

Protein Expression Profiling in the African Clawed Frog *Xenopus laevis* Tadpoles Exposed to the Polychlorinated Biphenyl Mixture Aroclor 1254[§]

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Exposure to environmental pollutants such as polychlorinated biphenyls (PCBs) is now taken into account to partly explain the worldwide decline of amphibians. PCBs induce deleterious effects on developing amphibians including deformities and delays in metamorphosis. However, the molecular mechanisms by which they express their toxicity during the development of tadpoles are still largely unknown. A proteomics analysis was performed on developing *Xenopus laevis* tadpoles exposed from 2 to 5 days postfertilization to either 0.1 or 1 ppm Aroclor 1254, a PCB mixture. Two-dimensional DIGE with a minimal labeling method coupled to nanoflow liquid chromatography-tandem mass spectrometry was used to detect and identify proteins differentially expressed under PCBs conditions. Results showed that 59 spots from the 0.1 ppm Aroclor 1254 condition and 57 spots from the 1 ppm Aroclor 1254 condition displayed a significant increase or decrease of abundance compared with the control. In total, 28 proteins were identified. The results suggest that PCBs induce mechanisms against oxidative stress (peroxiredoxins 1 and 2), adaptative changes in the energetic metabolism (enolase 1, glycerol-3-phosphate dehydrogenase, and creatine kinase muscle and brain types), and the implication of the unfolded protein response system (glucose-regulated protein, 58 kDa). They also affect, at least at the highest concentration tested, the synthesis of proteins involved in normal cytogenesis (α -tropomyosin, myosin heavy chain, and α -actin). For the first time, proteins such as aldehyde dehydrogenase 7A1, CArG binding factor-A, prolyl 4-hydroxylase β , and nuclear matrix protein 200 were also shown to be up-regulated by PCBs in developing amphibians. These data argue that protein expression reorganization should be taken into account while estimating the toxicological hazard of wild amphibian populations exposed to PCBs. *Molecular & Cellular Proteomics* 8:596–611, 2009.

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Over the last few decades, many populations of amphibians have declined in a number of geographical locations worldwide (1–3). Causes of this decline are assumed to result from man-made alterations of the environment, and exposure to environmental pollutants such as polychlorinated biphenyls (PCBs)¹ is now taken into account (4). PCBs were manufactured in the 1950s for use in electrical insulators, plasticizers, and carbonless copy paper (5). Twenty years after their production ban in most industrialized countries, PCBs are still persistent and widely distributed in the environment (6).

It has already been reported that PCBs induce deleterious effects on wild organisms. In developing amphibians, they cause mortality (7), developmental deformities (8–11), delays in metamorphosis (12), immunological effects (13), and disruption of gonad development (14–16).

It is admitted that PCBs exert part of their toxicity by binding to the cytosolic aryl hydrocarbon receptor (AhR). In the nucleus, the activated AhR forms a heterodimer with the aryl hydrocarbon nuclear translocator, and the complex binds to the xenobiotic-responsive elements or aryl hydrocarbon response element I (AHREI), which regulates the expression of numerous genes involved in physiological and developmental processes (17, 18). The AhR-aryl hydrocarbon nuclear translocator heterodimer also acts as a coactivator of the transcription of responsive genes via the interaction with another response element, AHREII (19). However, in developing tadpoles of numerous frog species,

¹ The abbreviations used are: PCB, polychlorinated biphenyl; AhR, aryl hydrocarbon receptor; AHRE, aryl hydrocarbon response element; ARE, antioxidant response element; EpRE, electrophile response element; ALDH, aldehyde dehydrogenase; Ckb, creatine kinase brain type; CBF-A, CArG binding factor-A; FETAX, frog embryo teratogenesis assay for *Xenopus*; GRP, glucose-regulated protein; LAP3, leucine aminopeptidase 3; Ckm, creatine kinase muscle type; Nmp200, nuclear matrix protein 200; PDI, protein-disulfide isomerase; Prx, peroxiredoxin; P4hb, prolyl 4-hydroxylase β ; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; UPR, unfolded protein response; pf, postfertilization; 2D, two-dimensional; ENO, enolase; Grhpr, glyoxylate reductase/hydroxypyruvate reductase; GPD1, glycerol-3-phosphate dehydrogenase 1; Hnrpab, heterogeneous nuclear ribonucleoprotein A/B; ER, endoplasmic reticulum.

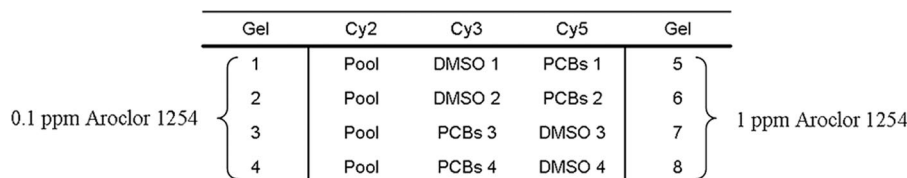


FIG. 1. **Schematic overview of the experimental conditions.** Control DMSO and test PCB samples were labeled with either Cy3 or Cy5, reversing the labeling for half of the samples. The internal standard corresponding to a mixture of equal amounts of control and test samples was labeled with Cy2. Four replicates were used per experimental condition.

an age-dependent insensitivity to chlorinated compounds linked to a low affinity for the AhR has been reported. The AhR machinery is present but requires high levels of inducer to provoke physiological changes (20–22). So far, the molecular mechanisms by which PCBs induce their toxicity during the development of tadpoles are still largely unknown. This hampers the risk assessment for developing tadpoles when they are environmentally exposed to these pollutants.

Proteomics is one of the possible strategies to gain better insight into the molecular responses to PCBs. Proteomics has been initially used successfully in drug discovery, biomarker identification, and protein-protein interaction studies in human disease processes (23, 24). This approach has been recently applied in ecotoxicology. It has been reported that environmental stresses such as variations of salinity and temperature and exposure to environmental contaminants like heavy metals, xenoestrogen, and chlorinated compounds have an impact on protein expression in different tissues of relevant aquatic organisms (25–31). Nevertheless such studies are scarce, and most of them focused on non-model organisms with the consequence of low output of protein identification (31).

The alteration of the genes expression in *Xenopus laevis* tadpoles exposed to PCBs has been explored in different studies (10, 32, 33). For example, 18-day postfertilization (pf) tadpoles exposed for 3 days to 50 ppb Aroclor 1254 showed significant up-regulation of several genes such as nerve growth factor, glyceraldehyde-3-phosphate dehydrogenase, interleukin-1 β -converting enzyme, proopiomelanocortin, and p53 (32). However, no correlation between the mRNA and protein levels has been reported so far as the impact of PCBs on protein expression profiles in developing organisms has not been documented. Because the understanding of the molecular mechanisms by which PCBs interact with normal amphibian development is of special interest, the potential effects of a mixture of these environmental pollutants on the protein expression of developing *X. laevis* tadpoles were evaluated. To achieve this goal, the 2D DIGE minimal labeling approach coupled to nanoflow LC-MS/MS was applied to detect and identify proteins differentially expressed in PCB conditions. Identification of these proteins provides insight into the potential molecular mechanisms by which PCBs are interfering with amphibian development and will eventually

lead to the proposal of candidate biomarkers for environmental pollution assessment.

MATERIALS AND METHODS

Animals, Breeding, and Housing—Adult African clawed frogs (*X. laevis*) were obtained in 2004 from the National Breeding Laboratory of *Xenopus*, University of Rennes, France. Animals were maintained in dechlorinated water at 22 ± 1 °C with a 12:12 hour photoperiod schedule. Fresh water was changed every other day. Animals were fed three times a week with commercial trout food (Trouw) or chironomid larvae. Breeding was induced by subcutaneous injection of adults with 750 IU of human chorionic gonadotropin (Sigma). Cleaving embryos of stage 8–13 (34) were placed in FETAX medium (625 mg of NaCl, 96 mg of NaHCO₃, 30 mg of KCl, 15 mg of CaCl₂, 60 mg of CaSO₄·2H₂O, and 75 mg of SO₄·7H₂O/liter of distilled water) until they hatched (48 h pf).

Chemical Exposure—Normally developing tadpoles (stage 35/36) were placed in glass bowls filled with 200 ml of FETAX medium. Each experimental condition included three replicates of 20–25 tadpoles. The PCB mixture Aroclor 1254 (Alltech Associates Inc.) was added to the medium using DMSO (Sigma) (final concentration of 0.05%) as a solvent, resulting in nominal concentrations of 0.1 and 1 ppm. A medium control group and a DMSO solvent control group (0.05%) were included in each experiment. During the assay, the temperature was maintained at 22 ± 1 °C, the solutions were changed every day, and dead tadpoles were removed daily. When tadpoles reached stage 45 (4 days pf), they were fed a mixture of *Spirulina* algae. After 72 h of exposure, the survival rate was recorded, and tadpoles were pooled and weighed, snap frozen, and stored at -80 °C. For each treatment, one replicate was assigned for proteomics analysis, and another was assigned for chemical analysis. Each experiment was repeated four times with tadpoles obtained from independent spawnings. Animals and tadpoles used in the present work were treated in accordance with an animal use protocol (code FUNDP 07/089) approved by the Ethic Commission of the Facultés Universitaires Notre-Dame de la Paix.

Protein Extraction and CyDye Labeling—Proteins were extracted in 1:3 (w/v) lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris; GE Healthcare) and solubilized by sonication on ice. Samples were then centrifuged for 15 min at $12,000 \times g$. The pH of the soluble protein extract was adjusted to 8.5 by addition of 50 mM NaOH, and protein concentration was measured using the Bio-Rad protein assay. For DIGE minimal labeling, 25 μ g of protein sample were labeled with 200 pmol of CyDye (GE Healthcare). Protein samples from DMSO control and PCB conditions (0.1 and 1 ppm Aroclor 1254) were labeled with Cy3 and Cy5. The reverse labeling of the test samples with Cy3 and of the control DMSO with Cy5 was done as well. A mixed sample composed of equal amounts of proteins from both Aroclor 1254-contaminated groups and DMSO control was minimally labeled with Cy2 and used as an internal standard (Fig. 1). Four independent replicates (tadpoles obtained from four independent spawnings) were used for each experimental condition. Labeling was

performed on ice for 30 min in the dark and quenched with 1 mM lysine for 10 min on ice. The labeled mixtures were combined, and the total proteins (75 μ g) were mixed v/v with the reduction solution (7 M urea, 2 M thiourea, 2% DTT, 2% CHAPS, 2% IPG 4–7 buffer; GE Healthcare) for 15 min at room temperature.

Separation of Proteins by 2D DIGE—Prior to electrofocusing, IPG strips (24 cm, pH 4–7; GE Healthcare) were passively rehydrated overnight with 450 μ l of a standard rehydration solution (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG 4–7 buffer, 2% DTT). The eight sample sets containing the labeled mixtures were then cup-loaded onto the IPG strips, and isoelectric focusing was performed with an Ettan™ IPGphor II isoelectric focusing unit (GE Healthcare). The electrophoresis conditions were as follows: 20 °C for 18 h; step 1, 300 V for 3 h; step 2, 1000 V for 6 h; step 3, 8000 V for 6 h; step 4, 8000 V for 6 h for a total of 68,000 V-h. Focused IPG strips were reduced (1% DTT) and alkalinized (2.5% iodoacetamide) in equilibration buffer (50 mM Tris, 6 M urea, 30% glycerol, 2% SDS, pH 8.8) just before loading onto a 12.5% 24-cm, 1-mm-thick acrylamide gel. The strips were overlaid with 1% agarose in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) and run in an Ettan DALTsix electrophoresis unit (GE Healthcare) at a constant 3 watts/gel at 15 °C until the blue dye front had run off the bottom of the gels.

Image Analysis and Statistics—Labeled CyDye gels were scanned with the Typhoon 9400 scanner (GE Healthcare) at wavelengths specific to the CyDyes. Resolution was 100 μ m. Image analysis was carried out with DeCyder software (GE Healthcare). The differential in-gel analysis module co-detected and differentially quantified the protein spots in each image using the internal standard sample as a reference to normalize the data. At a second step, biological variation analysis was used to calculate ratios between samples and internal standard abundances by performing a gel-to-gel matching of the internal standard spot maps from each gel. Protein spots that showed a statistically significant ($p < 0.01$) Student's *t* test for an increased or decreased intensity were accepted as being differentially expressed between Aroclor 1254-contaminated and DMSO control groups.

Mass Spectrometry and Protein Identification—For peptide sequencing and protein identification, preparative gels including 350 μ g of proteins of mixed samples were performed following the protocol described above except that they were poststained with ruthenium(II) tris(bathophenanthroline disulfonate) overnight (7 μ l of ruthenium/1 liter of 20% ethanol) after 6 h of fixation in 30% ethanol, 10% acetic acid and 3 \times 30 min in 20% ethanol at 20 °C (35).

Peptides were analyzed by using a nanoflow LC-ESI-MS/MS (Waters) instrument on a CapLC Q-TOF2 mass spectrometer (Waters). Spots were excised from preparative gels by using the Ettan Spot Picker (GE Healthcare), and proteins were cleaved with trypsin by in-gel digestion. The gel pieces were twice washed with distilled water and then shrunk with 100% acetonitrile. The proteolytic digestion was performed by the addition of 3 μ l of modified trypsin (Promega) suspended in 100 mM NH_4HCO_3 cold buffer. Proteolysis was performed overnight at 37 °C. The supernatant was collected and combined with the eluate of a subsequent elution step with 5% formic acid. The eluates were kept at –20 °C prior to analysis.

The digests were separated by reverse phase liquid chromatography using a 75- μ m \times 150-mm reverse phase NanoEase column (Waters) in a CapLC (Waters) liquid chromatography system. Mobile phase A was 95% 0.1% formic acid in water and 5% acetonitrile. Mobile phase B was 0.1% formic acid in acetonitrile. The digest (1 μ l) was injected, and the organic content of the mobile phase was increased linearly from 5% B to 40% in 40 min and from 40% B to 100% B in 5 min. The column effluent was connected to a PicoTip emitter (New Objective) inside the Q-TOF source. Peptides were analyzed in data-dependent acquisition mode on a Q-TOF2 (Waters) instrument. In a survey scan, MS spectra were acquired for 1 s in the

m/z range between 450 and 1500. When the intensity of 2+ or 3+ ions increased above 20 counts/s there was an automatic switch to the MS/MS mode. The CID energy was automatically set according to *m/z* and charge state of the precursor ion. Acquisition in MS/MS mode was stopped when the intensity fell below 5 counts/s or after 15 s. Q-TOF2 and CapLC systems were piloted by MassLynx 4.0 (Jasco). For the electrospray survey, background was subtracted with a threshold of 35%, polynomial 5. For smoothing, the Savitzky-Golay method with two iterations and a window of three channels was used. Finally we assigned the mass of peaks with a threshold of 3%, a minimum peak with four channels, and a centroid top method at 80%. For MS/MS raw data, a rigorous deisotoping method with a threshold of 3% was performed. Peak lists were created using ProteinLynx Global Server 2.2.5 (Waters) and saved as a PKL file for use with Mascot 2.1 (Matrix Science). Enzyme specificity was set to trypsin, and the maximum number of missed cleavages per peptide was set at 1. Carbamidomethylation was allowed as a fixed modification, and oxidation of methionine was allowed as a variable modification. Mass tolerance for the monoisotopic precursor peptide window and MS/MS tolerance window were set to ± 0.3 Da. We also specified ESI-Q-TOF as instrument. The peak lists were searched against the *Xenopus* subset of the National Center for Biotechnology Information non-redundant (NCBI nr) database (15,569 entries in September 2007). Control searches of all the files against the whole NCBI nr database (5,454,477 entries in September 2007) was used to confirm the identification.

For all protein identifications, a minimal individual peptide score of 20 (below this score no identity or homology was found for the analyzed peptides) and expect value below 1 were used for the initial identification criteria (all peptide sequences linked to protein identification are reported in Table I). For single peptide-based protein identifications, the sequence identified and the precursor *m/z* and charge observed as well as the score for this peptide are given in the supplemental data. In the case of redundant protein identifications, the protein identification with the highest score was selected. However, if all the individual peptides completely matched to more than one UniProt database accession number, we aligned the sequences using BLAST (basic local alignment search tool). If the alignment showed 100% sequence identity, the UniProt accession number with the best description was chosen. When peptides matched to different isoforms or to different members of the same protein family, the following criteria were applied for selecting which isoform to report; if one peptide with a high score matched exclusively to a specific isoform or protein member, the identification could be made unambiguously. Moreover the correlation between theoretical pI and molecular mass of the protein with the position of the corresponding spot in the 2D gel was also taken into account.

Polychlorinated Biphenyl Analysis—PCBs were extracted according to a slight modification of Environmental Protection Agency method 608 as described previously with modifications for analysis with tadpoles (36, 37). Twenty-four PCB congeners (from di- to nonachlorinated) (IUPAC numbers 28, 44, 52, 66, 70, 87, 95, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194, 195, 206, and 209) were identified and quantified. PCB concentrations were transformed in Aroclor 1254 equivalent and expressed in μ g/g of lipids and in μ g/g of body weight. All the tadpoles assigned for chemical analysis were pooled to obtain about 100 mg of lipid after extraction. Extraction of lipids was performed with hexane using an ASE 200 Accelerated Solvent Extractor (Dionex). All the extracts were used for lipid content determination: solvent was evaporated using a Turbovap LV (Zymark) until a constant weight was obtained. Samples were then diluted in 3 ml of *n*-hexane, and a surrogate (PCB 112 with a final concentration of 50 μ g/ μ l) was added to quantify possible loss of PCBs during the procedure. The extracts were subjected to cleanup

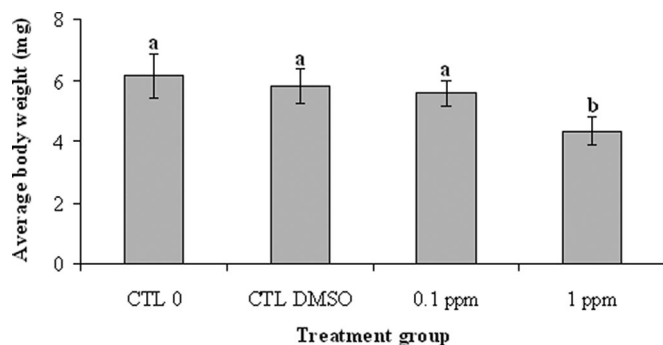


FIG. 2. Final average body weight (mg) of *X. laevis* tadpoles (3 days posthatching) following a 3-day exposure to Aroclor 1254. Data are presented in all figures as mean ($n = 4$) \pm standard deviation (error bars) of the mean. Columns sharing at least one common superscript letter (a or b) are not significantly different, whereas the other differ at $p < 0.05$. CTL, control.

with sulfuric acid to remove organic matter (lipids, lipoproteins, and glucides), and 2 ml of a mixture of concentrated (95%) and fuming (30%) sulfuric acid (3:1; v/v) were added to the extract. The mixture was shaken and centrifuged at $1750 \times g$. The supernatant was removed, and 3 ml of *n*-hexane were added to the decanted acid. Shaking, centrifugation, and removal were performed a second time before evaporation of the solvent. A cleanup column (Superclean™ ENVI Florisil solid phase extraction tubes (6 ml), Supelco) was also used to remove polar molecules. Columns were eluted with 5 ml of acetone, 5 ml of acetone-hexane, and 12 ml of hexane before the extracts were eluted with 6 ml of hexane. After the addition of 125 μ l of a surrogate (PCB 30; 100 pg/ μ l diluted in hexane) and 125 μ l of an internal standard (Mirex; 100 pg/ μ l diluted in hexane), the extracts were analyzed using a high resolution gas chromatograph (Thermoquest) equipped with a ^{63}Ni electron capture detector. PCBs were separated by progressive temperature increase. Congeners were identified and quantified according to their retention time using the software Chrom-Card for Windows 4.0. The quantification limit of PCBs in tissue was 1 ng/g (w/w) and 200 ng/g of lipids.

Statistical Analysis—Results for the survival and growth parameters were expressed as the mean ($n = 4$) \pm S.D. Normality analysis of data was assessed by Kolmogorov-Smirnov test. Homogeneity of variances was tested by Bartlett test. Differences between groups were compared using one-way analysis of variance followed by post hoc least significant difference multiple comparison test at a 5% significance level. All statistical analyses were performed using Statistica™ software for Windows (StatSoft).

RESULTS

General Impact on Animals and Level of PCBs in Tissues

The percentage of surviving tadpoles was $92.5 \pm 4.5\%$ for the medium control group and $90.9 \pm 3.6\%$ for the DMSO control group. Exposure to 0.1 and 1 ppm Aroclor 1254 had no effect ($p = 0.84$) on the survival of tadpoles (93.1 ± 5.2 and $89.7 \pm 5.1\%$, respectively). Regarding their final average body weight (Fig. 2), tadpoles exposed to 1 ppm Aroclor 1254 weighed 4.3 ± 0.5 mg, which was significantly ($p < 0.05$) lower compared with the average body weight of tadpoles from the control 0, the DMSO control, and the 0.1 ppm-treated groups (6.2 ± 0.7 , 5.8 ± 0.5 , and 5.5 ± 0.4 mg, respectively). Exposure to PCBs did not impact the develop-

mental stages as all tadpoles from the different experimental conditions reached stages 44/45 by the end of the experiment.

Levels of PCBs in tadpoles exposed to 0.1 and 1 ppm Aroclor 1254 increased in a dose-dependent manner and were as high as 13,693 $\mu\text{g/g}$ of lipids in the 0.1 ppm group and 39,653 $\mu\text{g/g}$ of lipids in the 1 ppm group. Low quantities of PCBs were also found in tissues of untreated control and DMSO control groups (2669 and 1737 $\mu\text{g/g}$ of lipids, respectively).

Proteome Analysis

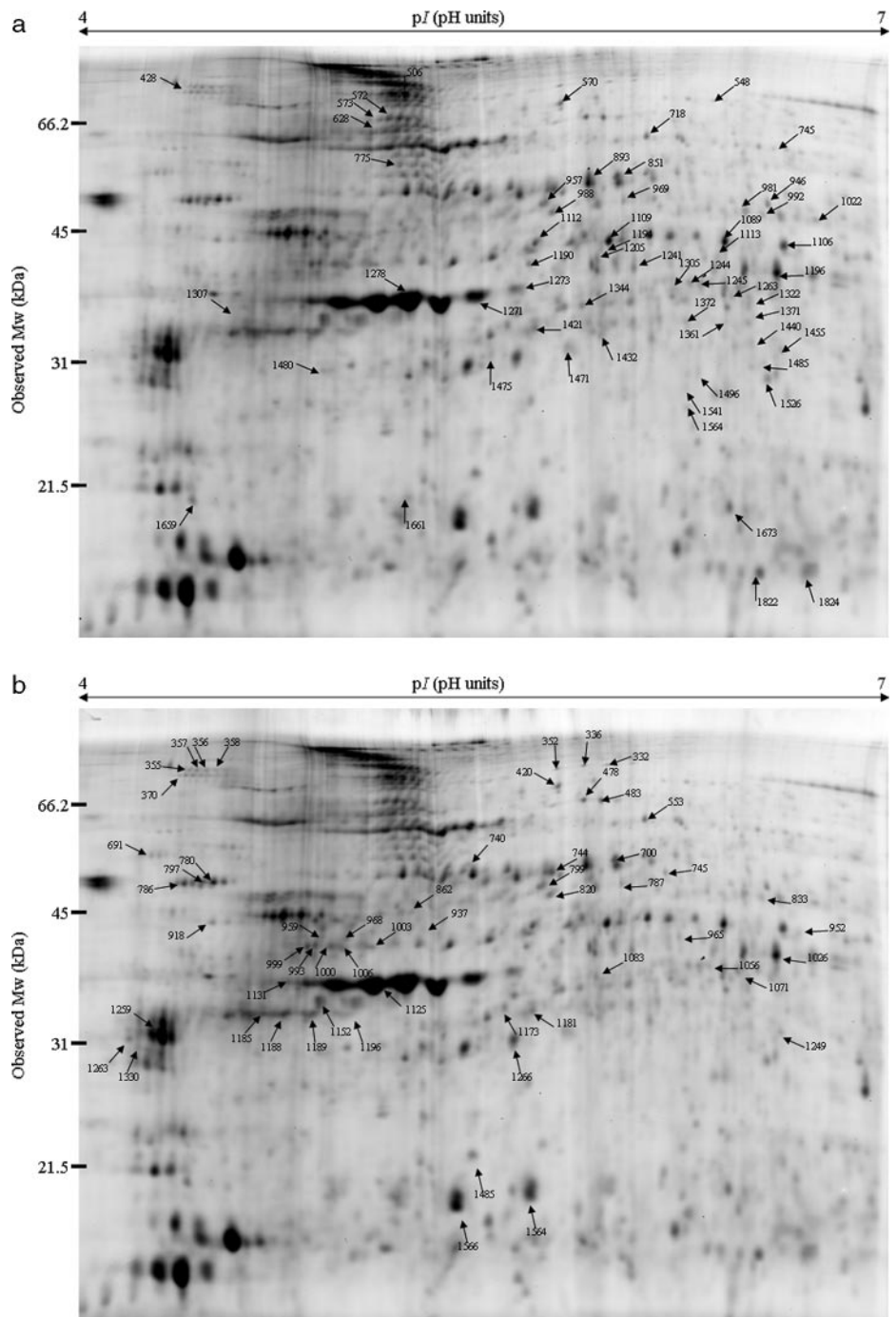
Protein Expression—To understand how PCBs could affect amphibian development, the effects of these pollutants on the protein expression pattern in developing *X. laevis* tadpoles was investigated. 2D DIGE technique was used to compare tadpoles from the DMSO control group with tadpoles exposed for 72 h to 0.1 and 1 ppm Aroclor 1254. The number of spots detected in the four gels of the 0.1 ppm group was 1659 ± 170 , whereas 1622 ± 159 spots were detected in the 1 ppm group. Only the 1083 spots from the 0.1 ppm group and the 937 spots from the 1 ppm group that were commonly matched between the four gels were selected for further statistical analysis. Protein spots that showed significant differences ($p < 0.01$) in intensity between tadpoles exposed to PCBs and DMSO were selected for MS/MS identification.

Changes in the protein expression pattern in tadpoles exposed to Aroclor 1254 are presented in Fig. 3. In the 0.1 ppm condition, 59 spots (Fig. 3a) showed significant differences ($p < 0.01$) in intensity in all gels corresponding to a change in protein abundance. An increase in abundance was observed for 83% of the protein spots (Fig. 4a). The increase was between 1.2 and 2 for 39 spots and between 2 and 2.5 for 10 spots. 17% of protein spots showed a decrease in abundance with stronger variations because the -fold decrease reached 2–4 for four spots and even more for one spot.

The second experimental group of tadpoles exposed to 1 ppm Aroclor 1254 showed a similar profile with 57 protein spots (Fig. 3b) displaying significant differences ($p < 0.01$) in intensity. Among these spots, 20 corresponded to a decreased abundance of proteins (35%), and 37 (65%) corresponded to an increased abundance after PCB exposure compared with the control tadpoles (Fig. 4b). The -fold increase ranged between 1.15 and 2. For the 35% of spots with a decreased abundance, the -fold change corresponded again to stronger variations with the -fold decrease ranging between 1.3 and 2 for 10 spots, between 2 and 4 for seven spots, and over 4 for three spots. The comparative analysis of protein data sets enabled selecting six protein spots commonly up-regulated between both experimental conditions.

Protein Identification—For the mass spectrometry analysis, preparative gels were run and stained with ruthenium (ruthenium(II) tris(bathophenanthroline disulfonate)). In these gels,

FIG. 3. Representative 2D gels showing the protein expression profiles obtained from *X. laevis* tadpoles exposed for 72 h to the PCB mixture Aroclor 1254. Proteins of the samples obtained for the different experimental conditions were differentially labeled with Cy3 and Cy5. An internal standard composed of equal amounts of each sample and labeled with Cy2 was added. Labeled samples (25 μ g of each of the Cy3 and Cy5 labeled samples and of the Cy2 labeled internal standard) were loaded on 24-cm pH 4–7 non-linear IPG strips and subjected to IEF. Proteins were further separated by SDS-PAGE (12.5%) in the second dimension. Arrows and numbers allocated by the DeCyder software indicate spots with significant changes in intensity ($p < 0.01$, Student's *t* test in four independent gels). *a*, 2D gel image with proteins differentially expressed in the 0.1 ppm condition. *b*, 2D gel image with proteins differentially expressed in the 1 ppm condition.



15–18% of the protein spots could not be matched with certainty in comparison with the 2D DIGE pattern. Only 48 and 47 protein spots clearly identified in the 0.1 and 1 ppm condition, respectively, were selected and excised for mass spectrometry analysis.

In total, the analysis of 45 protein spots allowed the identification of 28 different proteins (Table I). Single peptide-based protein identifications are illustrated in the supplemental data. The identified proteins can be divided into different

groups, the first one corresponding to the six protein spots up-regulated within both PCB-exposed groups. Spots 957/799 and 988/820 (first and second spot numbers correspond to 0.1 and 1 ppm conditions, respectively) were commonly identified in *X. laevis* as cytokeratin type 2 that forms intermediate filaments of the cytoskeleton. Spot 992/833 was identified by homology to a *Xenopus tropicalis* protein as aldehyde dehydrogenase 7A1 (ALDH7A1). Spots 1196/1026 and 1263/1071 corresponded to *X. laevis* creatine kinase

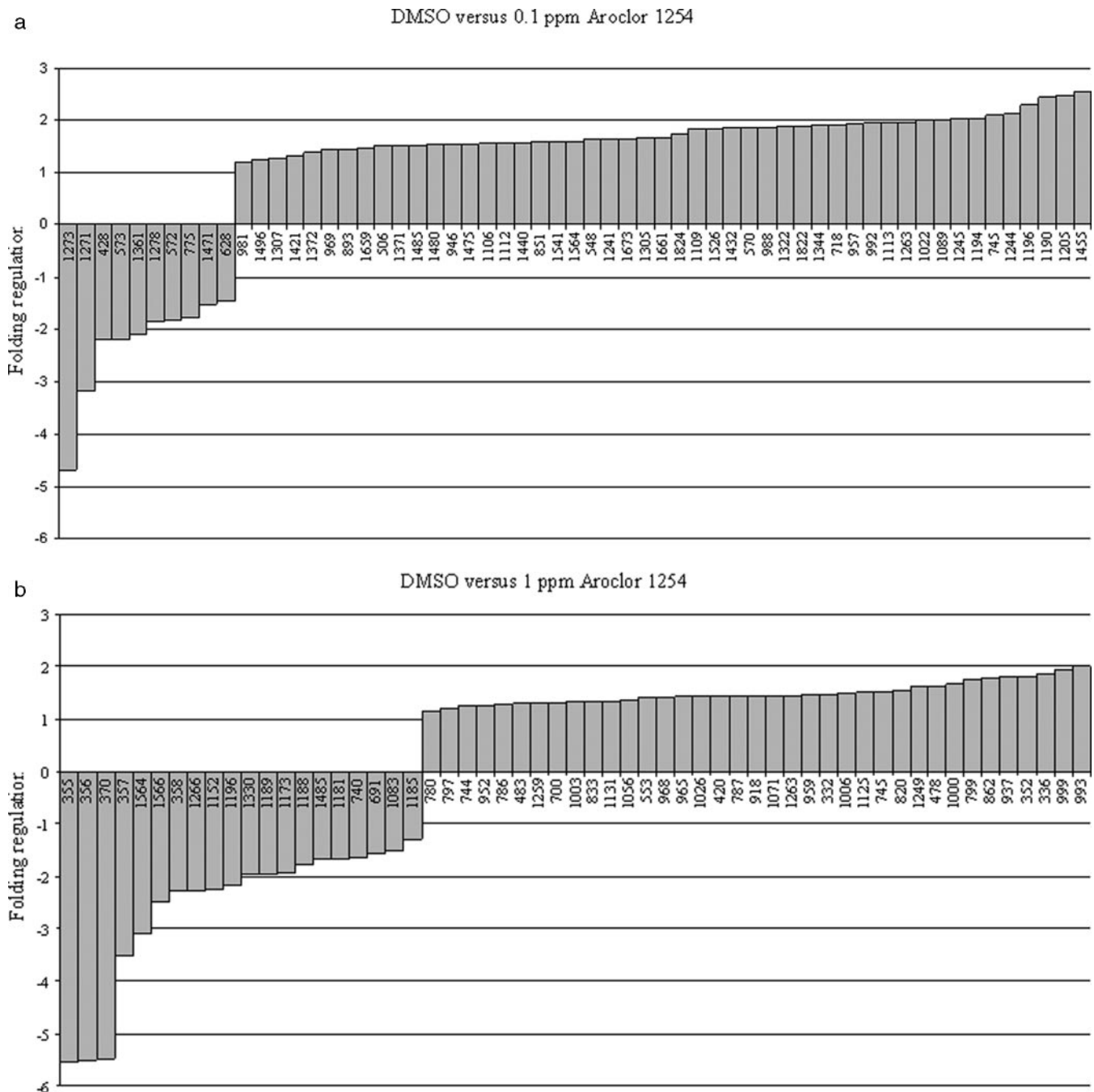


FIG. 4. Sets of protein spots showing differences in intensity between the PBC experimental groups and the DMSO control group. The y axis represents the -fold change intensity of the protein spots where a positive value indicates an increase in abundance and a negative value indicates a decrease in abundance. Data are organized on the x axis with the down-regulated proteins on the left side and the up-regulated proteins on the right side. a, tadpoles exposed to 0.1 ppm Aroclor 1254 versus control DMSO. b, tadpoles exposed to 1 ppm Aroclor 1254 versus DMSO control group.

muscle type (Ckm) and brain type (Ckb), respectively. The creatine kinase isoenzymes catalyze the synthesis of phosphocreatine and its subsequent use in the regeneration of ATP. Finally spot 851/700 was identified in *X. laevis* as glucose-regulated protein, 58 kDa (GRP58); GRP58 has a protein-disulfide isomerase (PDI)-like activity and plays an impor-

tant role in oxidative protein folding in the endoplasmic reticulum.

The second group included 16 protein spots up-regulated after the exposure of tadpoles to 0.1 ppm Aroclor 1254. Spot 1563 was identified in *X. laevis* as capping protein β subunit; capping protein binds to the barbed ends of actin filaments

Proteome of *X. laevis* Tadpoles Exposed to Aroclor 1254

TABLE I

List of the responsive proteins showing different abundance in tadpoles exposed to 0.1 and 1 ppm Aroclor 1254 versus control DMSO

No., spot number as given by the DeCyder software on the 2D gels; Accession no., accession number in UniProt/TrEMBL; Peptide fragments, unique peptides analyzed by Mascot ion search software, charge state always equal to 1, M represents oxidized methionine; Score, Mascot probability based on Mowse score calculated for MS/MS results, significance is reached for score ≥ 20 ; Expect, the expect value reflects the probability that the sequence match is a random event, significance is reached for expect value < 1 ; SC, sequence coverage (in %); M_r , molecular weight; Fc, -fold change where a positive value indicates an increase in protein spot intensity and a negative value indicates a decrease in intensity in tadpoles exposed to PCBs versus control DMSO.

No.	Accession no.	Identification	Peptide fragments	Score	Expect	SC	pI	M_r	Fc
0.1 ppm Aroclor 1254									
Cytoskeleton									
957	P16878	Keratin, type 2 cytoskeletal (<i>X. laevis</i>)	GKLEGELR FLEQQNR TEISELNR ALYEAELR SVSYGVSSGR LQAEIESVK WELLQNQK YEDEINKR LAELEAALQK SAVPNAGFSQMR ALDMSIIAEVK	23 35 40 62 30 50 23 21 71 35 81	0.23 0.013 0.0044 2.4e-005 0.028 0.00043 0.18 0.25 2.3e-006 0.0088 2.3e-007	19	5.5	55,287	1.93
988	P16878	Keratin, type 2 cytoskeletal (<i>X. laevis</i>)	FLEQQNR LLEGEENR TEISELNR ALYEAELR SVSYGVSSGR LQAEIESVK AQYEDIANK WELLQNQK ANAESAYQSK LAELEAALQK KLLEGEENR FQELQAAAGR EYQELMNVK SYSVTTTSSSR TGAENEFVVLK NMQDLVEDFK STKTEISELNR SAVPNAGFSQMR ALDMSIIAEVK	34 46 41 28 30 37 49 29 57 69 34 46 45 52 34 42 44 48 79	0.018 0.001 0.0035 0.056 0.032 0.0082 0.00042 0.044 5.7e-005 3.5e-006 0.017 0.0011 0.001 0.00021 0.014 0.002 0.0013 0.00044 3.7e-007	32	5.5	55,287	1.85
1563	A1DPL0	Capping protein β subunit (<i>X. laevis</i>)	LVEDMENK RLPPQIEK TGSGTMNLGGSLTR	20 39 102	0.43 0.0043 2e-009	8	5.7	30,864	1.72
Protein synthesis and degradation									
851	Q7ZWU3	Glucose-regulated protein, 58 kDa (<i>X. laevis</i>)	QAGPASVDLR LADDPNIVIAK LAPEYEIAATK VDCTANSNICNK	43 25 25 76	0.0016 0.089 0.1 6.3e-007	9	5.7	56,486	1.57
893	Q7ZWU3	Glucose-regulated protein, 58 kDa (<i>X. laevis</i>)	LNFAVANR SADGIVSTMK QAGPASVDLR SADGIVSTMKK LADDPNIVIAK FVMQEEFSR LAPEYEIAATK DGEDSGSYDGPR KLAPEYEIAATK VDCTANSNICNK EATNPPVKEDEKPK	33 54 75 70 66 56 58 58 40 95 22	0.023 0.00013 1.1e-006 3e-006 7.8e-006 8.1e-005 4.8e-005 5.3e-005 0.003 7.4e-009 0.14	19	5.7	56,486	1.56

TABLE I—continued

No.	Accession no.	Identification	Peptide fragments	Score	Expect	SC	pl	M_r	Fc	
946	Q5XGB9	Leucine aminopeptidase 3 (<i>X. tropicalis</i>)	TLIEFATR	24	0.14	8	8.4	53,788	1.54	
			FAEIFEQK	50	0.00033					
			SGGACTAAAFK	89	4.1e-008					
			GVLVAEGQNLAR	47	0.00058					
			TIQVDNTDAEGR	81	2.2e-007					
1659	Q68A89	Proteasome subunit α type (<i>X. laevis</i>)	GVNTFSPEGR	41	0.0028	4	4.8	26,613	1.48	
Glucose metabolism, neoglucogenesis										
1089	Q7SZ25	Enolase (<i>X. laevis</i>)	IEEELGSK	54	0.00019	9	6.2	47,817	2.01	
			ACNCLLLK	48	0.00066					
			AREIFDSR	35	0.012					
			NLNVVEQEK	38	0.0055					
			IGAEVYHNLK	62	2.5e-005					
1106	Q7SZ25	Enolase (<i>X. laevis</i>)	IEEELGSK	58	6.2e-005	10	6.2	47,817	1.56	
			ACNCLLLK	45	0.0013					
			AREIFDSR	22	0.25					
			NLNVVEQEK	28	0.049					
			GAEVYHNLK	27	0.079					
1109	Q7SZ25	Enolase (<i>X. laevis</i>)	IEEELGSK	49	0.00051	10	5.9	47,930	1.83	
			ACNCLLLK	48	0.00067					
			DGKYDLDFK	26	0.072					
			IGAEVYHNLK	68	5.7e-006					
			LMIEMDGTENK	52	0.00021					
1432	Q66KM4	Glyoxylate reductase/hydroxypyruvate reductase (<i>X. tropicalis</i>)	RLPPEGQK	41	0.0028	7	5.9	35,326	1.85	
			TAVFINTSR	38	0.0053					
			VPEAMEEVR	25	0.12					
			RVPEAMEEVR	33	0.018					
			EAFGMSLIK	20	0.36					
1440	Q7ZYM3	GPD1 protein (<i>X. laevis</i>)	GVDEGPEGLR	41	0.003	8	6.3	38,342	1.56	
			LISDIIQER	35	0.012					
Oxidative stress										
1190	Q7ZX44	Txndc5 (<i>X. laevis</i>)	EFGMSDVK	20	0.34	9	5.8	45,889	2.45	
			NGEKVDQYK	23	0.16					
			LFKPGQEAVK	37	0.0074					
			IAKVDCTAER	45	0.00098					
			SKEYFNK	21	0.27					
1787	Q5XH88	Peroxiredoxin 1 (<i>X. tropicalis</i>)	AVMPDGGFK	23	0.17	13	5.9	22,640	1.92	
			IGQPAPDFTAK	47	0.00072					
1824	Q6P8F2	Peroxiredoxin 2 (<i>X. tropicalis</i>)	DSKEFFSK	52	0.06	9	5.9	22,640	1.74	
			QITINDLPVGR	34	0.32					
Metabolism										
1196	Q7ZYQ9	Ckm (<i>X. laevis</i>)	FEEILTR	37	0.0073	4	6.2	42,905	2.28	
			GQTIDMMPAQK	58	4.7e-005					
1244	Q8AVH2	Ckb (<i>X. laevis</i>)	TDINSANLK	29	0.048	5	6.1	42,442	2.42	
			GGNMKEVFNR	56	6.7e-005					
1245	Q8AVH2	Ckb (<i>X. laevis</i>)	VLTLDMYK	41	0.0025	7	6.1	42,442	2.03	
			GGNMKEVFNR	55	9.3e-005					
			LSTEEYPDLSK	59	3.4e-005					
1263	Q8AVH2	Ckb (<i>X. laevis</i>)	GGNMKEVFNR	34	6.7e-005	5	6.1	42,442	1.97	
			TDINSANLK	29	0.048					
Other function										
992	Q28GS6	Aldehyde dehydrogenase 7 family member A1 (<i>X. tropicalis</i>)	QGLSSSIFTK	64	1.2e-005	8	6.2	55,139	1.94	
			STCTINYSK	30	0.039					
			CEGGTVVCGGK	40	0.0036					
			GAPTTSLTSVAVTK	40	0.0034					
1344	Q7ZYE9	Hnrpab (<i>X. laevis</i>)	DLKDYFAK	26	0.083	5	5.7	35,785	1.9	
			FGEVSDCTIK	46	0.00078					
1372	Q98UD3	CArG-binding factor A (<i>X. laevis</i>)	FGEVSDCTIK	53	0.00014	21	5.7	35,785	1.5	
			GAGGGQNDAEGDQINASK	64	1.1e-005					

Proteome of *X. laevis* Tadpoles Exposed to Aroclor 1254

TABLE I—*continued*

No.	Accession no.	Identification	Peptide fragments	Score	Expect	SC	pI	M_r	Fc
1 ppm Aroclor 1254									
Cytoskeleton									
799	P16878	Keratin, type 2 cytoskeletal (<i>X. laevis</i>)	GKLEGLER FLEQQNR TEISELNR ALYEAELR SVSYGVSSGR LQAEIESVK WELLQNQK YEDEINKR LAELEAALQK SAVPNAGFSQMR ALDMDSIAEVK	23 35 40 62 30 50 23 21 71 35 81	0.23 0.013 0.0044 2.4e-005 0.028 0.00043 0.18 0.25 2.3e-006 0.0088 2.3e-007	19	5.5	55,287	1.75
820	P16878	Keratin, type 2 cytoskeletal (<i>X. laevis</i>)	FGSGGSSGVK FLEQQNR LLEGEENR TEISELNR ALYEAELR SVSYGVSSGR LQAEIESVK AQYEDIANK ANASAYQSK LAELEAALQK FQELQAAAGR EYQELMNVK SYSVTTTSSSR STKTEISELNR SAVPNAGFSQMR	45 38 31 36 27 22 45 34 53 70 24 35 48 77 59	0.001 0.0074 0.035 0.011 0.074 0.2 0.0013 0.012 0.00015 2.8e-006 0.17 0.0094 0.00061 6.3e-007 3.6e-005	26	5.5	55,287	1.56
968	Q7SY65	Keratin, type 1 cytoskeletal 18-B (<i>X. laevis</i>)	ESELVQVR NSVTELR AQYDGLAQK LIDDTNISR VVAESNDTEVLKA	26 30 24 44 38	0.12 0.04 0.15 0.0013 0.0055	10	5.2	47,974	1.41
989	Q05AX6	Keratin 19 (<i>X. laevis</i>)	LAADDFR IVLQIDNAR TLETANSGLELK	32 26 46	0.029 0.076 0.00083	6	4.9	45,326	1.52
1000	Q28IM9	Keratin 12 (<i>X. tropicalis</i>)	LAADDFR LATYLEK SEITELRR FENELTLR ADYEVLAEK VLDELNLAR TIVEEVVDGK ALEAANAELVK	27 29 43 32 30 38 55 66	0.088 0.042 0.0021 0.021 0.035 0.0047 0.00012 8.5e-006	18	4.9	41,846	1.67
Protein synthesis and degradation									
744	Q7ZWU3	Glucose-regulated protein, 58 kDa (<i>X. laevis</i>)	QAGPASVDLR LADDPNIVIAK	65 32	1e-005 0.018	4	5.7	56,486	1.46
700	Q7ZWU3	Glucose-regulated protein, 58 kDa (<i>X. laevis</i>)	QAGPASVDLR LADDPNIVIAK LAPEYEIAATK VDCTANSNICNK	43 25 25 76	0.0016 0.089 0.1 6.3e-007	9	5.7	56,486	1.3
787	Q6DIK2	Chaperonin containing TCP1 subunit 2 (β) (<i>X. tropicalis</i>)	LAVEAVLR CDLLNISR ESVAMESFAK AGADEEKAETAR VAEIELAEKEK GATQQILDEAER TPGKESVAMESFAK	36 38 22 62 41 51 47	0.0078 0.0063 0.19 2e-005 0.0028 0.00023 0.0006	12	5.8	57,727	1.45

TABLE I—continued

No.	Accession no.	Identification	Peptide fragments	Score	Expect	SC	pI	M_r	Fc
Myofibrillogenesis and muscle contraction									
1152	Q6DIV8	Actin α 1 skeletal muscle (<i>X. tropicalis</i>)	IIAPPERK AGFAGDDAPR DLTDYLMK GYSFVTTAER EITALAPSTMK DSYVGDEAQS HQQVMVGMGQK DSYVGDEAQS	38 66 29 31 32 59 39 56	0.0057 8.9e-006 0.053 0.025 0.023 3.9e-005 0.0047 8.3e-005	18	5.2	41,988	-2.24
1173	Q5Y819	Myosin heavy chain α isoform (<i>X. laevis</i>)	ADIAESQVNK LDEAEQIAMK EQD TSAHLER	25 60 41	0.12 3.5e-005 0.0025	8	5.6	39,382	-1.94
1330	Q01173	Tropomyosin-1 α chain (<i>X. laevis</i>)	SLEAQA EK ATDAEGDVASLNR LEEAEKAADESER	37 72 49	0.0084 2e-006 0.00041	11	4.4	32,630	-1.97
420	Q5M901	Myosin-binding protein h (<i>X. tropicalis</i>)	DCAF I KK FTQALANR ALENFVQIR AINSLGEASVDCR IQLNLTGDKVTVR	20 38 26 68 59	0.33 0.0058 0.09 3.9e-006 3.5e-005	9	5.4	56,235	1.43
Metabolism									
1026	Q7ZYQ9	Ckm (<i>X. laevis</i>)	FEEILTR GQTIDMMPAQK	37 58	0.0073 4.7e-005	4	6.2	42,905	1.42
1056	Q8AVH2	Ckb (<i>X. laevis</i>)	FCTGLTK FGEILKR LLVEMEK LLVEMEKR	22 55 21 20	0.19 0.00015 0.31 0.34	5	6.1	42,442	1.55
1071	Q8AVH2	Ckb (<i>X. laevis</i>)	GGNMKEVFNR LLVEMEKR FCTGLTK	34 20 22	0.011 0.34 0.19	4	6.1	42,442	1.55
Other function									
478	Q802B7	NADH-ubiquinone oxidoreductase 75-kDa subunit (<i>X. laevis</i>)	VAGVLQGVQ GK SATYVNT EGR	32 26	0.019 0.07	2	6.1	79,575	1.62
483	Q802B7	NADH-ubiquinone oxidoreductase 75-kDa subunit (<i>X. laevis</i>)	SNYLLNSR VAGVLQGVQ GK SATYVNT EGR LQEVSPNLVR GNEMQVGTVEK	34 49 33 27 61	0.012 0.00034 0.013 0.066 2.1e-005	7	6.1	79,575	1.29
553	Q8JHX7	Dihydroliipoamide acetyltransferase precursor (<i>X. laevis</i>)	ILVAEGTR	30	0.043	1	7.2	66,849	1.41
745	Q7ZXW4	Nmp200 (<i>X. laevis</i>)	FLASTGMDR	31	0.023	1	5.9	54,772	1.52
780	Q7ZTJ5	P4hb protein (<i>X. laevis</i>)	VVDYNGER LITLEEEMTK MDSTANEIEAVK	37 43 82	0.0082 0.0014 1.9e-007	5	4.6	57,980	1.55
786	Q7ZTJ5	P4hb protein (<i>X. laevis</i>)	ALAPEY EK VADYNGER LITLEEEMTK	26 36 39	0.09 0.008 0.0035	5	4.6	57,980	1.57
797	Q7ZTJ5	P4hb protein (<i>X. laevis</i>)	ALAPEY EK VVDYNGER LITLEEEMTK MDSTANEIEAVK	21 26 46 80	0.3 0.094 0.00069 2.8e-007	7	4.6	57,980	1.18
833	Q28GS6	Aldehyde dehydrogenase 7 family member A1 (<i>X. tropicalis</i>)	QGLSSSIFTK STCTINYSK CEGGTVCGGK GAPTTSLTSVA VTK	64 30 40 40	1.2e-005 0.039 0.0036 0.0034	8	6.2	55,139	1.54

and is involved in the cytoskeleton regulation. Spots 893 and 1659 were identified in *X. laevis* as GRP58 and proteasome subunit α type, respectively. Proteasomal α subunits are major components of the 20 S proteasome, which is involved in the cytosolic proteolytic machinery. Spot 946 was identified by homology to *X. tropicalis* as leucine aminopeptidase 3 (LAP3) which is a metallopeptidase that cleaves N-terminal residues from proteins and peptides. Spots 1089, 1106, and 1109 were identified in *X. laevis* as enolase (ENO). ENO catalyzes the dehydration of 2-phospho-D-glycerate to phosphoenolpyruvate in the glycolytic pathway and the reverse reaction in gluconeogenesis. Spot 1432 corresponded to glyoxylate reductase/hydroxypyruvate reductase (Grhpr), which is similar to the corresponding protein of *X. tropicalis*. Grhpr functions both as glyoxylate reductase and as hydroxypyruvate reductase and plays a key role in directing the carbon flux to gluconeogenesis by its ability to convert hydroxypyruvate to D-glycerate. Spot 1440 was identified as *X. laevis* glycerol-3-phosphate dehydrogenase 1 (GPD1) involved in the branch point of the glycolytic pathway by converting dihydroxyacetone phosphate into glycerol 3-phosphate. Spot 1190 corresponded to *X. laevis* thioredoxin domain-containing 5 (Txndc5) whose biological function is not well described. Spots 1787 and 1824 were identified by homology to *X. tropicalis* as peroxiredoxin 1 (Prx I) and peroxiredoxin 2 (Prx II), respectively. Peroxiredoxins are members of the thiol-specific antioxidant proteins that catalyze the reduction of hydrogen peroxide with the use of electrons provided by thioredoxin. Spots 1244 and 1245 were both identified as *X. laevis* Ckb. Finally spots 1344 and 1372 were identified, respectively, as *X. laevis* heterogeneous nuclear ribonucleoprotein A/B (Hnrpab) proteins and CARG binding factor-A (CBF-A). The Hnrpab proteins comprise numerous proteins with a general packing role in RNA processing and transport. More precisely, CBF-A belongs to the subfamily of Hnrpab proteins and functions in both transcriptional and post-transcriptional processes of gene regulation.

The third group was made up of 17 proteins up- or down-regulated after the exposure of tadpoles to 1 ppm Aroclor 1254. Spots 1152, 1173, and 1330 were the only down-regulated identified proteins. Spot 1152 was similar to *X. tropicalis* actin α 1 skeletal muscle, whereas spots 1173 and 1330 were identified as *X. laevis* myosin heavy chain α isoform and α -tropomyosin, respectively. Actin α , myosin heavy chain, and tropomyosin α are muscle-specific proteins that play essential roles in myofibril assembly and muscle contraction. Spot 420 was similar to *X. tropicalis* myosin-binding protein h, which appears to function in the assembly of thick filaments during myofibrillogenesis. Spots 989 and 968 corresponded to *X. laevis* keratin 19 and keratin type 1 cytoskeletal 18-B, respectively, whereas spot 1000 was identified by homology to *X. tropicalis* as keratin 12. Keratins are major components of the cytoskeleton intermediate filaments. Spot 744 was identified in *X. laevis* as GRP58. Spot 787 was

assigned to *X. tropicalis* chaperonin containing TCP1, subunit 2 that is involved in protein folding. Spots 478 and 483 were both identified in *X. laevis* as NADH-ubiquinone oxidoreductase 75-kDa subunit, which is a major component of the mitochondrial respiratory chain complex 1 and catalyzes electron transfer from NADH to ubiquinone. Spot 1056 was identified as *X. laevis* Ckb. Spot 553 was assigned to *X. laevis* dihydrolipoamide acetyltransferase E2 precursor that is a member of the pyruvate dehydrogenase complex controlling the conversion of pyruvate to acetyl-CoA and NADH. Spot 745 corresponded to nuclear matrix protein 200 (Nmp200) of *X. laevis* known in mammals to be involved in pre-mRNA splicing, ubiquitylation, and DNA double strand break repair. Finally spots 780, 786, and 797 were all identified in *X. laevis* as prolyl 4-hydroxylase β (P4hb). P4h is a $\alpha_2\beta_2$ tetramer in which the β subunits are multifunctional polypeptides identical to the enzyme PDI.

DISCUSSION

The molecular mechanisms by which PCBs induce their toxicity during the development of tadpoles remain largely unknown. The present study is the first to investigate the potential effects of relevant environmental concentrations of these pollutants on the protein expression profiles of developing *X. laevis* tadpoles.

PCBs are known to affect the survival of numerous species, including amphibians. In the present study, the exposure of 5-day pf *X. laevis* tadpoles to 0.1 and 1 ppm Aroclor 1254 for 72 h did not impair their survival. This observation is in agreement with the data of Fisher *et al.* (11) that established that the survival of 9-day pf *X. laevis* tadpoles was not affected by the exposure to 1 ppm Aroclor 1254. The same observation was made on 7-day pf *X. laevis* tadpoles (10). However, another study conducted on *X. laevis* highlighted that 18-day pf tadpole exposed to 0.7 ppm Aroclor 1254 for 48 h showed a survival rate around 55% (32). Moreover the incidence of mortality was 10 times higher in the study performed by Zhou *et al.* (38) in which all tadpoles of 9 days pf died after 4 days of exposure to 1 ppm Aroclor 1254. This heterogeneity of the reported survival rates could be explained by an age-dependent insensitivity to chlorinated compounds in developing tadpoles. Indeed it has been reported that the embryos and tadpoles of green frogs (*Rana clamitans*), leopard frogs (*Rana pipiens*), and American toads (*Bufo americanus*) are 100–1000-fold less sensitive to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced lethality than most fish species (20). The crux of this insensitivity is that TCDD binds with very low affinity to the frog AhR especially during early development (22). Despite the presence of the AhR machinery, high levels of inducer are required to provoke cytochrome P450 1A1 (CYP1A1) induction (21). The same reason could explain a low affinity of PCBs for the AhR during the early development of tadpoles.

The present study has brought to light that the exposure of *X. laevis* tadpoles, a relevant aquatic organism used as a

model in ecotoxicological and developmental studies, to 0.1 or 1 ppm Aroclor 1254 led to significant changes in the abundance of 59 and 57 protein spots, respectively. The use of mass spectrometry downstream of 2D DIGE allowed the identification of different sets of PCB-responsive proteins. The function of these proteins can provide new clues on the molecular mechanisms by which PCBs induce toxicity during the development of amphibians. The set of proteins commonly identified between both Aroclor 1254 concentrations was limited to six. This uncoordinated response to different concentrations of the same compounds could be explained by the principle that the severity, or the probability of the effect, must be related to the dose or exposure level (40). This is true for the toxicogenomics and toxicoproteomics fields in which investigations of toxicant exposure indicated that dose-dependent changes are currently highlighted. For example, Poynton *et al.* (41) showed that in *Daphnia magna* exposed to different concentration of heavy metals each concentration produced a distinct gene expression profile. At the protein level, it has been highlighted that in *Mytilus edulis* exposed to graded copper concentrations only 11 protein spots were jointly regulated between experimental conditions (27).

Oxidative stress has been postulated to play a role in the toxic manifestations of PCBs and could thus induce an antioxidant response in exposed cells and tissues (42–44). In a previous study, modification of the activity of antioxidant enzymes such as superoxide dismutases, catalase, and glutathione redox cycle enzymes in 5-day pf *X. laevis* tadpoles exposed to 0.1 and 1 ppm Aroclor 1254 could not be readily observed, but the data do not exclude the induction of other antioxidant systems in response to PCBs (45). This is indeed the case because it was shown in this study that Prx I and Prx II were up-regulated in tadpoles exposed to 0.1 ppm Aroclor 1254. Prx I and Prx II are known as stress-inducible antioxidant enzymes as various stress agents are able to up-regulate the genes encoding Prx I and Prx II both *in vitro* and *in vivo*. Moreover the stress-inducible Prx I gene is activated through the antioxidant/electrophile response element (ARE/EpRE) present in the promoter region (46). The ARE/EpRE is a *cis*-acting regulatory element found in the 5'-flanking regions of numerous genes such as detoxifying phase 2 enzymes (several GSTs, heme oxygenase I, and cyclooxygenase 2) and is activated by redox-cycling phenols via the production of reactive oxygen species (47, 48). To our knowledge, very few studies have linked PCBs with the up-regulation of genes activated through the ARE/EpRE. However, Aroclor 1254 significantly induced the expression of the glutathione S-transferase Mu1 (GSTM1) gene, which is regulated through the ARE/EpRE (49), in the gills and digestive tract of the abalone *Haliotis discus* (50). Moreover some studies on rat hepatoma cells have reported that TCDD, whose chemical structure is similar to co-planar PCBs, induced the expression of different proteins in an ARE-dependent manner (51, 52). Thus, the up-regulation of antioxidant enzymes such as Prx I with ARE-

containing gene promoters is compatible with the hypothesis that PCBs could induce oxidative stress.

Endoplasmic reticulum (ER) stress is induced when high levels of misfolded proteins accumulate in the ER, generating the unfolded protein response (UPR). The UPR results in the up-regulation of chaperones such as GRP58, GRP74, GRP98, and PDI to prevent protein aggregation and cell death (53). In the ecotoxicological field, very few studies have described a possible induction of the UPR by toxicants (31, 54–56). In the present report, GRP58, also known as PDIA3, was one of the proteins up-regulated in both Aroclor 1254 conditions, suggesting a possible induction of the UPR by PCBs. PDIA3 is known to be overexpressed in TCDD-sensitive Long-Evans rat (52) and in the thymus of male marmosets (*Callithrix jacchus*) exposed to dioxin (57). It has also been highlighted that the gene encoding for PDIA3 contains AHREI, AHREII, and ARE motifs (52). Moreover the up-regulation of the proteasomal subunit α type, whose gene contains AHREI-AHREII motifs (19), and LAP3 in the 0.1 ppm condition could be linked to an overproduction of oxidized proteins within the cells. During mild oxidative stress, the 20 S proteasome degrades modified proteins (58). Oxidized proteins are continuously produced in cells as a result of the aerobic metabolism, but the protein oxidation can be increased by xenobiotic exposure (59). However, the link between PCBs and an overproduction of oxidized proteins has not been described in detail. Livingstone (60) reported an increase in oxidative damages such as oxidized proteins in both fish and invertebrates exposed to single or mixed contaminants including PCBs. Our findings on GRP58/PDIA3, the proteasomal subunit α type, and LAP3 are compatible with the hypothesis that PCBs could favor protein oxidation within the ER and cytosol, launching the UPR and increased proteolysis to protect the cell against aggregated and damaged proteins that can provoke cell death.

After exposure to 0.1 ppm Aroclor 1254, tadpoles displayed up-regulation of ENO, Grhpr, and GPD1, all enzymes involved in glycolytic and/or gluconeogenesis pathways. The impact of PCBs on glycolysis and gluconeogenesis has been poorly studied. Glycerol-3-phosphate dehydrogenase is known to be up-regulated in the thymus of male marmosets exposed to TCDD (57). In mice, exposure to PCBs induced an up-regulation of lactate dehydrogenase (61). However, Weber *et al.* (62) showed a reduced activity of phosphoenolpyruvate carboxykinase in mice exposed to TCDD. Also Kraemer and Schulte (63) highlighted that the exposure of the common mummichog *Fundulus heteroclitus* to PCBs induced a down-regulation of the equilibrium enzymes of glycolysis and gluconeogenesis. In the reported cases, the down-regulation of the enzymes was hypothesized to play a role in the toxic effects of PCBs, particularly those related to the wasting syndromes. Presently the overexpression of such enzymes could be linked to the increased requirements of both energy and protein synthesis/degradation pathways (64). This hypothesis is strengthened by the fact that Ckm and Ckb are

up-regulated in both conditions. Creatine kinases are crucial enzymes for high energy-consuming tissues like the brain, heart, and muscle, and their abundance is commonly correlated with muscle injury. These enzymes work as a buffering system for cellular ATP levels playing a central role in energy metabolism (65). Except from the serum of KANEMI YOSHU patients where high levels of creatine kinase have been correlated with high concentration of PCBs (66), few studies investigated the impact of PCBs on rapid energy metabolism. Finally the effects of PCB exposure should also be evaluated not only on the abundance of these enzymes but also on their enzymatic activities.

In the present study, the average body weight of tadpoles was significantly reduced by the exposure to 1 ppm Aroclor 1254 in agreement with other studies highlighting the interferences of PCBs with the growth of tadpoles (8, 10, 11). In a previous study, the reduction of weight in contaminated tadpoles was linked to an impairment of the energetic pathways in response to an increased energy demand associated with stress (45). Another explanation of the observed body weight reduction might be that PCBs could affect muscle development by interfering with normal myogenesis as actin α , myosin heavy chain α , and α -tropomyosin were down-regulated in tadpoles exposed to 1 ppm Aroclor 1254. Coletti *et al.* (67) showed that *in vitro* exposure of a rat myogenic cell line to Aroclor 1254 resulted in a decreased differentiation and fusion of myoblasts into myotubes. This response suggested that developing muscles could also be targets of PCBs. This hypothesis was also expressed by Fisher *et al.* (11) to explain the obscured or absent myotomal boundaries in the tail muscle of tadpoles exposed to Aroclor 1254. Two general mechanisms can be involved to explain the synthesis of myofibrillar proteins in the immature skeletal muscle: regulation may occur at the level of transcription or at the level of translation (68). The global effect of PCBs on the muscle development regulation has been poorly studied at the molecular level. The gene encoding for cardiac α -actin was reported to be slightly suppressed in the cardiovascular system of zebrafish (*Danio rerio*) embryos exposed to TCDD (69). However, Borlak and Thum (70) reported an up-regulation of the genes encoding the skeletal α -actin and α -myosin heavy chain in primary cardiomyocytes exposed *in vitro* to PCBs. Our data suggest a possible down-regulation of some of these genes. However, further analyses monitoring the expression of cytoskeletal genes at both the mRNA and protein levels are required to get a better insight into the response of tadpoles to PCBs.

Other biological functions evenly seem to be affected in tadpoles exposed to PCBs. ALDH7A1, also known as antiquitin in humans, was up-regulated in both Aroclor 1254 conditions. Antiquitin is assumed to play a role in osmoregulation and/or detoxification, but these functions have not been experimentally substantiated in animals (71). However, in plants, antiquitin is known to play a role in detoxification as the enzyme catalyzes the oxidation of endogenous and exoge-

nous aldehydes to their corresponding carboxylic acids (72). Some authors have already reported that PCBs and TCDD are able to induce the expression of genes encoding for aldehydes (19, 52, 73, 74). Moreover those genes are known to contain AHREI, AHREII, and ARE elements and are responsive to the aryl hydrocarbon receptor (52, 75). The present up-regulation of ALDH7A1 might be linked to a detoxification function even if the latter has not yet been confirmed in animals. The data also suggest that even if developing tadpoles are less sensitive to chlorinated compounds because of a low affinity for the AhR putative enzymes involved in detoxification processes such as ALDHs are overexpressed in developing amphibians exposed to PCBs.

CBF-A was originally described as a ubiquitously expressed protein that binds to CARG box motifs (76). CBF-A is also able to bind the Ha-ras element sequence with high affinity in rat carcinoma cells (77). The Ha-ras proto-oncogene is constitutively expressed in all cell types and can be induced in response to a wide number of mitogenic stimuli (78). PCBs are assumed to promote and perhaps to initiate malignant tumor formation. Among other things, PCBs that are AhR agonists show tumor-promoting activity in rodent liver (79). It has already been demonstrated that PCBs increase the expression of genes encoding proto-oncogenes such as Ha-ras (80–82). The present results highlight that PCBs are able to increase the abundance of CBF-A in contaminated tadpoles. However, it would be worthwhile to investigate whether the CBF-A up-regulation could be linked with an up-regulation of Ha-ras mRNA expression promoting carcinogenesis.

In tadpoles exposed to 1 ppm Aroclor 1254, an increase in the abundance of the P4hb subunit was also observed. This subunit is required for the proper tetramer formation of P4h. An increase of the β subunit could thus promote the formation of the active enzyme, essentially favoring collagen biosynthesis (83). However, this hypothesis is in contradiction with previous studies highlighting a reduction of collagen synthesis in the presence of PCBs (84, 85). Because a protein-disulfide isomerase activity has also been assumed for prolyl 4-hydroxylase β (86, 87), the overexpression of the P4hb subunit is in agreement with the up-regulation of GPR58 and reinforces the hypothesis that tadpole cells and tissues exposed to PCBs initiate defensive responses for protection against modified and misfolded proteins.

Lastly the exposure of tadpoles to 1 ppm Aroclor 1254 also induced the overexpression of Nmp200. Nmp200 is up-regulated after exposure to genotoxic agents and seems to be involved in the repair of DNA double strand breaks (88, 89). PCBs are known to be genotoxic as they induce intrachromosomal recombinations *in vitro* and *in vivo*. This genotoxicity might be explained by an oxidative stress as oxidation activities linked to the presence of PCBs might induce DNA strand breaks (39). The up-regulation of Nmp200 that we observed supports the genotoxic role of PCBs.

In conclusion, the present study is the first to highlight impacts of environmentally relevant concentrations of a PCB mixture on the proteome of developing tadpoles. It has been shown that PCB toxicity could be related to interactions with well known mechanisms such as oxidative stress, energy metabolism, myogenesis, and UPR. It has also been found that proteins such as ALDH7A1, CBF-A, P4hb, and Nmp200 could be associated with detoxification and toxicity processes in developing amphibians. The comparative analysis of protein data sets enabled selecting only six protein spots commonly up-regulated between both experimental conditions. Those proteins are linked to the UPR, energy metabolism, and detoxification processes, suggesting that those responses are of preferential concern when tadpoles are facing PCB exposure. These data demonstrate that environmentally relevant exposure to PCBs can deeply modify the amphibian proteome and suggest that these changes have to be taken into account while estimating the toxicological hazard of wild amphibian populations exposed to those chemicals.

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