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Development and Use of Real-Time Reverse Transcription-Polymerase Chain Reaction Assay to Quantify Cytochrome P4501A1 Expression in American Mink

Nirmal K. Roy · Steve Bursian · David T. Mayack · Isaac Wirgin

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Abstract The distribution of natural populations of American mink is restricted to locales that are in proximity to aquatic ecosystems. Because of the lipophilicity and persistence of polychlorinated biphenyls (PCBs) and reliance of mink on aquatic-based diets, mink at contaminated locales often bioacccumulate high levels of PCBs. In addition, in controlled laboratory studies, mink are highly sensitive at reproductive and developmental end points to the toxic effects of environmental PCB mixtures. It is believed that most, if not all, toxic effects of PCBs occur through activation of the aryl hydrocarbon receptor (AHR) pathway. Transcription of cytochrome P4501A1 (CYP1A1) by PCBs is also mediated through activation of AHR. Thus, levels of CYP1A1 mRNA provide a quantitative assay of exposure to and early biologic effect of PCBs on mink and may be predictive of toxicity at higher levels of biologic organization. We developed polymerase chain reaction (PCR) primers to amplify CYP1A1 as well as identified a housekeeping gene from mink cDNA. We used real-time reverse transcription-PCR to quantify and compare levels of hepatic CYP1A mRNA among groups of ranched mink kits and juveniles, which were fed diets or exposed in utero to fish that were low in PCBs (Atlantic herring) or to diets that were contaminated with three different levels of PCBs (carp) from Saginaw Bay, Lake Michigan. We found significant differences in CYP1A1 mRNA expression between mink fed the control diet and those fed a PCB-contaminated carp diet at all three treatment levels and exposure times. CYP1A1 mRNA was significantly induced 5.3- to 6.6-fold and 3.7- to 4.7-fold at 6 and 27 weeks, respectively. In previous studies, dietary exposures to PCB-contaminated carp were shown to cause mild to moderate lesions in the mandible and maxilla of these animals. This study demonstrates that hepatic CYP1A1 mRNA may be a sensitive biomarker of exposure of mink to environmentally relevant levels of PCBs and may be predictive of their effects in natural populations.

Many aquatic ecosystems worldwide are polluted from industrial or municipal sources with a variety of contaminants, which often include aromatic hydrocarbon (AH) compounds, such as polychlorinated biphenyls (PCBs). PCBs are mixtures that are comprised of potentially up to 209 different congeners that often differ in their environmental persistence, bioavailability, and toxicity. Toxicities of some PCB congeners, i.e., those that are coplanar, are mediated by activation of the aryl hydrocarbon receptor (AHR) pathway. The molecular components of the AHR pathway are structurally conserved across all vertebrate taxa and are believed to function similarly in fish and in humans (Hahn 1998). Briefly, the AHR resides in the cytosol complexed with two molecular chaperones, two molecules of heat-shock protein 90, and AHR interacting protein. On exposure, PCBs diffuse into cells where they bind with cytosolic AHR, and AHR releases its chaperones

N. K. Roy · I. Wirgin (✉)
Department of Environmental Medicine, New York University School of Medicine, 57 Old Forge Road, Tuxedo, NY 10987, USA
e-mail: isaac.wirgin@nyumc.org

S. Bursian
Department of Animal Science, Michigan State University, East Lansing, MI 48824, USA

D. T. Mayack
Division of Fish, Wildlife and Marine Resources, New York State Department of Environmental Conservation, Gloversville, NY 12078, USA

and translocates to the nucleus where it binds with its heterodimerization partner, the aryl hydrocarbon nuclear translocator. Subsequently, the AHR-ARNT-ligand complex recognizes and binds to specific DNA motifs within DNA-enhancer elements in the regulatory region of genes in the AHR battery that are induced by PCB exposure. Binding of AHR complex to these DNA sequences results in disruption of nucleosome structure, which allows for access of transcription factors to proximal regulatory motifs and initiation of transcription.

Among the genes induced by PCBs through this mechanism, cytochrome P4501A1 (CYP1A1) has been most studied, and levels of its expression are often used in some taxa as biomarkers of PCB exposure and early biologic effect. Furthermore, it is believed that increased levels of CYP1A1-encoded enzymatic activities play a major role in some toxic responses to AH contaminants either through generation of highly reactive polycyclic aromatic hydrocarbon (PAH) metabolites that bind DNA or protein or through generation of reactive-oxygen species that also target these cellular macromolecules (Wirgin and Theodorakis 2002). Thus, although induced expression of CYP1A1 is often a sensitive and dose-responsive marker of exposure, it may also be predictive of higher-level organismic responses to PCBs and other AHs.

Individual PCB congers differ significantly in their potencies to induce CYP1A1. Those that are coplanar and most closely resemble 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) best bind and activate AHR and are most effective at inducing expression of CYP1A1 and presumably higher-level toxicities. Toxic equivalency factors (TEFs) provide an empirically derived numeric system to express and compare the toxicities of individual coplanar PCB congeners relative to that of TCDD. Toxic equivalency quotients (TEQs) provide a numeric system to express and compare the total toxicity of environmental samples through enumeration of their concentrations of individual coplanar PCBs and their individual TEFs (Van den Berg et al. 1998).

The American mink *Mustela vison* is a sensitive and environmentally relevant sentinel species to evaluate the exposures and toxic effects of PCB contamination on aquatic ecosystems (Basu et al. 2007). Mink are abundant throughout most of North America, especially in wetland-containing areas (Arnold and Fritzell 1990). Mink are highly piscivorous; it is estimated that fish comprise approximately 50% of their diet and probably are the primary route through which they are exposed to PCBs (Chan et al. 2003; Wiener et al. 2002). Also, because of their high trophic status and the environmental persistence of PCBs, mink are likely to bioaccumulate and biomagnify PCBs to high levels. Mink reside in small demes, which because of their limited 1- to 5-km linear home ranges (Larivière 1999), may be highly isolated from other nearby reproductive units. As a result, the exposure history of individuals is likely to represent local sources of prey, and the effects of contaminants are likely to be induced at the population level (Foley et al. 1988).

In the early 1970s, it became evident that ranched mink were sensitive to reproductive failure from consumption of diets of Great Lakes fish contaminated with PCBs (Aulerich et al. 1973; Jensen et al. 1977; Platanow and Karstad 1973; Aulerich and Ringer 1977; Bleavins et al. 1980; Hornshaw et al. 1983; Heaton et al. 1995); that these toxicities were related to PCB structure; and that they were AHR mediated (Aulerich et al. 1985; Tillitt et al. 1996). It was estimated that the adverse reproductive effects of PCBs occur at a TCDD TEQ of approximately 60 pg/g, a concentration that is often exceeded in natural populations of mink from contaminated locales (Bursian et al. 2006b). Reproductive toxicities observed included a decreased number of kits produced, decreased survivability of kits, and compromised whelping success. These reproductive deficits presumably would have adverse consequences on the viabilities of populations exposed in the wild (Wren 1991). Other toxicities included decreased growth, anorexia, increased liver and kidney weights, immunodysfunction, endocrine disruption, and decreased levels of vitamin A and thyroid hormones. Approximately 65% of total TCDD TEQs were derived from PCB 126, the congener with the highest TEF, suggesting that coplanar PCBs contributed most of the toxicity in the contaminated Great Lakes fish diet. In addition, controlled laboratory studies have demonstrated that consumption of PCB-contaminated fish diets with moderate TEQs (47 and 73 ng TCDD TEQs/kg feed) results in proliferation of maxillary and mandibular squamous epithelia, loosened teeth, and swollen jaws in mink (Bursian et al. 2006a, 2006b).

Thus, mink may bioaccumulate high levels of environmentally borne PCBs and may be unusually sensitive to their toxic effects, particularly those that are AHR mediated. However, no easily and rapidly quantifiable biomarker of PCB exposure and early biologic effect is currently available for mink. In this study, our objectives were to develop a PCR assay to reliably measure CYP1A1 mRNA expression in mink and then to evaluate its applicability in mink exposed under controlled laboratory conditions to known amounts of dietary PCB.

Materials and Methods

Treatment of Mink Samples

The mink used in this study for hepatic CYP1A1 expression were the same animals previously analyzed for PCB-induced jaw lesions (Bursian et al. 2006a). Mink were bred

and treated with dietary PCBs at the Michigan State University Experimental Fur Farm as previously described. Briefly, 40 first-year female mink were randomly assigned to four treatment groups (10 mink/group) and were fed diets daily that contained 30% fish. The negative control group's diet contained Atlantic herring *Clupea harengus*, whereas the three experimental groups were fed mixtures of herring and 10, 20, or 30% of contaminated carp *Cyprinus carpio* from Saginaw Bay, MI. Concentrations in the four feeds were expressed as total PCBs (tPCBs) or TCDD TEQs. There were 0.03, 0.83, 1.1, and 1.7 mg tPCBs/kg feed or 2.5, 28, 47, and 73 ng TCDD TEQs/kg feed. Non-*ortho* and mono-*ortho*–substituted PCBs comprised approximately 40–45% of total TCDD TEQs in the Saginaw Bay carp-supplemented diets.

After 2–4 weeks, these female mink were mated with untreated male mink. Kits received nourishment exclusively through nursing until 3 weeks of age after which nursing activity gradually decreased, and kits were allowed to consume solid negative control or fish-contaminated feeds as previously described for the remainder of the treatment period. At 6 weeks of age, 6 kits from each treatment group were killed, and the remainder of kits (n = 8/treatment group) were killed at 27 weeks of age. At killing, liver from each animal were frozen at −80°C and retained for analysis of total PCBs, individual PCB, PCDF, PCDD congeners, and expression of CYP1A1.

Development of Mink CYP1A1 PCR Primers

Four mammalian CYP1A1 cDNA sequences—cat (AB-199730), dog (XM544773), grey seal (AJ621378), and harp seal (AJ621380)—were compared using the pileup option in the GCG Version 11.1 data analysis package (Accelrys Inc., San Diego, CA). Four upstream primers (Catcyp1 5′-AAGAGTTTCTCCATTGCC-3′, Catcyp2 5′-CCCAACCCTGCCCTGGAT-3′, Catcyp3 5′-GTCTTTGTGAACCAGTGG-3, and Catcyp4 5′-CAGATCAACCATGACCAG-3′) and 3 downstream primers (Catcyp5 5′-CTGCAGCAGGATGGCCAG-3′, Catcyp6 5′-CTGGTCATGGTTGATCTG-3′, and Catcyp7 5′-CCACTGGTTCACAAAGAC-3′) were developed based on consensus sequences (Table 1) (IDT, Coralville, IA). Upstream and downstream primers were used in all possible combinations in RT-PCR reactions to generate mink CYP1A1 cDNA amplification products that were subsequently sequenced. The amplification reactions contained 4 μl first strand reaction, 0.1 μM each of the primers, 50 μM each dNTP (GE Healthcare, Piscataway, NJ), 1 × PCR reaction buffer (Roche Applied Science, Indianapolis, IN), and 1 U Taq DNA polymerase (Roche) in a total volume of 30 μl. Cycling parameters were denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min followed by a final extension at 72°C for 7 min. One μl of amplicons were subjected to sequencing reactions using 5 pmole each of the primers and 6 μl of DTCS reaction mix (Beckman Coulter, Fullerton, CA), EtOH precipitated, and sequenced on a Beckman Coulter CEQ8000 instrument. Of those PCR products sequenced, a 699-bp amplicon (GenBank accession no. FJ380050) generated using Catcyp2 and Catcyp5 was used to obtain mink-specific primers for real-time PCR assays to quantitate CYP1A1 mRNA levels.

Table 1 Primers used to obtain a mink CYP1A amplicon based on conserved sequences among cat, gray seal, harp seal, and dog

CYP1A	
Forward	
Catcyp1:	AAGAGTTTCTCCATTGCC
Catcyp2:	CCCAACCCTGCCCTGGAT
Catcyp3:	GTCTTTGTGAACCAGTGG
Catcyp4:	CAGATCAACCATGACCAG
Reverse	
Catcyp5:	CTGCAGCAGGATGGCCAG
Catcyp6:	CTGGTCATGGTTGATCTG
Catcyp7:	CCACTGGTTCACAAAGAC
β-actin	
Forward	
Minkactin1:	GGCGGGACCACCATGTAC
Minkactin3:	TGGACCTGGCTGGCCGGG
Reverse	
Minkactin2:	GGAAGGTGGACAGCGAGG
Minkactin4:	CGGTCAGCGATGCCGGGG

Development of Mink β-Actin PCR Primers

Mink β-actin primers were developed based on cat (AB051104), dog (AF021873) and domestic ferret (AF038150) β-actin sequences. The upstream primers tested were Minkactin1 (5′-GGCGGGACCACCATGTAC-3′) and Minkactin3 (5′-TGGACCTGGCTGGCCGGG-3′), and downstream primers were Minkactin2 (5′-GGAAGGTGGACAGCGAGG-3′) and Minkactin4 (5′-CGGTCAGCGATGCCGGGG-3′). The mink RT-PCR products were sequenced to generate a 525-base pair (bp) β-actin sequence (GenBank accession no. FJ380051). This sequence was used to generate primers for real-time PCR experiments as endogenous mRNA.

Isolation of RNA

RNAs were isolated from approximately 50 mg of mink livers using Ultraspec reagent (Biotecx, Houston, TX). Frozen tissues were homogenized in 500 μl Ultraspec

Table 2 PCR primers designed based on empirically derived mink CYP1A and β-actin sequences used in real time RT-PCR assays

CYP1A	
MCYPRTF:	CGAACCCCAATGTGCAGAA
MCYPRTR:	GGCCCTGCCAATTACTGTGT
β-actin	
MACTRTF:	GGAGATCGTGCGTGACATCA
MACTRTR:	GCCATCTCCTGCTCGAAGTC

reagent in 1.5-ml plastic microcentrifuge tubes, and total RNAs were isolated as described by the manufacturer. First-strand cDNAs were generated from 100 ng mink total RNA preparations. Five hundred nanograms random hexamers (IDT) were added to the RNAs in a total volume of 15 µl and incubated at 75°C for 5 min. The mixture was chilled on ice, and 10 µl of RT-mix was added, so that the final reaction mix contained 1 × MMLV reaction buffer, 20 U MMLV reverse transcriptase (Promega Life Science, Madison, WI), 0.05 µl RNasin RNase inhibitor (Promega), and 0.5 mM dNTPs (GE Healthcare). Reactions were incubated at 42°C for 1 h, and products were denatured at 98°C for 5 min and chilled on ice until PCR amplification.

An ABI7300 real-time PCR system was used to amplify and quantitate CYP1A1 mRNA expression using β-actin mRNA as endogenous control. The mink-specific CYP1A1 primers MCYPRTF 5′-CGAACCCCAATGTGCAGAA-3′ and MCYPRTR 5′-GGCCCTGCCAATTACTGTGT-3′, and mink-specific β-actin primers MACTRTF 5′-GGAGATCGTGCGTGACATCA-3′ and MACTRTR 5′-GCCATCTCCTGCTCGAAGTC-3′, were used (Table 2). Amplification of CYP1A1 and β-actin cDNAs was done separately using the same volumes of the reverse transcriptase reaction products. Reactions contained 2 or 4 µl first-strand cDNA reactions, 150 nM each of the primers, and 12.5 µl Power SYBR Green master mix (ABI, Foster City, CA) in final reaction volumes of 25 µl. Amplification parameters were initial incubation at 95°C for 10 min and then 40 cycles of 95°C for 15 s and 60°C for 1 min.

Statistical Analysis

The relative comparison ΔΔCt method for real-time RT-PCR, as described in Applied Biosystems User Bulletin No. 2 (Livak and Schmittgen 2001) was used to evaluate relative expression levels of CYP1A. This approach was used to calculate β-actin-normalized fold induction of CYP1A1 mRNA in the PCB-treated groups compared with that in the negative control group separately for animals killed at 6 and 27 weeks of age. Analysis of variance (ANOVA) initially was used to analyze whether significant differences exists in gene expression among all groups; those individual groups that differed in expression were identified by Tukey's multiple range test. MEGA version 4 was used to construct a dendogram of mammalian cytochrome P450 1A and 1B sequences.

Results

Mink-specific CYP1A1 and a β-actin PCR primers were developed based on conserved sequences among four mammalian species to allow for quantification of normalized expression of mink CYP1A1. Initially, primers based on conserved CYP1A1 sequence among harp seal, grey seal, cat, and dog were used to amplify a 699-bp product from mink cDNA (Fig. 1). Similarly, conserved sequences among domestic ferret, cat, and dog were used to obtain a 525-bp β-actin amplicon from mink cDNA (data not shown). The 699-bp CYP1A1 PCR product was completely sequenced in both directions and found to be highly similar to previously characterized CYP1A1 of all four mammalian species. UPGMA (unweighted pair group method with averages) analysis showed the mink CYP sequence to group with 100% certainty within the cluster including grey seal, mouse, cat, monkey, and human CYP1A1 (Fig. 2). Nucleotide similarity of putative CYP1A1 between mink and the three other species (harp seal excluded) was between 90% and 93%, whereas amino acid similarity was 91–96%. Based on these mink-specific

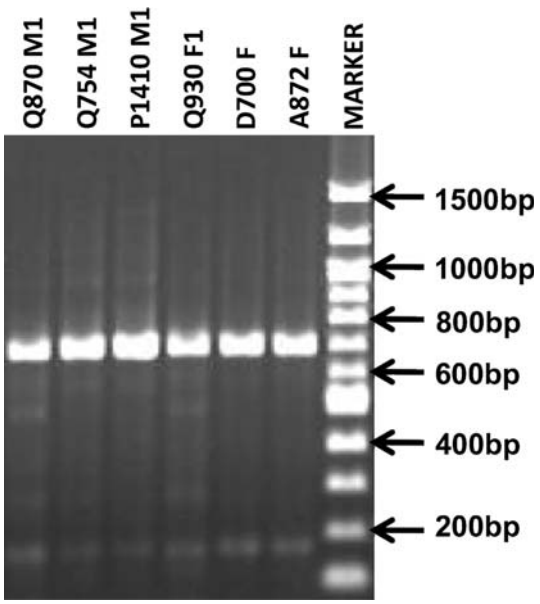

Fig. 1 Six hundred ninety-nine-base pair amplicon of mink CYP1A1 amplified with CATCYP2 and CATCYP5 primers. One hundred nanograms total hepatic RNA from 6 mink specimens were subjected to first-strand cDNA synthesis as described in Materials and Methods. CATCYP2 and CATCYP5 primers were then used to PCR amplify first-strand cDNA, electrophoresed in an 1.4% agarose gel, stained with ethidium bromide, and photographed under ultraviolet light

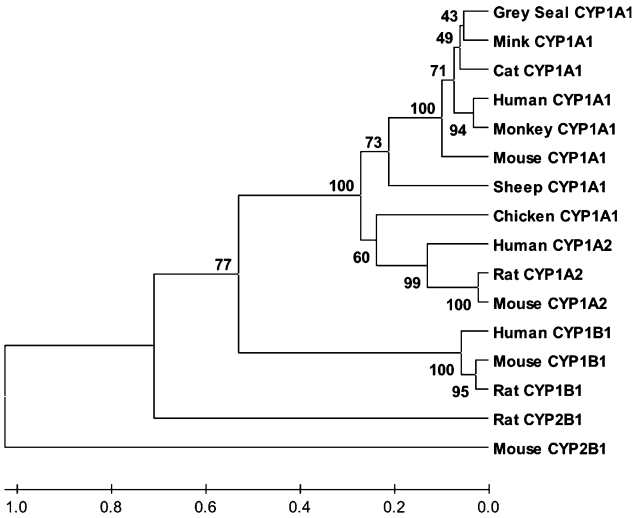

Fig. 2 Phylogenetic analyses of partial peptide sequences of 13 mammalian genes in the CYP1 family, including CYP1A1, CYP1A2, CYP1B1, and two genes in the CYP2 family that were used as outgroup (CYP2B1). The figure represents an unrooted tree generated by UPGMA analysis in Mega4 (Tamura et al. 2007), and bootstrap values are indicated on the tree's nodes. GenBank accession numbers of the analyzed sequences are grey seal CYP1A1 (AJ621378), cat CYP1A1 (AB199730), human CYP1A1 (BC023019), monkey CYP1A1 (NM_001040238), mouse CYP1A1 (NM_009992), sheep CYP1A1 (NM_001129905), chicken CYP1A1 (NM_205146), human CYP1A2 (AF182274), rat CYP1A2 (NM_012541), mouse CYP1A2 (BC054827), human CYP1B1 (DQ359216),), mouse CYP1B1 (NM_009994), rat CYP1B1 (NM_012940), rat CYP2B1 (NM_00001134844), and mouse CYP2B1 (NM_008898)

sequences, nested primers were developed for real time RT-PCR analysis of 59- and 66-bp mink products for CYP1A1 and β-actin. Similarly, CYP1A1 expression levels were compared using real-time RT-PCR among groups of mink kits that were exposed in utero under controlled laboratory conditions to three different diets of PCB-contaminated carp and noncontaminated carp and killed at 6 or 27 weeks of age.

Expression of CYP1A1 mRNA was dose-responsively induced by PCB treatment at both the 6- and 27-week time points (Fig. 3). Initial ANOVA analysis showed a significant difference in CYP1A1 mRNA expression among the four treatment groups at both time points, 6 weeks ($F_{3,20} = 16.6$–27.8, $p < 0.001$) and 27 weeks ($F_{3,17} = 23.05$–12.83, $p < 0.001$). We then used Tukey's multiple range test to perform pairwise comparisons at both time points to determine which treatment groups differed significantly from the negative control group. At 6 weeks of age, CYP1A1 expression in all three PCB treatment groups was significantly higher than in the group fed the control diet ($p < 0.05$). However, there was no significant difference among treatment groups in gene expression. At 27 weeks of age, CYP1A1 expression in all three PCB treatment groups was significantly higher than in the negative control group. However, at this age, CYP1A1 expression significantly differed ($p < 0.05$) among all PCB treatment groups except for those two that were exposed to the highest PCB levels.

Discussion

Mink can bioaccumulate high burdens of PCBs in highly contaminated natural environments (Basu et al. 2007), sometimes at levels within an order of magnitude of those that induce reproductive impairment (0.72–1.5 μg/g) (Tillitt et al. 1996; Bursian et al. 2006a), lesions of the mandible and maxilla (Bursian et al. 2006b), and hepatic lesions (Restum et al. 1998) in controlled laboratory exposures of ranched mink to PCBs. For example, populations of wild mink collected within one home range unit (6 km) of the Hudson River, NY, and downstream of two major sources of PCBs exhibited hepatic Aroclor concentrations as high as 139 (mean 13.0 μg total PCBs/g lipid; Mayack and Loukmas 2001).

However, the presence of contaminants, such as PCBs, in the environment and even within the tissues of exposed organisms does not confirm their biologic activity (Dickerson et al. 1994; Basu et al. 2007). This is the foundation on which the biomarker concept is built. Biomarker responses confirm the presence and biologic activity of stressors in receptor species and potentially in target organs, usually at a significantly decreased cost compared with analytic analysis of tissue contaminant burdens. Molecular biomarker responses, such as induced gene expression, offer the advantages of a rapid response, dose responsiveness, chemical specificity, and direct association with exposure (Vanden Heuvel and Davis 1999). However, there is no certainty that a positive molecular response is associated with impacts at higher levels of biologic organization. As a result, induced gene expression is sometimes viewed as a biomarker of exposure rather than effect.

Induction of CYP1A1 expression is a biomarker frequently used to indicate exposure to PCBs (and PCDDs and PAHs) in fishes because of its extreme sensitivity, dose responsiveness, and wide window of response (Wirgin and Theodorakis 2002). In this study, we report that significant 3.7- to 6.6-fold induction of hepatic CYP1A1 mRNA occurs in mink at environmentally relevant concentrations and routes of exposure to PCBs. We are aware of few studies that have evaluated CYP1A1 expression in terrestrial mammalian species, particularly at the transcriptional level. Ben-David et al. (2001), using species-specific primers, observed that CYP1A1 mRNA was significantly induced in peripheral blood mononuclear cells of wild river otters experimentally exposed to Prudhoe Bay crude oil.

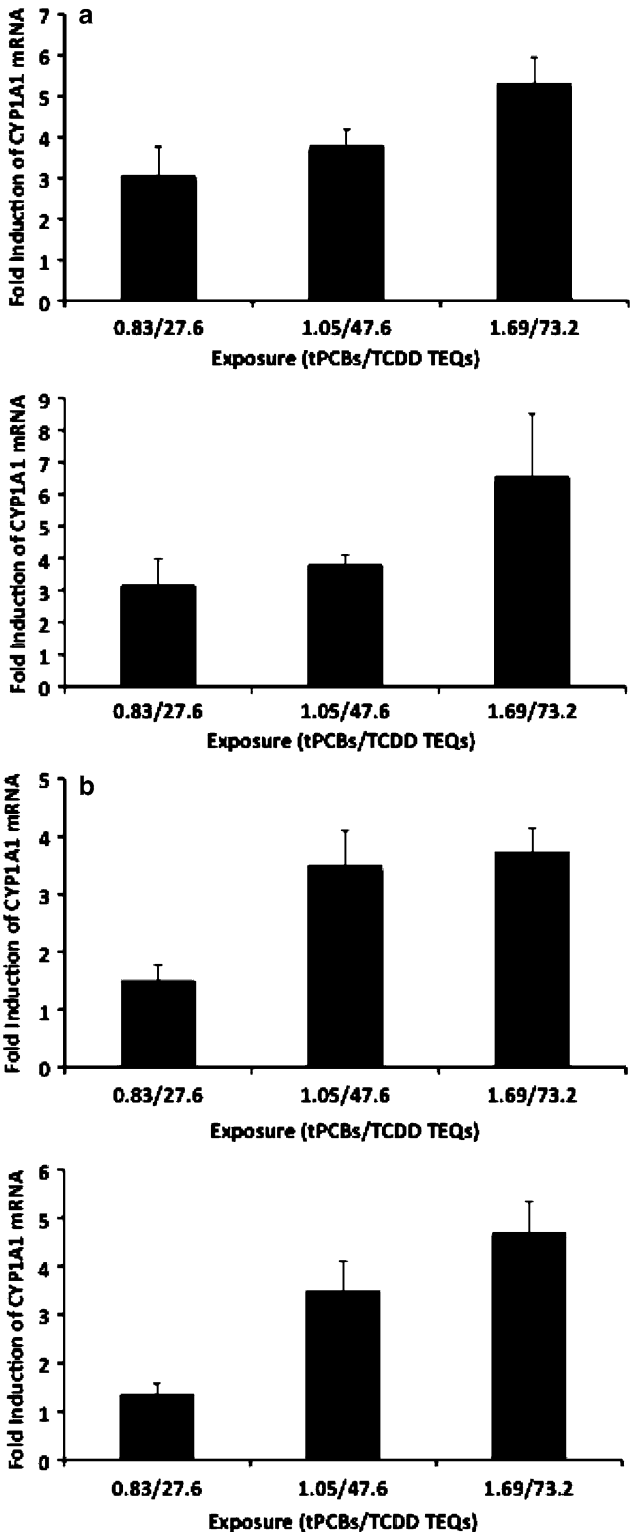

Fig. 3 Mean fold induction of CYP1A1 mRNA in groups of (**a**) 6-► week-old and (**b**) 27-week-old mink containing Atlantic herring (0.03 ng tPCBs/kg feed or 2.2 ng TCDD TEQs/kg feed) or three different amounts of PCB-contaminated carp (0.83, 1.1, and 1.7 mg tPCBs/kg feed or 28, 47, and 73 ng TCDD TEQs/kg feed) collected from Saginaw Bay, MI. CYP1A1 mRNA expression was determined by real time RT-PCR using mink-specific CYP1A1 and β-actin primers developed in this study. Fold induction of CYP1A1 mRNA in each of the three PCB treatment groups were determined compared with the expression of the Atlantic herring control group. Each comparison was done with 2 and 4 µl of first-strand cDNA reactions. Levels of CYP1A1 expression were normalized to β-actin expression. Bars indicate standard errors of the means

Schwartz et al. (2004) reported that American mink fed a diet supplemented with 500 ppm bunker C fuel oil exhibited 2.4-fold induction of CYP1A1 mRNA compared with those fed a control diet. Similarly, CYP1A1 mRNA was induced approximately 2-fold in rat liver after ingestion of oil-contaminated mussels (Chaty et al. 2008). These studies and ours suggest that the window of CYP1A1 induction in mammals exposed to AH contaminants is not nearly as great as in fishes, but the magnitude of gene induction can still be statistically significant at environmentally relevant levels of exposure.

The most informative use of molecular biomarker responses to stressors requires their linkage to effects at higher levels of biologic organization. Induced expression of CYP1A1 mRNA has previously been correlated in a variety of fish and avian models with a suite of early life-stage toxicities, often through impairment of cardiac function. In our study, we observed significant induction of CYP1A1 mRNA in mink at both 6 and 27 weeks of age, and this correlated with histologic alterations in the 27-week-old animals. Previously, Bursian et al. (2006a) reported an absence of alterations in 6-week-old kits fed contaminated carp but a high incidence of maxillary and mandibular squamous epithelial cell proliferation in the 27-week-old kits that had been fed diets containing 20% (4 of 7 kits mildly affected) and 30% (6 of 8 juveniles mildly to moderately affected) of Saginaw Bay–contaminated carp. Hepatic TCDD TEQs in the 27-week-old juveniles were 75 and 105 ng/kg in the 20 and 30% treatment groups, respectively, of which 31–35% were contributed by non-*ortho* and mono-*ortho* PCBs. Bursian et al. (2006a) speculated that these lesions could eventually lead to tooth loss, with probable detrimental effects on the health and survival of these animals. Thus, it is not difficult to envision that these effects of PCBs exposure may be induced at the population level in natural environments.

This study successfully developed an assay to measure normalized CYP1A1 mRNA expression in mink and validated its use in animals that were fed PCBs (and probably other contaminants) under controlled laboratory conditions. We envision that the most informative use of this assay will be to evaluate the exposure and early effects of AHs on natural populations of mink. However, as currently acquired and stored, tissues may not be of suitable quality for gene expression analysis. Typically, mink are not

collected directly by wildlife agency personnel but instead are secured from professional fur trappers. This arrangement usually entails the preservation of whole carcasses for extended periods under less than ideal storage conditions. However, recent innovations have included the development of a storage solution in which tissues can be preserved for short periods of time at room temperature and subsequently for extended times at −20°C. Studies have demonstrated that RNA from these tissues that have not degraded are then adequate for quantitative analysis of gene expression at the transcriptional level (Mutter et al. 2004). We foresee future efforts in which trappers are provided storage solution before the trapping season, which will allow for adequate preservation of archive tissue samples.

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