a clear separation in the first component (PC1) between them (Fig 2A). The pattern analyses  
301 showed a clustering in a hierarchical way, where protein maps corresponding to control  
302 group formed a cluster separated from those corresponding to the ivermectin treated group  
303 (Fig 2B)

304 The results of protein identification by PMF and/or LC-MS/MS and data base research  
305 are listed in Table 2. The 2D-DIGE analyses (spot n<sup>o</sup>, protein name, MW/pI, gi accession  
306 number, *p*-value, and the average ratio/fold change) are also indicated in Table 2. Ten  
307 differentially expressed proteins were identified. It was found that five of these proteins are  
308 related to the oxygen transport: beta globin (spots 3736, 3776, 3784, 3838 and 3841), two  
309 proteins are involved in the cholesterol metabolism: apo A-I (spot 3993 and 3992), and one  
310 protein is related in ATP synthesis: ATP synthase beta chain (spot 3913). All of them were  
311 down regulated. Also, ivermectin induced remarkable changes in two proteins that have  
312 unknown function and were down regulated (spots 3074 and 3760).

313 Among the proteins identified, the beta globin and apo A-I were selected for gene  
314 expression analyses. Transcript abundance of beta globin and apo A-I were determined by  
315 quantitative PCR. Figure 3 shows transcript hepatic expression levels of beta globin and apo  
316 A-I. The expressions of both genes apparently decreased as a result of the ivermectin  
317 treatment (relative mRNA expression of beta globin:  $1 \pm 0.69$  vs  $0.35 \pm 0.23$ , mean  $\pm$  std and  
318 relative mRNA expression of apo A-I:  $1.04 \pm 0.32$  vs  $0.62 \pm 0.29$ ), but there were not  
319 significant differences between control and treated fish ( $p > 0.05$ ).

320 Structural or morphological differences were not observed between control and  
321 ivermectin treated livers, and no pathological alterations were found in the livers from fish  
322 treated with ivermectin (Fig 4).

323

324

325 **Discussion**

326 As far as we know, this study represents the first proteomic approach to determine  
327 the potential hepatotoxicity of ivermectin in gilthead sea bream. Differential gel  
328 electrophoresis (2D-DIGE) allowed us to detect differences in the expression level of 36  
329 proteins, revealing the existence of effects of ivermectin treatment at recommended dose in  
330 gilthead sea bream.

331 The concept of altered protein expression signatures (PES), i.e. a set of proteins  
332 observed in bidimensional electrophoresis (2-DE) as state markers signalling for early  
333 pathological stages or stress exposure, was first described by Shepard and Bradley (2000)  
334 and Shepard et al. (2000), and successfully used by Vioque-Fernández et al. (2009) for  
335 assessing toxicity caused by environmental pollutants in the red crayfish (*Procambarus*  
336 *clarkii*). According to the cluster analysis, the changes in relative abundance of a set of 36  
337 proteins that we have found is sufficient to discriminate spot maps corresponding to  
338 ivermectin treated fish from those corresponding to control fish. These changes seem to be  
339 preliminary to hepatotoxicity, or transient, since the histological analysis did not show hepatic  
340 damage. Therefore changes in this set of proteins can be considered as a very early PES of  
341 ivermectin standard treatments in this species.

342 Eleven spots were characterised by PMF and /or LC-MS/MS and database research,  
343 From those, eight were positively identified and related to three main proteins associated to  
344 reverse transport of cholesterol and lipid metabolism (apoA-I) (2), oxygen transport (beta-  
345 globin) (5), and ATP synthesis and transport (ATP synthase subunit beta) (1). These proteins  
346 have been previously identified and analysed in other organisms, including fish.

347 ApoA-I is the major protein component of high-density lipoprotein (HDL), and in  
348 general, is synthesised predominantly in the liver and/or intestine in mammals, birds and fish.  
349 Although its typical role is the reverse transport of cholesterol from tissues to the liver, recent  
350 studies on fish apoA-I show that this protein is involved in many other functions. It is also  
351 known that, apo A-I has antimicrobial activity (Johnston et al., 2008) and is implicated in  
352 innate immunity in fish (Villarroel et al., 2007). However, the apoA-I responsible for this  
353 function is more abundantly expressed in the epidermis, gills and intestinal mucosa, than in  
354 the liver and plasma. Additionally, apoA-I has been involved in osmotic regulation in fish  
355 (Chen et al., 2009)

356 In the present work, apoA-I decreased significantly in liver as a consequence of  
357 ivermectin treatment. However, in the measured transcript significant differences were not  
358 found. Direct assumption of co-expression of mRNA leading to co-expression of protein has  
359 been recently questioned (Wang, 2008).

360 Reduced expression of apoA-I has been observed in previous studies carried out in  
361 fish. Kleveland et al. (2006) found a down-regulation of mRNA expression of apoA-I in  
362 salmon liver fed with 3-thia fatty acids, and Chen et al. (2009) obtained significant down-

363 regulation at both protein and mRNA of apoA-I in an ayu, *Plecoglossus altivelis*, when  
364 transferred from freshwater to brackish water. To our knowledge, apoA-I has not been  
365 implicated with ivermectin toxicity, but since the main role of apoA-I in liver is related to lipid  
366 transport and metabolism, a decrease in liver apoA-I could be important in the development  
367 of fatty livers in ivermectin treated fish at prolonged treatments or higher doses, as reported  
368 for many other compounds (xenobiotics) like hydrazine (Klenø et al., 2004). It is known that  
369 hydrazine is a compound that causes a marked rise in the level of hepatic triglycerides  
370 (steatosis) in vivo. It has been pointed out that fatty livers of animals treated with hydrazine  
371 may be the result of decreased transport of lipids from the liver as apolipoproteins, due to low  
372 protein synthesis (Waterfield et al., 1997). In fact, Klenø et al. (2004) found a down  
373 regulation in liver apolipoprotein A-IV (apo A-IV) in hydrazine treated rats, suggesting that the  
374 observed reduction of liver apo A-IV might be relevant to the development of fatty livers in  
375 rats after hydrazine treatment.

376       Beta globin (recommended name: haemoglobin subunit beta) is involved in oxygen  
377 transport and also possesses scavenging properties with free radicals and reactive oxygen  
378 and nitrogen species. It is believed that some of these reactions may be related to the  
379 defence of organisms against oxidative stress (Herold and Fago, 2005). In vivo, hepatic beta  
380 globin has been found over expressed in juveniles of the Senegalese sole, *Solea*  
381 *senegalensis* affected by gas bubble disease, and it was related to oxidative damage (Salas-  
382 Leiton et al., 2009). We have found a reduction in the expression of beta globin at protein  
383 level but not at mRNA level after ivermectin treatment. Our findings may suggest that to a  
384 certain extent, ivermectin treatment could have affected the protection of the liver from  
385 oxidative stress, as described by Olsvik et al. (2008) in Atlantic salmon treated with  
386 emamectin benzoate. These authors have also reported that a standard seven-day treatment  
387 has little effect on the transcription of genes in liver, although, this compound seems to  
388 produce a temporary oxidative stress response that affects protein stability and folding,  
389 followed by a secondary inflammatory response.

390       The ATP synthase subunit beta (spot 3913) identified by Mascot search in protein  
391 databases (SwissProt/Uniprot, NCBI) in the present study has been described in a bamboo  
392 palm, *Chamaedorea seifrizzi* as reference organism (see Table 1). However, a new search  
393 using BLASTP algorithm (protein-protein BLAST) revealed a sequence producing significant  
394 alignments of this protein with the annotation BAE45286.1 in GenBank, corresponding to an  
395 ATP synthase beta subunit described in rainbow trout, *Oncorhynchus mykiss*. The  
396 expression level of ATP synthase subunit beta was down regulated after ivermectin  
397 treatment. This protein is found mostly at the inner side of the mitochondrial membrane, but it  
398 has also been located at the cell surface (Martinez et al., 2003; Bae et al., 2004; Kim et al.,  
399 2004). ATP synthase subunit beta is involved in ATP synthesis from ADP and phosphate in

400 the presence of a proton gradient across the membrane. Additionally, this protein is known to  
401 work as an apoA-I receptor in the plasma membrane to uptake high-density lipoprotein (HDL)  
402 into hepatocytes (Martinez et al., 2003). Previous studies have reported changes in the  
403 expression of ATP synthase subunit beta in relation to various toxicants (Chandra et al.,  
404 2005; Kim et al., 2008; Qui et al., 2008). The decrease in the level of ATP synthase subunit  
405 beta protein found may reflect an effect of the mitochondrial respiration and thus an  
406 expression of alteration in the cellular energy metabolism as described by Chandra et al.  
407 (2005). These authors found a reduction in ATP synthase subunit beta in mouse  
408 macrophage cells treated with anthrax lethal toxin, suggesting that this toxin alters the  
409 mitochondrial respiration and acts as an uncoupler of oxidative phosphorylation, in  
410 consequence targeting cellular energy metabolism and eventually leading to cell death. Also,  
411 it is possible that the decrease in liver ATP synthase subunit beta may be directly involved in  
412 oxidative stress responses of liver to ivermectin treatment, since this protein is considered a  
413 part of the oxidative stress responses (Ding and Ong, 2003; Malécot et al., 2009). Besides,  
414 the decrease in ATP synthase subunit beta could be the cause of the reduced levels of  
415 apoA-I found, and both proteins may be related with a transient oxidative stress response of  
416 liver to ivermectin treatment.

417 In conclusion, this study has shown that a habitual ten-day ivermectin treatment at  
418 the recommended dose of 0.2 mg kg<sup>-1</sup> fish induces some effects on the liver proteome of  
419 gilthead sea bream. The change in protein expression of a set of 36 proteins was able to  
420 separate medicated and control groups of fish, and may be considered as PES for  
421 hepatotoxicity of ivermectin in this species. Hepatic proteins identified are involved in lipid  
422 metabolism (apoA-I), oxidative stress responses and energy generation (beta-globin, ATP  
423 synthase subunit beta). The down regulation of the identified proteins suggested that  
424 ivermectin might induce a reduced capacity to protect liver from oxidative damage. Overall,  
425 the PES obtained and the proteins identified suggest a potential hepatotoxicity of ivermectin,  
426 at molecular level, after a standard treatment. The proteomic approach used in this study  
427 was found to be a very sensitive tool to pinpoint unapparent subtle effects of xenobiotics at a  
428 molecular level. These effects may be possibly regarded as transient as described for other  
429 avermectins (Olsvik et al., 2008). Further studies are however necessary to confirm this fact.

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441

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569 **Figure legends**

570 **Fig. 1.** Representative 2D-DIGE gel of soluble liver proteins extracted from gilthead sea  
571 bream (*S. aurata*). 3-10NL pH range were used for IEF. Protein spots differential expressed  
572 and identified are numbered as in table 2. In green dow-regulated spots and in red up-  
573 regulated spots.

574

575 **Fig. 2.** Multivariate analyses of liver proteomic data. (A) Principal Component Analysis (PCA)  
576 and hierarchical cluster analyses (B) of the proteins differentially expressed. PCA and the  
577 dendrogram after hierarchical analyses show a good separation of the spots maps  
578 corresponding to the different experimental groups (control in blue vs treated in red).

579

580 **Fig. 3.** Relative liver gene expression of beta-globin and apoA-I in control and ivermectin  
581 treated fish. Values are means  $\pm$  sd (n = 4-6). Statistical differences were checked by t-test  
582 with a p-value of 0.05.

583

584 **Fig. 4.** Histological sections of gilthead sea bream (*S. aurata*) from the liver of (A) control and  
585 (B) ivermectin treated fish.

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**Table 1**[Click here to download Table: Table 1.doc](#)**Table 1.** Forward and reverse primers used in the real-time quantitative PCR assay.

| Gene               | Accession number | Primer sequence  | Position           |
|--------------------|------------------|--|--------------------|
| Apolipoprotein A-1 | AF013120         | F GAA TAC AAG GAG CAG ATG AAG CAG ATG<br>R TGG TGA CGG AGG CAG CGA TG    | 664-690<br>808-789 |
| $\beta$ -globin    | AJ277207         | F TCA ATA TGG TCC AGT GGT CAG ATG C<br>R AGC CTG AGA AGT GTC TTT GAG TCC | 58-82<br>198-175   |
| $\alpha$ -tubulin  | AY326430         | F GAC ATC ACC AAT GCC TGC TTC<br>R GTG GCG ATG GCG GAG TTC               | 514-534<br>647-630 |

Table 2

[Click here to download Table: Table 2.doc](#)

**Table 2** – Protein identities differentially expressed in the gilthead seabream (*Sparus aurata*) liver after treatment with 0.2 mg Kg<sup>-1</sup> ivermectin for 10 days. p-value represents the significance level of t-test performed by the image analysis software DeCyder of up or down regulated spots.

| Spot n° | Protein name                          | Theo. MW/pi | Accession no.          | 2D-DIGE |                            | Protein identification |                  |          |               |        | Function    | Reference organism     |                              |
|---------|---------------------------------------|-------------|------------------------|---------|----------------------------|------------------------|------------------|----------|---------------|--------|-------------|------------------------|------------------------------|
|         |                                       |             |                        |         |                            | PMF (MALDI TOFTOF)     |                  |          | LC MS/MS      |        |             |                        |                              |
|         |                                       |             |                        | p-value | Av. Ratio (fold induction) | Score#                 | matched peptides | %seq cov | MSMS peptides | Score# | N° peptides |                        |                              |
| 3736    | Beta-globin (hemoglobin subunit beta) | 16340/9,07  | gil736322 (P56251)     | 0,0074  | -1,65                      | 193                    | 6                | 48       | 4             |        |             | Oxygen transport       | <i>Sparus aurata</i>         |
| 3776    |                                       |             |                        | 0,0133  | -2,1                       | 245                    | 7                | 60       | 3             |        |             |                        |                              |
| 3784    |                                       |             |                        | 0,0011  | -2,4                       | 183                    | 8                | 69       | 3             |        |             |                        |                              |
| 3838    |                                       |             |                        | 0,0090  | -1,84                      | 266                    | 8                | 61       | 3             |        |             |                        |                              |
| 3841    |                                       |             |                        | 0,0021  | -1,94                      | 172                    | 9                | 85       | 2             |        |             |                        |                              |
| 3993    | Apolipoprotein A-1                    | 29615/5,21  | gil2511712             | 0,0183  | -1,8                       | 95                     | 13               | 72       | 2             |        |             | Cholesterol metabolism | <i>Sparus aurata</i>         |
| 3992    |                                       |             |                        | 0,0011  | -1,41                      | 131                    | 9                | 49       | 1             |        |             |                        |                              |
| 3074    | unnamed protein product               | 27847/5,41  | gil47211357            | 0,0020  | -1,51                      |                        |                  |          |               | 51     | 1           | unknown                | <i>Tetradon nigroviridis</i> |
| 3760    | unnamed protein product               | 85031/9,32  | gil47228876            | 0,0035  | -1,45                      |                        |                  |          |               | 47     | 1           | unknown                | <i>Tetradon nigroviridis</i> |
| 3913    | ATP synthase subunit beta             | 53795/5,22  | gil34582342 (Q9MU80.1) | 0,0007  | -1,53                      | 66                     | 11               | 38       | 1             |        |             | ATP synthesis          | <i>Chamaedorea seifrizii</i> |
|         |                                       |             |                        |         |                            |                        |                  |          |               |        |             |                        |                              |

Accession n°: NCB n° (SWISS PROT n°)

Fig. 1

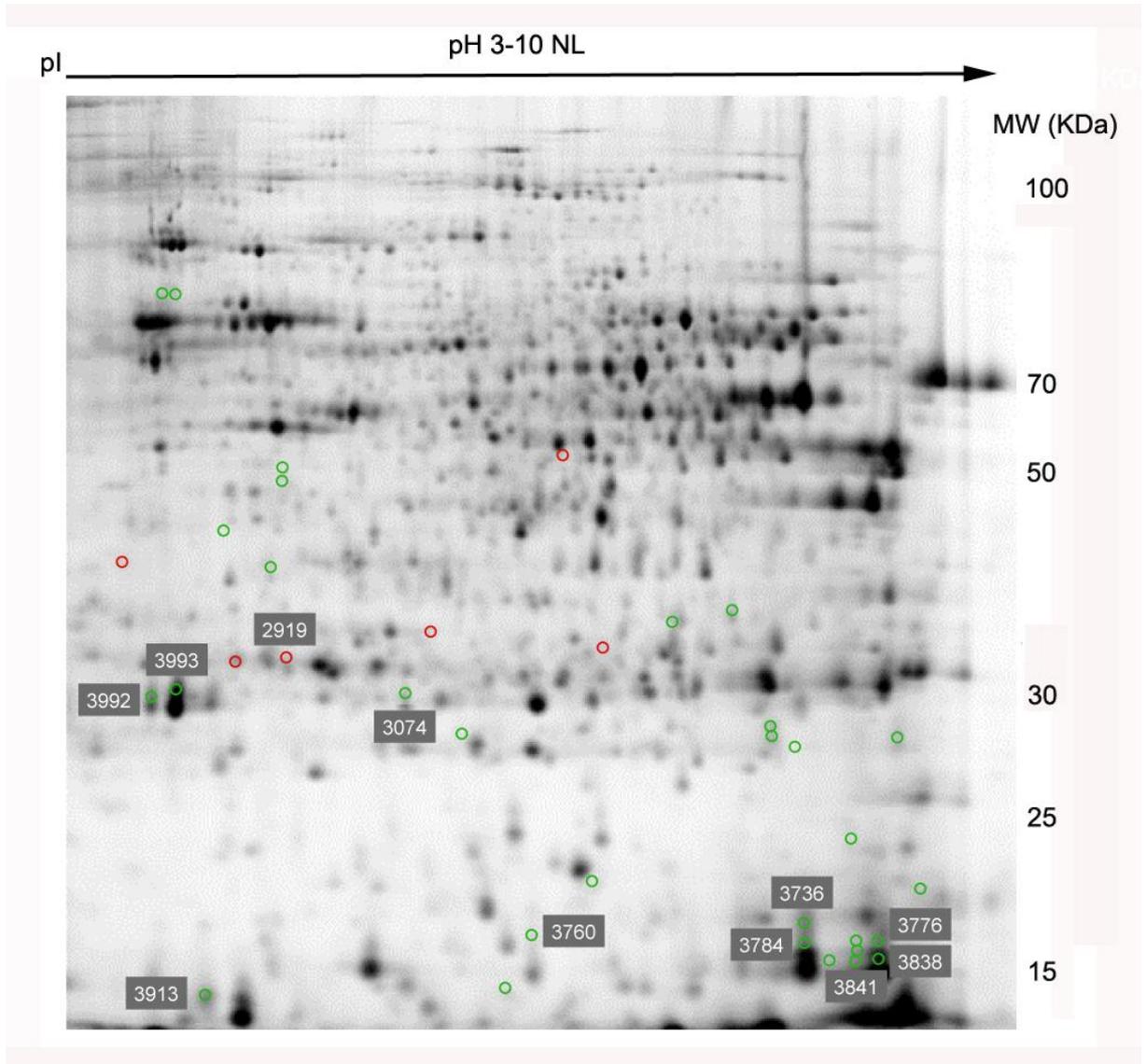


Fig. 2 (A)

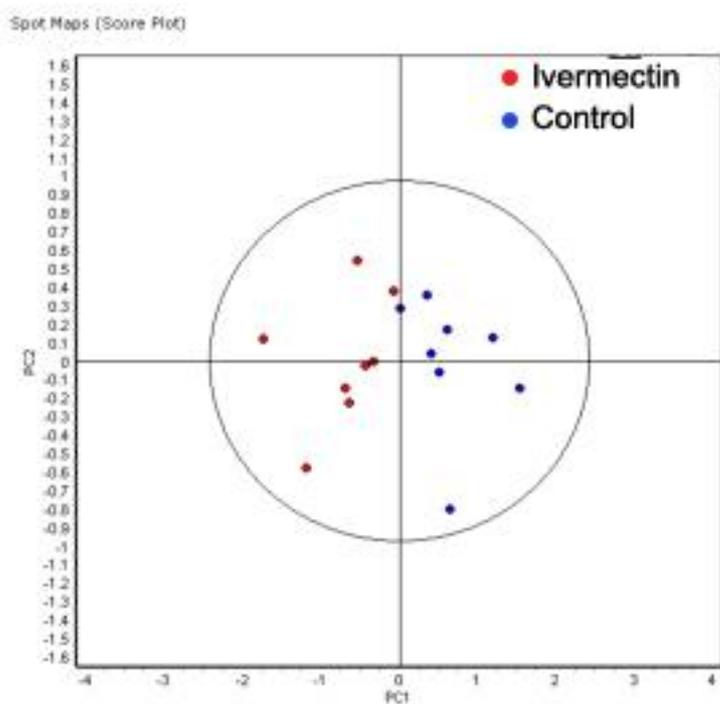


Fig. 2 (B)

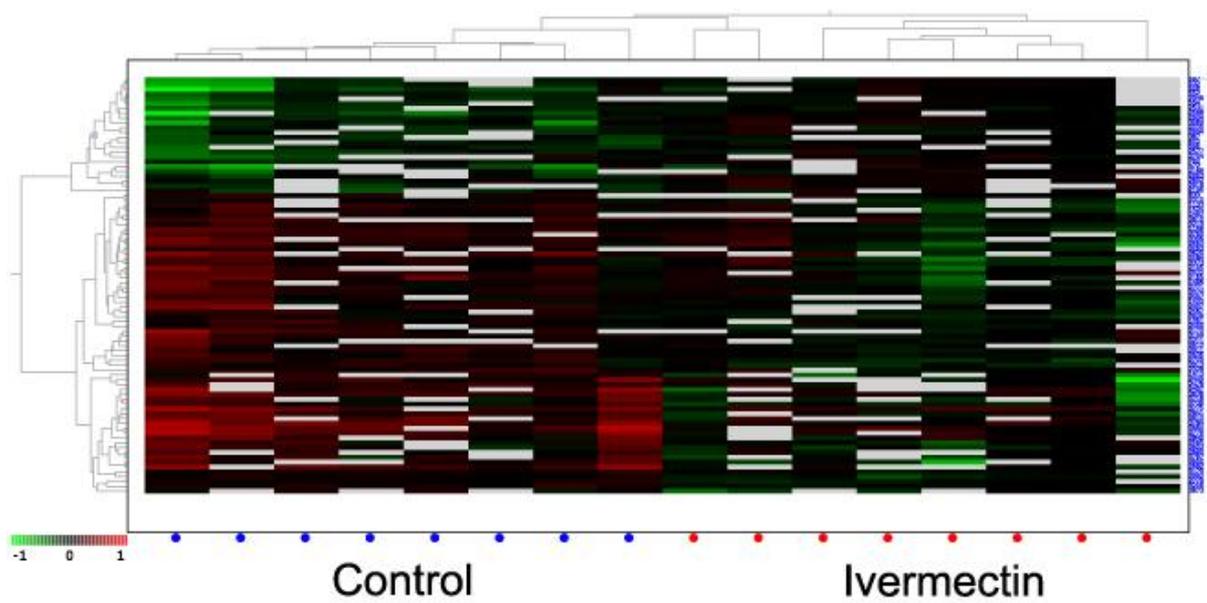


Fig. 3

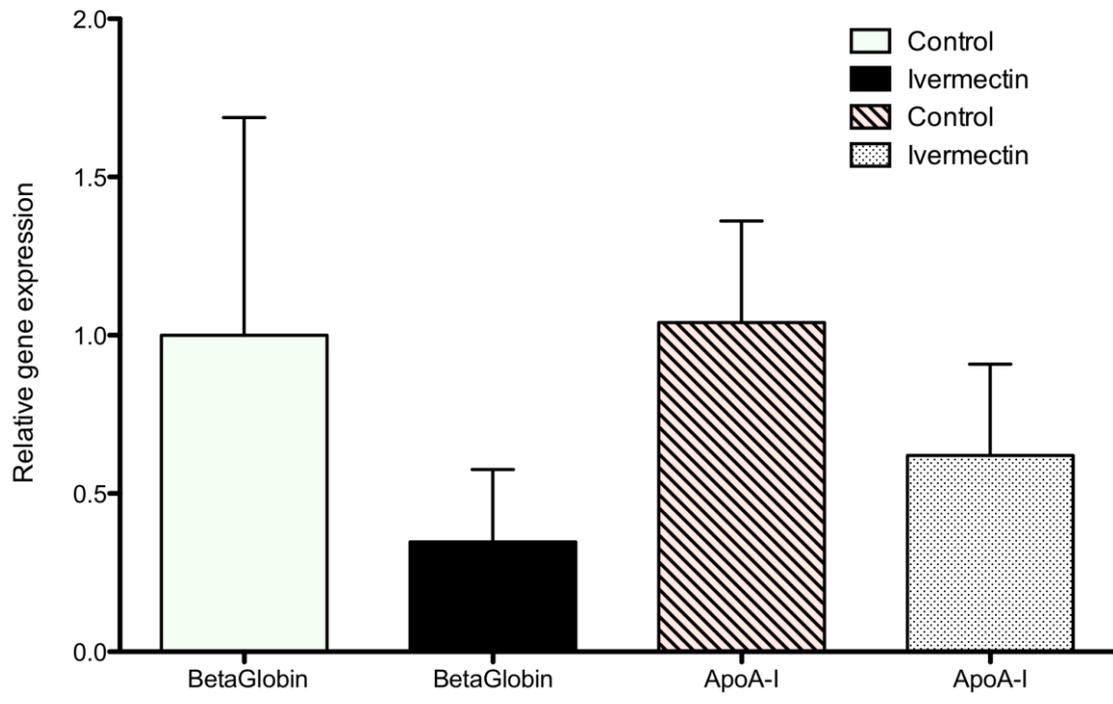


Fig. 4

