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Hepatic Proteome Analysis of Atlantic Salmon (*Salmo salar*) After Exposure to Environmental Concentrations of Human Pharmaceuticals*[S]

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Pharmaceuticals are pseudopersistent aquatic pollutants with unknown effects at environmentally relevant concentrations. Atlantic salmon (*Salmo salar*) were exposed to Acetaminophen: 54.77 ± 34.67; Atenolol: 11.08 ± 7.98, and Carbamazepine: 7.85 ± 0.13 $\mu g \cdot L^{-1}$ for 5 days. After Acetaminophen treatment, 19 proteins were differently expressed, of which 11 were significant with respect to the control group (eight up-regulated and three down-regulated). After Atenolol treatment, seven differently expressed proteins were obtained in comparison with the control, of which six could be identified (four up-regulated and two down-regulated). Carbamazepine exposure resulted in 15 differently expressed proteins compared with the control, with 10 of them identified (seven up-regulated and three down-regulated). Out of these, three features were common between Acetaminophen and Carbamazepine and one between Carbamazepine and Atenolol. One feature was common across all treatments. Principal component analysis and heat map clustering showed a clear grouping of the variability caused by the applied treatments. The obtained data suggest (1) that exposure to environmentally relevant concentrations of the pharmaceuticals alters the hepatic protein expression profile of the Atlantic salmon; and (2) the existence of treatment specific processes that may be useful for biomarker development. ***Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.045120, 371–381, 2015.***

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Molecular approaches in ecotoxicology have greatly enhanced mechanistic understanding of the impact of aquatic pollutants in organisms. High throughput "omic" technologies, including quantitative proteomics methods such as 2D differential in-gel electrophoresis (DIGE), are now being acknowledged to be a promising tool to evaluate the effects of contaminant exposure on organisms and are becoming more widely used in ecotoxicology (1). Information on altered protein expression, including post-transcriptional modifications, can provide protein expression signatures, sets of proteins specific to different stressors, and insight into the possible mode of action (MoA)[1] of chemical pollutants and their higher level toxicological effects (2–5). Contaminant specific MoAs and protein expression signatures may be used for monitorization, especially because the use of omic techniques allows earlier identification of effects than traditional endpoints. However, ecotoxicoproteomic studies are still relatively uncommon and in their infancy (6).

Human and veterinary pharmaceuticals are being released into the environment in extremely large quantities on a regular basis. Millions of prescription and nonprescription drugs are purchased and ingested by, or applied on individuals every day and eventually excreted through urine or feces, ultimately entering the effluent of wastewater treatment plants and aquatic environments (7). Frequently, sewage treatment does not affect the chemical structure, and therefore the concentrations of drugs that enter aquatic environments can be sufficient to exert toxicity on nontarget species. Most pharmaceuticals are relatively stable to avoid being biologically

[1] The abbreviations used are: MoA, Mode of Action; APAP, Acetaminophen; AT, Atenolol; CBZ, Carbamazepine; CSIC, Spanish National Council for Scientific Research; CAS N°, Chemical Abstracts Service Number; LOD, Limits of detection; LOQ, limits of quantification; DIA, Differential In-gel Analysis; BVA, Biological Variation Analysis; NCBI, National Center for Biotechnology Information; MASCOT, software search engine that uses mass spectrometry data to identify proteins from primary sequence databases; PCA, Principal Component Analysis; CTRL, Control; EDA, Extended Data Analysis; PC, Principal Components; LC_{50}, 50% Lethal Concentration; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; MPT, mitochondrial permeability transition; EF, Elongation Factor; cAMP, 3′-5′-cyclic adenosine monophosphate; NOEC, no observed effect concentration.

inactivated before carrying out their intended biological function (8). However, this stability means that they also persist and accumulate in abiotic and biotic compartments of ecosystems, potentially creating environmental problems (9). In fact, some major pharmaceutical compounds are now considered to be included in the priority list of the Water Framework Directive of the European Union (2000/60/EC). The exact effects that exposure to environmentally relevant concentrations of pharmaceuticals is having on ecosystems, biota, and also humans who may consume contaminated water or organisms are still not completely understood. Risk assessments have shown that some pharmaceuticals have the potential to cause adverse human and environmental effects from indirect exposure (8, 10–12). For the vast majority however, including most metabolites, important knowledge gaps still exist concerning long-term effects on nontarget organisms.

Some of the most pervasive groups of pharmaceuticals that are currently found in aquatic environments are analgesics, β-blockers and anti-epileptics (13–17). In this study, we have chosen Acetaminophen (APAP; analgesic), Atenolol (AT; β-blocker), and Carbamazepine (CBZ; anti-epileptic) as model compounds to assess the effects on the liver proteome of Atlantic salmon, *Salmo salar*, after exposure to environmentally relevant concentrations. APAP is a non-steroid anti-inflammatory and analgesic drug, used as painkiller and to reduce inflammations and fevers. The exact molecular processes are not known yet, and only a limited number of pathways have been identified (18, 19). Effects of APAP on gene and protein expression in rodents have been published elsewhere (20–22). AT is a selective β-adrenergic receptor antagonist or β-blocker for the treatment of angina, glaucoma, heart failure, high blood pressure, and other related conditions (23–25). An extensive review about the comparative physiology, pharmacology, and toxicology of β-blockers, including AT, in fish has been published recently (26). CBZ is a mood-stabilizing treatment for bipolar affective disorder. The molecular mechanisms underlying the actions of CBZ and the cause of the illness itself are unknown. However, several biochemical pathways have been postulated as possible targets of mood stabilizing drugs (27–31).

Salmonids are frequently employed in effects evaluation of environmental contaminants, and Atlantic salmon are a culturally and economically important sentinel species in many North-West European rivers. However, wild stocks of Atlantic salmon are increasingly endangered (32). A variety of ecological and climatic reasons for this have been proposed, but there is little doubt that declines in water quality are a major threat. The juvenile stages of Atlantic salmon spend a year or more in rivers, before migrating to the sea, and during these stages salmon may be particularly vulnerable to contamination by pharmaceuticals, and other chemicals, especially when river levels are low. The purpose of this study is to determine sets of proteins, in the liver of Atlantic salmon parr, whose expression is changed in response to environmentally relevant concentrations of APAP, AT, or CBZ. An ontologic analysis of the salmon proteome associated with pharmaceutical treatment can provide in-depth mechanistic information of the molecular MoA and the function of the altered proteins. Furthermore, those proteins that are highly regulated will be useful as candidates for development as biomarkers in environmental monitoring exercises, with potential for indicating exposure to specific pharmaceuticals, and thus providing an early indication of the ecological risk posed by pharmaceutical contaminant discharge.

EXPERIMENTAL PROCEDURES

Ethics Statement—All procedures were performed under license to, and in accordance with United Kingdom Home Office regulations governing animal experimentation, and following oversight by an institutional ethics review committee.

Exposure—APAP (CAS N°: 103-90-2), AT (CAS N°: 29122-68-7), and CBZ (CAS N°: 298–46-4) were purchased from Sigma. Atlantic salmon parr (approximately one year old) were purchased from the Stirling University aquaculture facility (Howietown Fish Farm, Stirling, UK). The fish were acclimated to laboratory conditions for 14 days. After that, ~15 fish per treatment were exposed to environmentally relevant concentrations of the pharmaceuticals for 5 days under continuous flow through conditions. The concentrations were chosen based on maximum levels detected in various European freshwater bodies (13–17) and the exposure time of 5 days was selected as an approximation of low water conditions in a natural riverine environment. The experiments were carried out in duplicate and control trials were run simultaneously. A peristaltic pump supplied the compounds from daily renewed working stock solutions. Water flow through the system was adjusted to 360 $L \cdot d^{-1}$. Water samples (250 ml) were collected from each tank at days 1, 3, and 5, and stored at 4 °C not longer than 24 h until their pretreatment for posterior analysis by high performance liquid chromatography (HPLC). After 5 days of exposure, all fish were killed by a blow to the head, sexed, and final weights, tissue weights, and length were measured. Liver tissues were immediately frozen and stored at −70 °C.

Exposure Concentration Analysis—Exposure concentrations were measured as described elsewhere (33). After solid phase extraction (OASIS HLB; 60 mg, 3 ml; Waters, Milford, MA), analytes were separated under isocratic conditions with acetonitrile and 50 mM potassium dihydrogen phosphate solution. APAP and CBZ were measured using the UV signal at 250 nm, AT using the fluorescence signal at 271 nm. Compounds were identified and quantified by comparing retention times and peaks in samples and standard solution chromatograms. Limits of detection (LOD) and limits of quantification (LOQ) were calculated by using a signal-to-noise ratio of 3 and 10, respectively.

Proteomic Analysis—Frozen liver tissues were homogenized in lysis buffer (7 M urea, 2 M Thiourea, 4% CHAPS, and 30 mM Tris, pH 8.5) on ice using a glass mortar with pestle for protein solubilization. Following homogenization, the tissue lysates were centrifuged at 14,000 × *g* for 10 min at 4 °C to remove any insoluble particles. The supernatant was then transferred into 50 μL aliquots and stored at −80 °C until gel electrophoresis was performed. Protein concentration was determined by the method based on Bradford (Bio-Rad Protein Assay, Hercules, CA) and adjusted to 5 $\mu g \cdot \mu L^{-1}$ by dilution with lysis buffer. 24 cm long immobilized pH gradient (pH 3–11NL, GE Healthcare) IPG strips were rehydrated overnight in 450 μL DeStreak with 2.25 μL IPG buffer (pH 3–11NL) added (both GE Healthcare). After rehydration, five individual protein samples (biological replicates; 50 μg for each sample) per treatment were labeled with 400

pmol Cy3 or Cy5 minimal NHS ester dyes (GE Healthcare) allocated randomly to provide both Cy3 and Cy5 labeled samples for each treatment. A pool of material from all 20 samples was labeled with Cy2 (GE Healthcare). Different pairs of Cy3- and Cy5-labeled samples (randomly allocated and each containing 50 μg of protein) were combined and mixed with a 50-μg aliquot of the Cy2-labeled pooled standard. These mixtures were diluted 1:1 with lysis buffer containing 0.5% IPG buffer (pH 3–10) and then applied onto the IPG strips. Isoelectric focusing (IEF, first dimension) was carried out on an IPGphor system (GE Healthcare) in four stages with a ramped voltage change between each step: step and hold: 30V, 12 h; gradient 300V, 1 h; step and hold: 300V, 1 h; gradient: 1000V,1 h; step and hold: 1000V, 1 h; gradient: 8000V, 2 h; step and hold: 8000V, 8 h. Focusing was stopped after a total of 70,000 Vh accumulated. After IEF, the IPG strips were equilibrated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.05 M Tris-Cl at pH 8.8, 50% glycerol, and 2% DTT) followed by 15 min in equilibration buffer II (same as buffer I but containing 2.5% iodacetamide instead of DTT and 0.02% bromphenol blue). The second dimensional separations were carried out on 12.5% SDS-polyacrylamide gels on the Ettan DaltSix system (GE Healthcare) at 1W/gel for one hour and subsequently at 15W/gel.

Image Analysis—Labeled proteins were visualized using the Typhoon 9000 series imager (GE Healthcare) and the Cy2, Cy3, and Cy5 components of each gel were individually imaged using excitation/emission wavelengths specific for Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). PMT was varied in order to equalize fluorescence intensities between channels and to prevent over saturation of the signal. Background subtraction, quantitation, normalization, and first-level-of matching (within gel) was performed by Differential In-gel Analysis (DIA) using DeCyder 2D Differential Analysis Software v6.5 (GE Healthcare). For each gel, normalized spot volumes (area multiplied by density of the spot) were calculated as the ratio of each spot volume to total spot volume in the gel. Within the Biological Variation Analysis (BVA) module, each drug treatment set was combined, compared with the control treatment set (*t* test) and spots with a p value <0.05 returned.

In-gel Digestion and Mass Spectrometry—A preparative gel with 300 μg of protein was used for spot picking and visualized with silver staining to pick spots of interest. The gel pieces were destained and digested by trypsin (Promega, Madison, WI). Upon concentrating and desalting tryptic fragments using Millipore C18 ZipTips (Millipore, Bedford, MA), samples were mixed in a 1:1 ratio with α-cyano-4-hydroxycinnamic acid matrix (saturated solution in 50% ACN and 2.5% TFA) and spotted onto the target plate. MS/MS analyses were performed on a 4800 MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA). Measurements were taken in the positive ion mode between 900 and 3000 *m*/z. Sequences were automatically acquired by scanning first in peptide mass fingerprint (MS) mode. A database search (NCBI nr) was performed, combining the results of peptide mass fingerprint (MS) with subsequent fragmentation (MS/MS) of up to twelve peptides from each spot according to the quality of the MS spectrum, using MASCOT (Version 2.0.00, release date: 19.02.2007) as a search engine. Scores greater than the given cutoff value for MS/MS fragmentation data were taken as significant ($p < 0.05$). Protein lists were submitted to Venn analysis (http://bioinfogp.cnb.csic.es/tools/venny/) to detect treatment specific and/or common features.

Data Evaluation by Multivariate Statistical Analyses—Multivariate analyses were performed on datasets constituted by the relative levels of all the spots that were consistently matched between the 20 gels, in order to avoid the replacement of null values by inference. Principal component analysis (PCA) was based on the obtained spots of interest where hierarchical clustering was performed on all differently expressed spots. PCA and cluster analysis were carried out using the DeCyder software package (DeCyder 2D Differential Analysis Software v6.5; GE Healthcare).

RESULTS

Exposure Concentration—The measured average concentrations of the three selected pharmaceuticals were: APAP: 54.77 $\pm$ 34.67 $\mu g \cdot L^{-1}$; AT: 11.08 $\pm$ 7.98 μg/L, and CBZ: 7.85 $\pm$ 0.13 μg/L. All fish survived the 5-day trial.

Protein Expression Analysis—In order to identify sets of proteins that respond to different pharmaceutical exposure in Atlantic salmon, we compared the liver proteome of the four experimental groups (CTRL, APAP, AT, and CBZ) using 2D-DIGE. One representative gel for each experimental condition is shown in Fig. 1*A*–1*D*. A total of 53 significantly differently expressed spots were acquired in a reproducible way across all gels and after statistical analysis (*t* test, $p \leq 0.05$) significant differences in several spots were detected between the experimental groups. For the APAP treatment, 19 spots were differently expressed, of which 11 were significant with respect to the control group. Eight of these 11 spots were up-regulated, whereas only three were down-regulated. After AT treatment, seven differently expressed spots were obtained in comparison with the control of which six could be identified. Four of them were up-regulated and two down-regulated. CBZ exposure resulted in 15 differently expressed spots compared with the control, with 10 of them identified. In this last treatment, seven features were up-regulated and three down-regulated. Tables I, II, and III show the results of protein spot identification for the APAP, AT, and CBZ treatment, respectively. The 78kDa glucose regulated protein observed in the AT treatment (Table II) is the feature with the highest absolute change, down-regulated 2.469-fold. Three features were common between different treatments (Fig. 2): the phosphoglycerate kinase 1 (gi 197631857) was up-regulated in all three treatments (APAP: 1.722; AT: 1.307, and CBZ: 1.754 and 1.491). Two other features were represented both in the APAP as well as in the CBZ treatment: acetyl-CoA acetyltransferase, cytosolic (gi 213513638), which was up-regulated during the APAP treatment 1.412 times and during the CBZ treatment 1.279 times, and the glyceraldehyde-3-phosphate dehydrogenase (gi 209737954), which was up-regulated during the APAP treatment 1.484 times, whereas during the CBZ treatment it was down-regulated 1.704 times. Obtained fold changes throughout all the treatments were relatively low, and absolute values comprised between 1.2 (threshold setting) and 2.5.

Multivariate Analyses—Data were analyzed by two different multivariate analysis methods using the DeCyder-Extended Data Analysis (EDA) module. Principal component analysis (PCA) was applied to visualize the differences in expression patterns in the different spot maps from treated and untreated organisms (Fig. 3).

By analyzing the first two PCs a total separation of the different spot maps into the four different groups is observed,

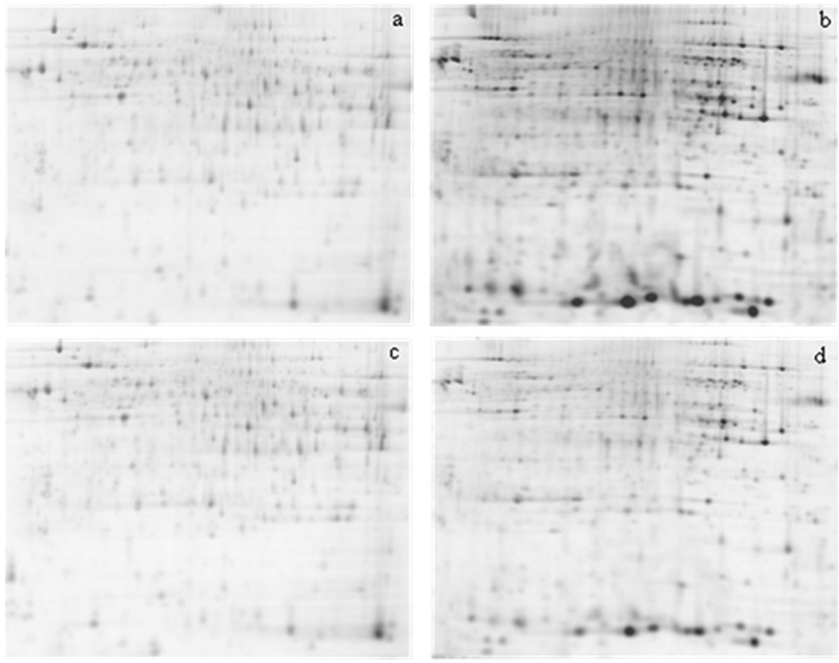

FIG. 1. **Representative 2D DIGE gel images for the three treatments a) APAP, b) AT, c) CBZ and d) control.** Each multiplexed gel was charged with 50 μg of protein per dye.

TABLE I
Identification of significantly altered proteins after APAP treatment by Mass Fingerprint approach

# Master spot	Putative identification	Homology to protein (Genebank)	Protein score[a]	Protein score C.I. %	Protein MW	Protein PI	Peptide count	Total ion score	Total ion C.I. %	T-test value	Fold change
556	Transketolase	*Salmo salar* gi 213511480	114	100	68896.6	6.18	15	24	0	0.0151	1.978
899	ATP synthase H+ transporting mitochondrial F1 complex beta	*Salmo salar* gi 198285477	105	99.998	52910.6	4.87	11	33	56.937	0.0061	1.639
952	Elongation factor 1-gamma	*Salmo salar* gi 213515528	232	100	34931.8	9.19	11	164	100	0.0081	−1.414
1052	Phosphoglycerate kinase 1	*Salmo salar* gi 197631857	315	100	44917.1	8.31	11	257	100	0.0004	−1.722
1053	RNA polymerase beta subunit protein	*Rhododendro n Simsii* gi 290489550	73	97.18	156861.6	9.05	18	–	–	0.0458	−1.534
1061	unknown	*Arabidopsis thaliana* gi 116830485	69	92.582	8922.7	9.3	6	–	–	0.0055	−1.423
1122	Acetyl-CoA acetyltransferase, cytosolic	*Salmo salar* gi 213513638	243	100	41722	8.07	8	196	100	0.0024	−1.412
1177	OsmC-like family protein	*Dichelobacter nodosus VCS1703A* gi 146329847	73	97.428	16299	5.22	8	–	–	0.0359	−1.649
1284	Glyceraldehyde-3-phosphate dehydrogenase-1	*Salmo salar* gi 209737954	468	100	36097.4	7.12	21	238	100	0.0309	−1.51
1408	Caspase-3	*Salmo salar* gi 213513742	94	99.975	31376.4	5.97	10	–	–	0.0109	1.541
1788	Peptidyl-prolyl cis-trans isomerase B precursor	*Salmo salar* gi 209735348	325	100	23993.6	9.18	11	222	100	0.004	−1.484

[a] Mascot score: Probability Based Mowse Score: Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 83 are significant ($p < 0.05$). MALDI-TOF-TOF peak lists were searched against a NCBInr 10997816 protein database using MASCOT ™ software with the following settings: Type of search: Sequence Query; Enzyme: Trypsin; Fixed modifications: Carbamidomethyl (C); Variable modifications: Oxidation (M); Mass values: MONOISOTOPIC; Protein Mass: Unrestricted; Peptide Mass Tolerance: ± 100 ppm; Fragment Mass Tolerance: ± 0.2 Da; Max Missed Cleavages: 1; Instrument type: MALDI-TOF-TOF.

with the treatments APAP and CBZ more separated from each other than the AT and Control group. These latter treatments, although also clearly separated, show a slight overlap and are positioned closer to the coordinate intersection than the other two treatments. The control group was separated from the rest of the treatments in the first component (PC1) occupying the most negative position. The rest of the treatments are more placed toward positive values, with a clear vertical sep-

TABLE II
Identification of significantly altered proteins after AT treatment by Mass Fingerprint approach

# Master spot	Putative identification	Homology to protein (Genebank)	Protein score	Protein score C.I. %	Protein MW	Protein PI	Peptide count	Total ion score	Total ion C.I. %	T-test value	Fold change
105	Elongation factor 2	*Salmo salar* gi 223647986	212	100	96406.4	6.19	21	76	99.995	0.0462	−1.748
265	78kDa glucose/regulated protein Salmo salar	*Salmo salar* gi 213511032	538	100	72539.3	5	28	288	100	0.0042	2.469
813	GL16441	*Drosophila persimilis* gi 195164237	76	98.619	21738.9	10.33	10	–	–	0.0121	−1.498
1052	Phosphoglycerate kinase 1	*Salmo salar* gi 197631857	315	100	44917.1	8.31	11	257	100	0.0002	−1.307
1539	Branched chain amino acid aminotransferase	*Zunongwangia profunda SM-A87* gi 295134966	73	97.244	40105.2	5.63	11	–	–	0.0359	1.423
1782	hypothetical protein	*Salmo salar* gi 281416447	69	92.751	6919.7	9.4	4	29	0	0.0414	−1.526

Mascot score: Probability Based Mowse Score: Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 83 are significant ($p < 0.05$). MALDI-TOF-TOF peak lists were searched against a NCBInr 10997816 protein database using MASCOT ™ software with the following settings: Type of search: Sequence Query; Enzyme: Trypsin; Fixed modifications: Carbamidomethyl (C); Variable modifications: Oxidation (M); Mass values: MONOISOTOPIC; Protein Mass: Unrestricted; Peptide Mass Tolerance: ± 100 ppm; Fragment Mass Tolerance: ± 0.2 Da; Max Missed Cleavages: 1; Instrument type: MALDI-TOF-TOF.

TABLE III
Identification of significantly altered proteins after CBZ treatment by Mass Fingerprint approach

# Master spot	Putative identification	Homology to protein (Genebank)	Protein score	Protein score C.I. %	Protein MW	Protein PI	Peptide count	Total ion score	Total ion C.I. %	T-test value	Fold change
272	Elongation factor 2	*Salmo salar* gi 223647986	212	100	96406.4	6.19	21	76	99.995	0.0087	−1.644
395	Contactin 1a precursor	*Danio rerio* gi 136256388	70	94.749	115044.4	6.19	15	–	–	0.0168	−1.576
404	TenA family transcriptional activator	*Marinomonas sp. MWYL1* gi 152997829	71	95.632	25638.9	5.16	9	–	–	0.0285	−1.652
559	Transketolase	*Salmo salar* gi 213511480	165	100	68896.6	6.18	20	–	–	0.0406	1.445
1020	Phosphoglycerate kinase 1	*Salmo salar* gi 197631857	315	100	44917.1	8.31	11	257	100	0.0272	−1.754
1027	Fumarylacetoacetase	*Osmerus mordax* gi 225706644	71	95.829	49875.9	6.27	5	53	99.485	0.0211	1.87
1052	Phosphoglycerate kinase 1	*Salmo salar* gi 197631857	315	100	44917.1	8.31	11	257	100	0.0272	−1.754
1061	unknown	*Arabidopsis thaliana* gi 116830485	69	92.582	8922.7	9.3	6	–	–	0.0281	−1.206
1122	Acetyl-CoA acetyltransferase, cytosolic	*Salmo salar* gi 213513638	243	100	41722	8.07	8	196	100	0.0057	−1.279
1323	Glyceraldehyde-3-phosphate dehydrogenase	*Salmo salar* gi 209737954	468	100	36097.4	7.12	21	238	100	0.0006	1.704

Mascot score: Probability Based Mowse Score: Ions score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 83 are significant ($p < 0.05$). MALDI-TOF-TOF peak lists were searched against a NCBInr 10997816 protein database using MASCOT ™ software with the following settings: Type of search: Sequence Query; Enzyme: Trypsin; Fixed modifications: Carbamidomethyl (C); Variable modifications: Oxidation (M); Mass values: MONOISOTOPIC; Protein Mass: Unrestricted; Peptide Mass Tolerance: ± 100 ppm; Fragment Mass Tolerance: ± 0.2 Da; Max Missed Cleavages: 1; Instrument type: MALDI-TOF-TOF.

aration (PC2). The second component (PC2) separated the rest of the experimental groups that were also clustered individually, although a slight overlap between the treatments AT and CBZ could be observed. Also, a pattern analysis generating a heat map was carried out (Fig. 4) with manual base set where including those spots that were present in > 75% of the spot maps. Unassigned spots were removed and the applied normalization comprised scaling corresponding to the subtraction of the internal reference from the control. This resulted in 1273 spots that were represented in the heat map. The pattern analysis revealed a hierarchical clustering where the control group and the APAP treatment showed the most pronounced difference. Control and AT, as well as APAP and CBZ showed a closer relationship to each other than to the rest, forming, in both groups, two separate clusters.

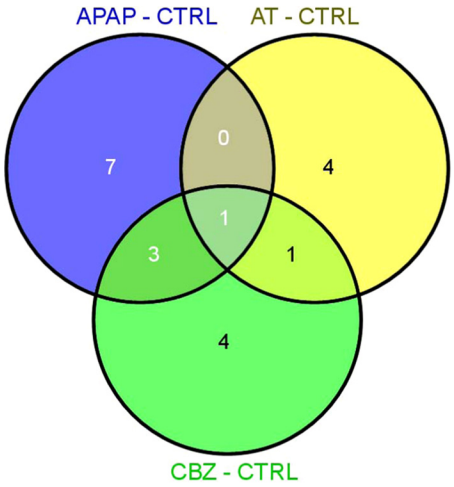

FIG. 2. Venn diagram representation of differentially expressed proteins for each treatment in comparison to the control. Individuals were exposed to 54.77 ± 34.67, 11.08 ± 7.98 and 7.85 ± 0.13 $\mu g \cdot L^{-1}$ APAP, AT and CBZ, respectively for 5 days. Venn diagram shows the overlaps of differentially expressed proteins based on at least a 1.2-fold filter change with a $p = 0.05$.

DISCUSSION

Because of their wide distribution and pseudo persistence, an increasing number of studies are focusing on the toxicological effects of exposure to pharmaceuticals in mammalian (34–37) and fish model species (26, 38–39). To better understand the effects of these potentially hazardous chemicals on ecologically relevant fish species, the present study aimed at evaluating the toxicity of short-term exposure to three highly environmentally prevalent pharmaceuticals in the liver of the Atlantic salmon parr, *Salmo salar*, a candidate sentinel species. We showed that short-term, subacute exposure to the three pharmaceuticals induced significant alterations in the hepatic protein expression profile. To date, effects of exposure to pharmaceuticals at environmentally relevant concentrations on nontarget organisms in general and, especially in Atlantic salmon remain largely unknown. Experiments with both mammals and teleost fish have shown high 50% lethal concentration (LC_{50}) values (exposure concentration that produced mortality in 50% of the exposed organisms), suggesting that acutely exposed animals can tolerate quite high levels of these pharmaceuticals (40). In the present study, the tested concentrations were chosen according to environmental levels reported in the literature (13–17) and no mortality was observed. Using a proteomic analysis as means of evaluation of low level subacute effects, the present study reported a number of proteins displaying significant changes in abundance following exposure to the selected pharmaceuticals and of which most of them were successfully identified and validated through MASCOT from primary sequence databases using Peptide Mass Fingerprint, sequence query and MS/MS ion search. The function of these proteins may provide new clues on the molecular mechanisms by which APAP, AT, and CBZ induce effects in liver tissue and help to identify effective biomarkers of this kind of pollutants.

Regarding PCA (Fig. 3), the horizontal distance (PC#1) between the treated and control group is slightly larger than the distance between samples within one group, indicating that the most important difference between groups is because of the application of different treatments. The vertical distance (PC#2) between samples in the plot represents biological and technical variation. Proteins from individual samples were extracted and processed for each gel, so differences in resistance or sensitivity toward adverse conditions are to be expected. Additionally, technical variation induced by protein extraction, labeling, separation, spot picking, and digestion procedures may also provide a source of variation within a treatment group. The spot maps with the largest PC#1 values between each other are those who presented most differences in expression patterns. These are in our case the maps from the APAP and the Control treatments, whereas the AT and CBZ treatment present the least distance and lowest PC#1 value. This is in concordance with the fact that most significantly different proteins (this study) and also transcripts (41) have been found after APAP treatment where the supplied dose was 10 and 5 times higher than the one for CBZ and AT as found in the environment. Moreover, the target organ for APAP is the liver, the test tissue employed in this experiment, whereas the target tissues for AT and CBZ are the heart and the brain, respectively. Nevertheless, and although CBZ and AT are not pharmaceutically targeted to liver, these pharmaceuticals induced changes in hepatic protein expression in previous studies, such as hepatic cytochrome P450 subfamily members in the case of CBZ (42, 43, 44) or general hepatic dysfunction in the case of AT (45).

Hierarchical Cluster Analysis result representation as heat map (Fig. 4) visualizes the pattern of expression of proteins across treatments as compared with the internal standard. The trees to the left of the heat map and above the heat map show the relationships between the proteins and between the treatments, respectively. Proteins more distantly placed within branches show greatest differences in expression patterns between treatments. All three drug treatments varied significantly from the expression pattern observed in the control (with respect to the internal standard), with the largest difference occurring between the Control and APAP treatment. This is consistent with the PCA, as observed above (Fig. 3).

Expression of Proteins Involved in Energy Metabolism—In the present study, the proteins transketolase, mitochondrial ATP synthase, acetyl-CoA acetyltransferase, phosphoglycerate kinase 1, and glyceraldehydes-3-phosphate dehydrogenase were modulated by low level exposure within all the three treatments. All these enzymes play important roles in energy metabolism. Several ATP synthase mitochondrial precursors, a CoA isomerase precursor, as well as several heat shock proteins have also shown to be altered in the liver proteome of APAP treated mice (21), indicating similar responses in these

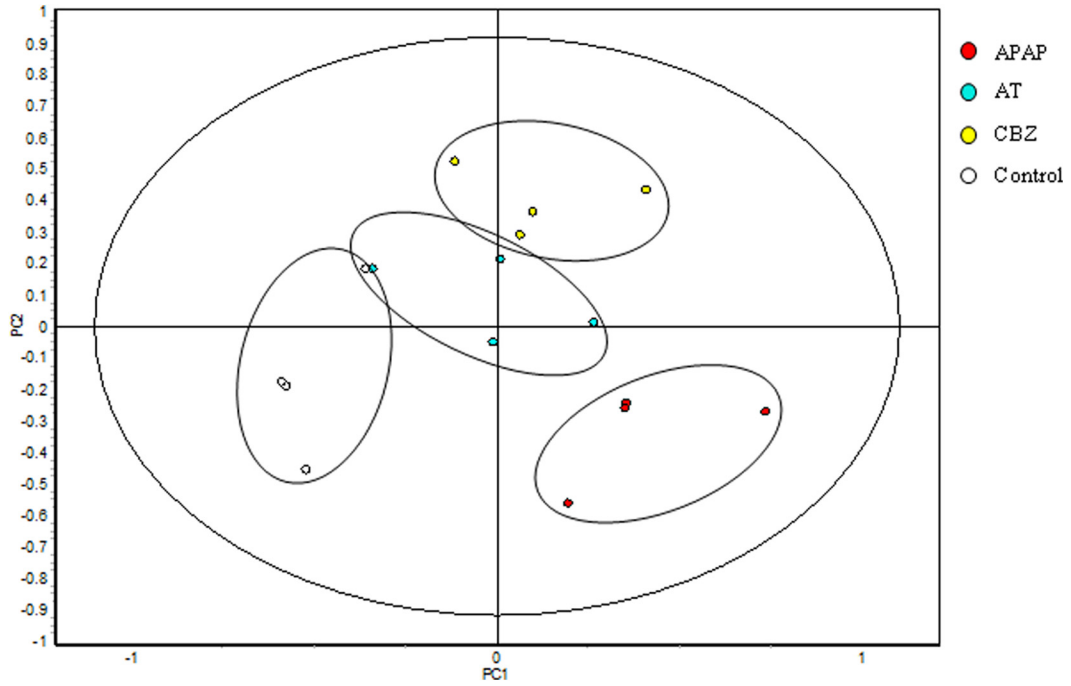

FIG. 3. **Principal component analysis (PCA) of the protein spot maps.** PC#1 represents the variability because of the applied treatments; PC#2 represents the variability because of biological and technical variations between samples. The proteins that are included in the PCA were present in at least 75% of the spot maps and passed the filter of the one-way ANOVA ($p < 0.01$) test.

different vertebrates. However, the doses applied to mice in these previous experiments were significantly higher that may explain the higher total number of altered proteins. One of the first responses of adaptation to stress in fish is the mobilization of energetic reserves, including the activation of liver glycogenolysis and gluconeogenesis in order to maintain a continuous supply of glucose to essential organs such as brain and muscle (46). In all three treatments phosphoglycerate kinase 1, an enzyme of the gluconeogenic and glycolytic pathways is increased. In APAP glyceraldehyde-3-phosphate dehydrogenase (GAPDH), another gluconoegenic/glycolytic enzyme is also increased, although this enzyme is decreased in CBZ. Transketolase, important for NADPH production in the pentose phosphate shunt, is also increased in APAP and CBZ. At the same time, we observed a 1.4 and 1.3-fold abundance increase of acetyl-CoA acetyltransferase in organisms exposed to APAP or

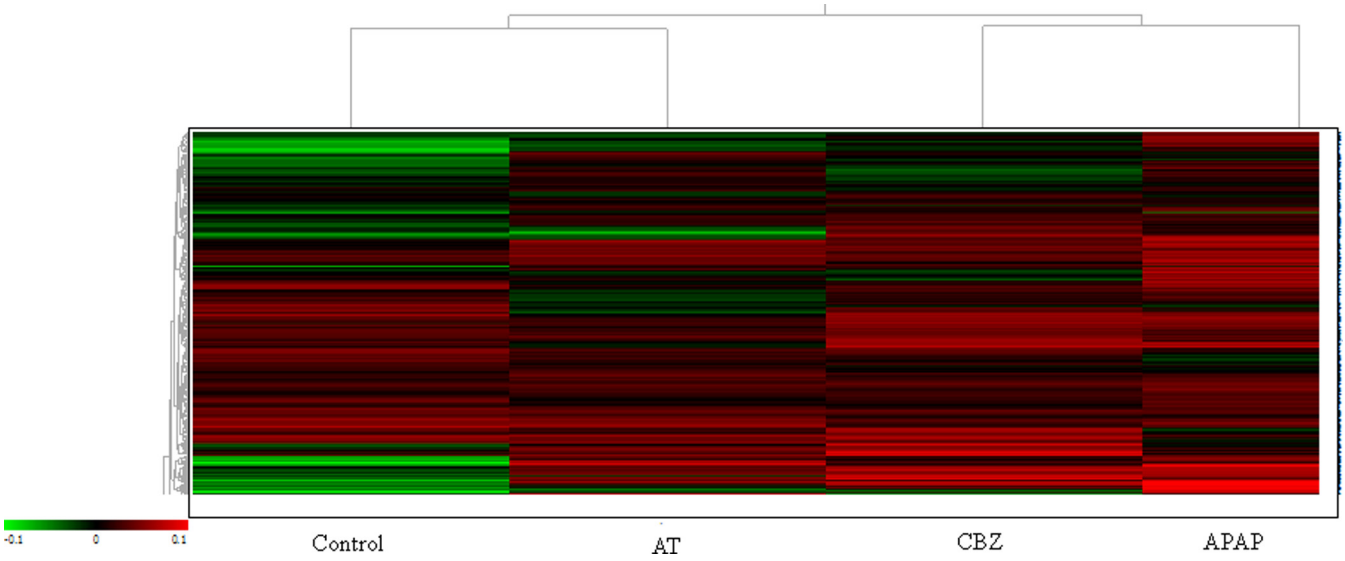

FIG. 4. **Hierarchical cluster analysis.** Heat map showing the separation of the different experimental groups taking into account all altered proteins present in at least 75% of the spot maps and filtered by one-way ANOVA ($p < 0.01$). Every colored box represents a protein that is up-regulated (red) or down-regulated (green) in a certain treatment. The brighter the color, the more intense is the change.

CBZ. Acetyl-CoA acetyltransferase is a major enzyme of ketone body production in liver, and increases suggest responses to stressors, as well as, gluconeogenesis and, thus, glucose production. Overall these results suggest an ametabolic shift from glycolysis to gluconeogenesis fueled by an increase in NADPH production and concurrent with up-regulation of the ketogenic pathway producing metabolic fuel for the liver and peripheral tissues (47). However, as no significant changes in direct lipid metabolic enzymes or pathways were observed we did not see any evidence for mobilization of energy stores. The 1.639-fold decrease in ATP synthase H+ transporting mitochondrial F1 complex beta (gi 198285477) protein concentration upon APAP treatment may indicate mitochondrial toxicity and a loss of cellular energy production in line with mitochondrial damage. Previous studies indicated that mitochondrial permeability transition (MPT) is the principal mechanism in APAP-induced injury, with a potential to open the transition pore (48). MPT is recently focused as a mechanism for drug-induced hepatocyte injury (49–53) and the observed up-regulations of different energy related features could be a compensatory mechanism. Notably induction of MPT is a major signal for activation of apoptotic cell death pathways, and the major executioner of apoptosis, caspase 3, was also increased after APAP treatment.

Other Proteins—OsmC-like family proteins: in bacteria, this kind of proteins have shown to be induced by different types of stress, particularly high osmotic pressure and starvation (54). The test specimens employed in our experiments were salmon parr, which were about one month before smoltification, still adapted to fresh water. However, undergoing adaptation processes may have been already under way as the process of smoltification implies an adaptation from fresh to salt water with the accompanying physiological changes required. It is possible that some osmotic mechanism was already working in the fish. However, this feature was only detected in one (APAP) of the three treatments which were carried out under identical conditions, where its expression was down-regulated by 1.65 ($p = 0.0359$) indicating an interaction of APAP with normal osmotic adaptation processes in the liver.

Elongation factor: In eukaryotes, peptide chain elongation is mediated by elongation factors EF1 and EF2. EF2, which was up-regulated in our AT and CBZ treatments, catalyzes the translocation of peptidyl-tRNA on the ribosome. Elongation factors are highly conserved among different species and may be involved in functions other than protein synthesis, such as organization of the mitotic apparatus, signal transduction, developmental regulation, aging and transformation (55). EF2 can be modulated by reversible phosphorylation. Increased levels of phosphorylated EF2 reduce elongation rates presumably because phosphorylated EF2 fails to bind the ribosomes. Treatment of mammalian cells with agents that raise the cytoplasmic Ca^{2+} and cAMP levels reduce elongation rates by activating the kinase responsible for phosphorylating EF2. One of the therapeutic actions of CBZ is a result of its interaction with the adenylyl cyclase system and the consequent reduction of intracellular cAMP levels (56, 57). Possibly, at least after the CBZ treatment, the expression of EF2 has been induced to compensate for the reduction in elongation rates because of activation of the kinase responsible for phosphorylating EF2. In relation to AT, it was shown that beta-adrenergic receptor blockers can inhibit β adrenoreceptor-mediated cAMP accumulation in living cardiac rat cells (58).

TenA family transcriptional activator: this protein is a heme oxygenase related feature that was significantly changed under the CBZ treatment. Effects of CBZ on heme oxidase, glutathione-S-transferase and cytochrome P450 3A-like have been observed in the crustacean *T. platyurus* and the cnidarian *H. attenuate*, and lipid peroxidation was reduced in both organisms suggesting redox activity of the lipophilic CBZ molecule (59). In a related study where we analyzed the effects of the exposure trials on the liver transcriptome, we observed altered electron carrier activity and heme binding activity after CBZ treatment by Blast2Go analysis within the top 10 GO terms related to molecular function (41). In human, CBZ is well known to have various hematological toxic effects, such as aplastic anemia, leukopenia, eosinophilia, agranulocytosis and thrombocytopenia with often selective failure of red cell production during CBZ monotherapy (60).

The number of significantly differentially expressed features we obtained in our three exposure experiments was slightly lower or at the lower threshold of the average amount found in proteomic studies carried out in human and rodents (61). In a study compiling the results from about 200 proteomic experiments in human and rodents, these authors found that a typical published 2D-based expression proteomics experiment features 400–1500 spots and reports between 10 and 40 identified up- or down-regulated proteins which in many cases are repeated across the whole range of examined studies (61). Two features of their top 15 protein list from humans and rodents were also present in one or more of our identified protein lists: elongation factors and ATP synthase beta subunit. In relation with the microarray studies carried out within the same experimental trial, we can observe that tissue samples from the same individuals have shown to induce far more features at transcriptome level than at protein level. The number of differently expressed features per treatment we detected in these experiments was significantly higher (order of several hundred) than the differently expressed proteins detected in this study (41). Whereas at transcriptome level the main induced pathways were also those related to energy metabolism, genes belonging to other processes were altered, in many cases similar to the responses observed in mammalian studies of similar treatments. These are for instance inflammatory responses after APAP treatment, iron ion related processes after AT treatment and Ca^{2+} channel activity after CBZ treatment. However, when analyzing the same tissues at protein level, the main effect was alteration of proteins belonging to energy related processes (41). The si-

multaneous measurement of thousands of proteins in a cell has high potential impact in toxicology, as cellular effects and thus modes of action are more relevant at protein than at mRNA level. However, as all proteins have different properties (mass, isoelectric point, solubility, stability, *etc.*), and may exist in multiple forms, the accurate measurement of thousands of proteins in a sample is a very complicated task. Several approaches have been followed to study the problem of the complexity of the cellular proteome and the difficulty to isolate homogeneous samples for this type of studies (62). It is important to realize that the relationship between mRNA and protein content is heavily dependent on time after treatment, cellular localization as well as stability of the molecules (63).

The lack of obvious congruence in differently expressed features after transcriptomic and proteomic analysis, and the results observed by Petrak *et al.* (61) that the same proteins seem to be observed after widely varying treatments, raises the concern that with current proteomic techniques, only the highly abundant and soluble proteins are detected giving rise to technical artifacts, limitations or biases of the method. However, the combination of techniques, transcriptomic and proteomic, offers the opportunity to reliably quantify expression changes and identify previously unknown features which can be useful tools in ecotoxicology, if followed by further studies to confirm potential as biomarkers of contaminant specific contamination.

Environmental Relevance—Traditional risk assessment is based on the derivation of no observed effect concentrations (NOECs) from laboratory derived dose-response curves for comparison with measured or estimated exposure concentrations. Although proteomic data does not provide additional information for the derivation of NOECs, the function of the altered proteins provides in-depth mechanistic information of the molecular processes related with the exposure to environmental contaminants. In addition, proteins that are found to be specifically induced by one (group of) contaminant could be used as a base to develop contaminant specific biomarkers for monitoring environmental exposure in wildlife.

It is yet to be determined if the exposure to the selected concentration ranges would directly produce an adverse response, as proteome change alone does not necessarily equate to toxicity or adversity. An ecologically relevant adverse response is the impairment of functional capacity (morphology, development, lifespan, growth) because of an insult that exceeds an organism's homeostatic range and is likely to have consequences finally at community and ecosystem level. However, energy used for alteration of the proteome and metabolic adaptation in response to contamination will not be available for other critical physiological processes, and may thus have indirect adverse responses and consequent knock-on ecosystem effects.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (64) via the PRIDE partner repository with the dataset identifier PXD001354.

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S This article contains supplemental Files S1 to S4.

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