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

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ABSTRACT

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

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

Introduction

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

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

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

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

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

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

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

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

Materials and Methods

Animals and treatment

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

continuous aeration under room temperature (19 ± 0.5 ºC) in a closed circuit, and acclimated for 1 week before starting the experiment.

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

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

2-D difference gel electrophoresis (2D-DIGE)

Liver homogenization and protein labelling

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

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

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

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

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

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

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

Data analysis

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

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

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

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

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

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

The digestion mixture was dried in a vacuum centrifuge, resuspended in 7 µL of 0.1% TFA (trifluoroacetic acid, Sigma), and 1 µL was spotted onto the MALDI target plate. After the droplets were air-dried at room temperature, 0.5 µL of matrix (5 mg mL^{-1} CHCA (α-cyano-4-hydroxycinnamic acid, Sigma) in 0.1% TFA-ACN/H2O (1:1, v/v) was added and allowed to air-dry at room temperature. The resulting 576 fractions were analyzed in a 4700 Proteomics Analyzer (Applied Biosystems, Foster City, USA) in positive reflection mode (2000 shots every position). Five of the most intense precursors (according to the threshold criteria: minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, maximum fraction gap: 4) were selected for every position for the MS/MS analysis. And, MS/MS data corresponding to the control was acquired using the default 1 kV MS/MS method.

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

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

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

Quantitative RT-PCR of selected proteins

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

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

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

Histology

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

Results

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

experiment (pH = 3-11NL) and with a molecular weight from approximately 10 to 250 kDa were resolved, and 3993 spots in the master gel were detected using the DeCyder BVA software. Each protein spot was assigned an average ratio (i.e. change in expression level due to ivermectin treatment) and *p*-value (Student´s t-test) to indicate the level of significance. A total of 36 protein spots showed significant changes in the expression compared to the control group (standardized average volume ratio ≥ 1.3, t-student ≤ 0.02). This represented about 1% of the total protein spots analysed. Among these protein spots, 6 were up regulated and 30 down regulated by ivermectin. The positions of those differentially expressed protein spots in the 2D-DIGE are shown in Figure 1**.** The PCA and hierarchical cluster analyses of data are shown in Figure 2. The PCA results obtained indicated that two components are enough to cluster the different experimental groups (control *vs* treated), with a clear separation in the first component (PC1) between them (Fig 2A). The pattern analyses showed a clustering in a hierarchical way, where protein maps corresponding to control group formed a cluster separated from those corresponding to the ivermectin treated group (Fig 2B)

The results of protein identification by PMF and/or LC-MS/MS and data base research are listed in Table 2**.** The 2D-DIGE analyses (spot nº, protein name, MW/pI, gi accession number, p-value, and the average ratio/fold change) are also indicated in Table 2. Ten differentially expressed proteins were identified. It was found that five of theses proteins are related to the oxygen transport: beta globin (spots 3736, 3776, 3784, 3838 and 3841), two proteins are involved in the cholesterol metabolism: apo A-I (spot 3993 and 3992), and one protein is related in ATP synthesis: ATP synthase beta chain (spot 3913). All of them were down regulated. Also, ivermectin induced remarkable changes in two proteins that have unknown function and were down regulated (spots 3074 and 3760).

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

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

Discussion

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

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

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

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

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

Reduced expression of apoA-I has been observed in previous studies carried out in fish. Kleveland et al. (2006) found a down-regulation of mRNA expression of apoA-I in 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 invermectin 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

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

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

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Figure legends

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

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

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

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

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	664-690
		R	TGG TGA CGG AGG CAG CGA TG	808-789
ß-globin	AJ277207	F	TCA ATA TGG TCC AGT GGT CAG ATG C	58-82
		R	AGC CTG AGA AGT GTC TTT GAG TCC	198-175
α-tubulin	AY326430	F	GAC ATC ACC AAT GCC TGC TTC	514-534
		R	GTG GCG ATG GCG GAG TTC	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^{-1} 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 nº	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#	Nº peptides		
3736	Beta-globin (hemoglogin subunit beta)	16340/9,07	gi|736322 (P56251)	0,0074	-1,65	193	6	48	4			Oxygen transport	*Sparus aurata*
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	gi|2511712	0,0183	-1,8	95	13	72	2			Cholesterol metabolism	*Sparus aurata*
3992		29615/5,04		0,0011	-1,41	131	9	49	1				
3074	unnamed protein product	27847/5,41	gi|47211357	0,0020	-1,51					51	1	unknown	*Tetradon nigroviridis*
3760	unnamed protein product	85031/9,32	gi|47228876	0,0035	-1,45					47	1	unknown	*Tetradon nigroviridis*
3913	ATP synthase subunit beta	53795/5,22	gi|34582342 (Q9MU80.1)	0,0007	-1,53	66	11	38	1			ATP synthesis	*Chamaedorea seifrizii*

Accession nº: NCB nº (SWISS PROT nº)

Figure 1

Fig. 1

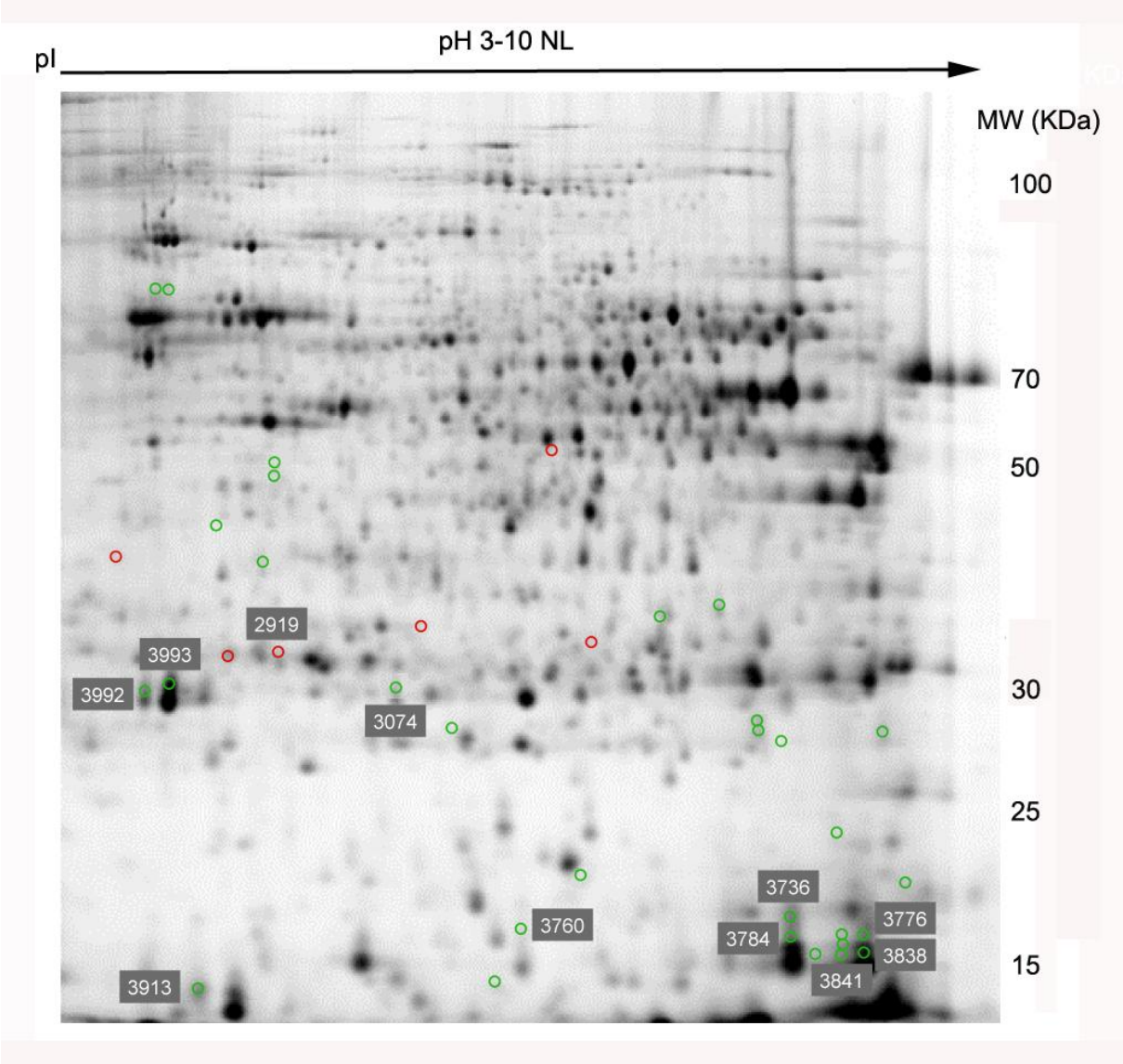

Figure 2

Fig. 2 (A)

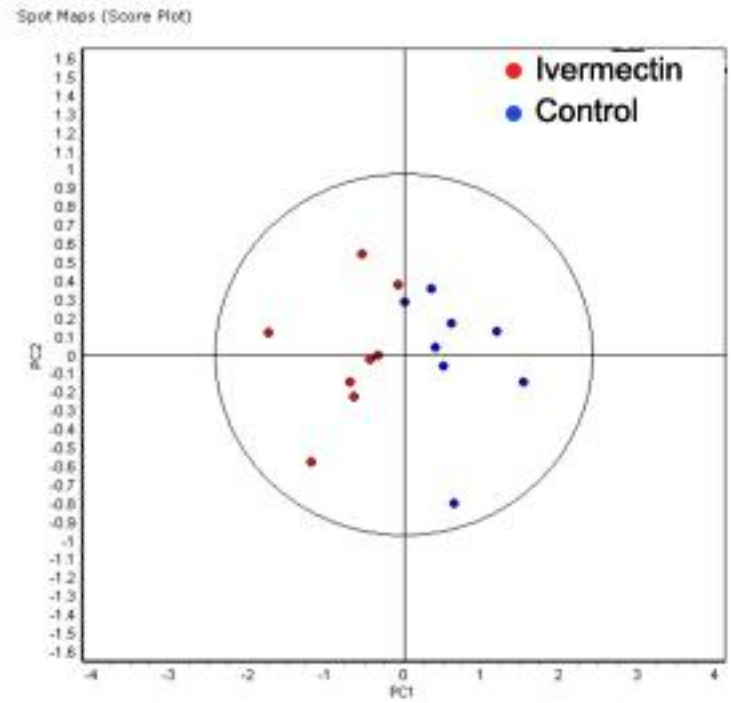

Fig. 2 (B)

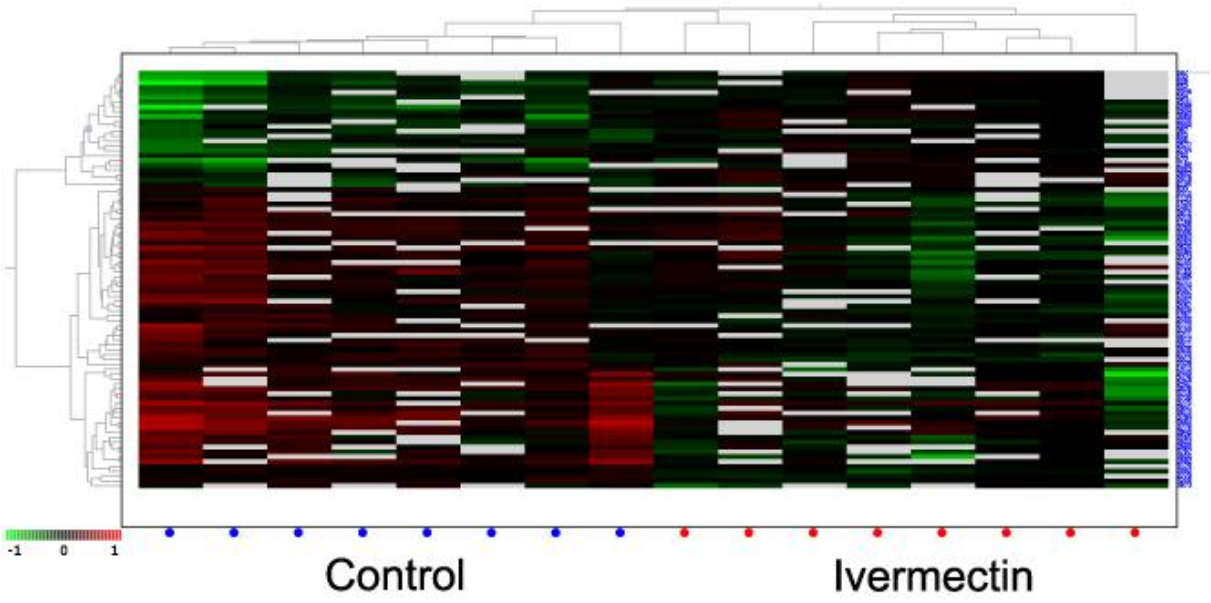

Figure 3

Fig. 3

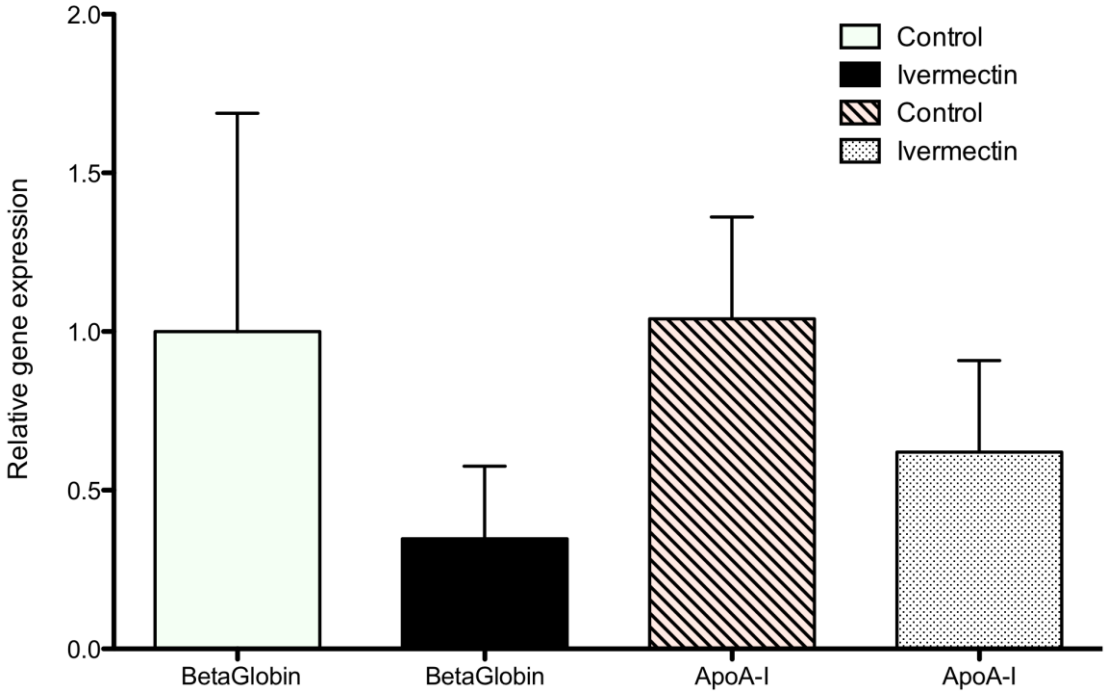

Figure 4

Fig. 4