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INDUCTION OF CYTOCHROME P450-ASSOCIATED MONOOXYGENASES IN NORTHERN LEOPARD FROGS, *RANA PIPIENS*, BY 3,3′,4,4′,5-PENTACHLOROBIPHENYL

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Abstract—Northern leopard frogs (*Rana pipiens*) were injected intraperitoneally either with a solution of polychlorinated biphenyl (PCB) 126 in corn oil at a concentration of 0.2, 0.7, 2.3, or 7.8 mg/kg body weight or with corn oil alone. Appropriate assay conditions with hepatic microsomes were determined for four cytochrome P450-associated monooxygenases: ethoxyresorufin-*O*-dealkylase (EROD), methoxy-ROD (MROD), benzyloxy-ROD (BROD), and pentoxy-ROD (PROD). One week after PCB administration, the specific activities of EROD, MROD, BROD, and PROD were not elevated at doses $\leq$0.7 mg/kg ($p > 0.05$) but were significantly increased at doses $\geq$2.3 mg/kg compared to the control groups ($p < 0.05$). The increased activities of these four enzymes were 3 to 6.4 times those in the control groups. The increased activities were maintained for at least 4 weeks. Because of a lack of induction at low doses of PCB 126, which were still relatively high compared to currently known environmental concentrations, we suspect that EROD, MROD, BROD, and PROD activities are not sensitive biomarkers for coplanar PCB exposure in leopard frogs.

Keywords—*Rana pipiens* Polychlorinated biphenyls Cytochrome P450-associated monooxygenase Biomarker

INTRODUCTION

In the past decade, biochemical and physiological characteristics such as hepatic detoxifying system, DNA adducts, thyroid malfunction, and acetylcholinesterase inhibition have been used extensively as biomarkers for contaminant exposure [1]. Among these biomarkers, the induction of hepatic detoxifying enzymes has been proposed as a promising early warning system for animal exposure to polyaromatic hydrocarbons [2]. In general, environmental toxicants can be categorized as either 3-methylcholanthrene-type (3-MC-type) or phenobarbital-type monooxygenase inducers. For instance, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD), 2,3,4,7,8-tetrachlorodibenzofuran, and coplanar polychlorinated biphenyls (PCBs) 77 and 126 belong to 3-MC-type inducers, and dichlorodiphenyl trichloroethane (DDT) and 2,2′,4,4′-tetrachlorobiphenyl are phenobarbital-type inducers [3,4]. The majority of monooxygenase studies have focused on fish [5], birds [6], laboratory mammals [7], and one reptile species [8]. Although a few studies on amphibian monooxygenases exist [9–11], to our knowledge, there is no published study on induction of hepatic monooxygenases by PCBs in adults of any amphibian species. This is unfortunate because induction can be used as an ecological biomarker for exposure [1,2].

To be a useful biomarker for exposure of environmental contaminants, hepatic monooxygenases in amphibian species should (1) respond to pollutants in a dose-dependent manner over a concentration range that is environmentally meaningful and (2) persist over a period of time after pollutant exposure [1]. Therefore, our objectives in this study were (1) to test the sensitivity of monooxygenase induction in the northern leopard frog, *Rana pipiens*, to a coplanar PCB congener; (2) to test the persistence of monooxygenase induction following a single PCB injection; and (3) to evaluate several candidate monooxygenase activities for assessing PCB exposure in amphibians. The northern leopard frog was chosen because it is a common species in many wetlands in North America. We chose coplanar PCB 126, 3,3′,4,4′,5-pentachlorobiphenyl, because (1) coplanar PCBs have caused adverse effects on a wide spectrum of species, including fish [12], birds [13], and mammals [14] and (2) PCB 126 is the most potent of the PCBs that act as inducers of aryl hydrocarbon hydroxylase in rodents [15]; thus, it is the prototype PCB for studying monooxygenase induction.

MATERIALS AND METHODS

Chemical source and animal care

3,3′,4,4′,5-Pentachlorobiphenyl (PCB126) (purity, >99%) was purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Its purity was analyzed by the Department of Water Sciences, University of Wisconsin–Madison (Madison, WI, USA) as described below (see section on tissue residue analyses) and was determined to be >99.5% of the total PCB quantity found in the stock solution. Enzyme substrates and standard resorufin were purchased from Molecular Probes (Eugene, OR, USA).

All procedures for animal housing, handling, and dissection adhered to guidelines provided by the Animal Use Committee of Research Animal Resources Center and the Office of Biological Safety of the University of Wisconsin–Madison.

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Table 1. Gender, initial body weight, and final body weight

Treatment	Gender		Body weight[a] (g)	
	F	M	Initial	Final
Week 1				
Control	1	4	27.90 ± 3.09	29.74 ± 2.64
0.2 mg/kg	3	3	25.93 ± 2.51	27.65 ± 2.56
0.7 mg/kg	1	5	23.72 ± 2.15	24.57 ± 2.31
2.3 mg/kg	2	3	26.24 ± 1.30	27.14 ± 1.85
7.8 mg/kg	2	3	27.28 ± 1.00	28.74 ± 1.33
Week 2				
Control	0	6	27.27 ± 0.84	27.08 ± 1.26
7.8 mg/kg	2	4	27.08 ± 1.51	28.58 ± 1.68
Week 3				
Control	1	4	20.56 ± 1.97	23.90 ± 2.41
7.8 mg/kg	0	4	22.30 ± 1.97	24.30 ± 2.01
Week 4				
Control	2	4	26.85 ± 0.65	29.02 ± 0.91
7.8 mg/kg	2	3	25.32 ± 1.51	27.14 ± 1.27

[a] Mean ± 1 SEM.

Sixteen female and 50 male leopard frogs (25.61 ± 4.39 g [mean ± SD], $n = 66$) were collected in mid-August 1993 at the Leopold Memorial Reserve (Baraboo, WI, USA). Until the experiment, they were housed for 2 months in four fish tanks with running dechlorinated water at 23°C in the Water Science and Engineering Building on the University of Wisconsin–Madison campus. The tanks were tilted so that frogs could swim in the water pool or sit on dry substrate. Frogs were fed crickets and mealworms ad libitum. The room temperature was set at 20°C under a 12-h-light/12-h-dark photoperiod.

Two weeks before the experiment was conducted, frogs were housed individually in Rubbermaid® tubs (58.4 × 42.5 × 22.9 cm) lined with heavy-density polyethylene plastic bags (Associated Bag, Milwaukee, WI, USA). The liners were changed every 6 d. Water was supplied in small plastic dishes and changed every other day. We fed each frog seven crickets every 3 d, which permitted maintenance of body weight or growth. Mealworms were also provided intermittently starting the second week. Vitamins and minerals were provided every 6 d by dusting them onto crickets. In both holding regimes, frogs ate and responded to humans normally, and no skin diseases were found.

On December 21, frogs that had been fasted 24 h were injected once intraperitoneally with 2 ml/kg of either a solution of PCB 126 in corn oil at a designated dose or corn oil alone.

Dose–response experiment

The dose levels were 0, 0.2, 0.7, 2.3, and 7.8 mg/kg, and frogs were killed 1 week after dosing. Twenty-seven frogs were used, with four to six individuals in each treatment group. Numbers of each gender in each group are presented in Table 1 (see Results).

Time course experiment

Frogs received 7.8 mg/kg PCB 126 in corn oil or corn oil alone and were killed 1, 2, 3, and 4 weeks after dosing, with four to six individuals in each treatment group.

Liver sample preparation and enzyme assay

At the indicated times, frogs were decapitated, and their livers were removed immediately. Each liver was placed in a cryotube with enough glycerol to cover the whole tissue, then stored at −110°C. One week after final dissection, all liver samples were shipped overnight in dry ice to the Patuxent Wildlife Research Center (Laurel, MD, USA) for enzyme analysis.

Microsomal preparation and enzyme assay followed the methods of Burke and Mayer [16] and Melancon [17] with slight modifications. The total volume for each reaction was 260 μl, consisting of 50 μl of buffer solution, 150 μl of substrate (5 μM, except 2.5 μM methoxyresorufin) in buffer, 50 μl of microsomes (from 2.6 mg of liver) in buffer, and 10 μl of reduced nicotinamide adenine dinucleotide phosphate (NADPH) (0.125 mM). After the reaction mixture was preincubated at 28.5°C for 10 min, NADPH was rapidly added, and the microwell plate was placed in a computer-coupled fluorescence microwell plate scanner (Fluoroskan II); eight readings were taken at 1.5-min intervals. The enzyme-specific activities were calculated from the change of fluorescence with time as compared to the fluorescence of known amounts of product added to a series of wells with all components present except NADPH. All enzyme activities were expressed as picomoles of product per minute per milligram of microsomal protein.

The microsomal protein concentration was measured by the method of Lowry et al. [18] using crystalline bovine plasma albumin as standard.

Tissue PCB residue analyses

Carcasses, except livers and kidneys that had been used for other purposes, from the same treatment groups were proportionally pooled and blended with dry ice. The dry ice was sublimed gradually in a −20°C freezer.

Sample extraction and cleanup for PCBs were achieved using method 1410 of the Organic Chemistry Section of the Wisconsin State Laboratory of Hygiene with slight modifications. All solvents were pesticide grade (EM Science, Cherry Hill, NJ, USA). Congener-specific PCB analyses were performed with a Hewlett-Packard 5890 gas chromatograph containing a DB-5 capillary column (0.25-mm inner diameter, 0.32-μm film thickness, and 30-m length) (J&W Scientific, Folsom, CA, USA) and an electron capture detector. The temperature program ran from 90 to 240°C at 1°C/min (with a ramp to 300°C at the end for 25 min). Injection volume was 2 μl. The calibration standard consisted of a 250:180:180 (w:w:w) mixture (610 ng/ml total, single-point calibration) of Aroclor® 1232, 1248, and 1262, and individual congener concentration data were those values used in the Green Bay Weight Balance Study [19]. The minimal detection levels for PCBs ranged from 0.4 to 3.0 ng/g.

Statistical analyses

The initial body weight was the weight when a fasted frog was administered the PCB solution. The final body weight was calculated by subtracting the weight of gut contents from the body weight measured immediately before killing. All frogs had food residue in their stomachs at the time of killing. The difference between initial body weight and body weight at killing was analyzed using repeated-measures analysis of variance (ANOVA) with dose and week as independent variables. The variation of liver weight at killing was analyzed using analysis of covariance (ANCOVA) with dose and week as independent variables and body weight as a covariate, followed by backward elimination analyses.

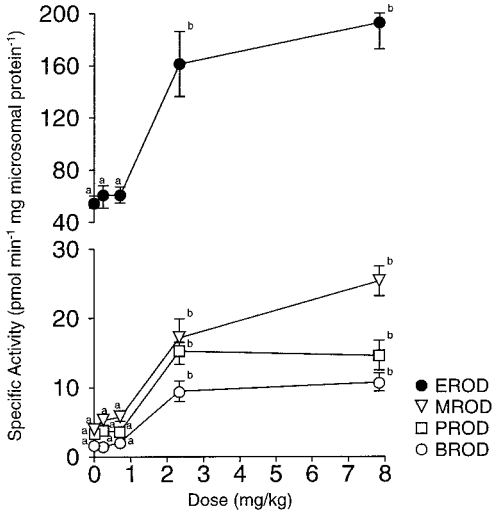

Fig. 1. Dose response of monooxygenase activity to administration of polychlorinated biphenyl 126. Within each treatment group (n = 4–6/group), those values with the same lowercase letter are not significantly different. EROD = ethoxyresorufin-*O*-dealkylase; MROD = methoxy-ROD; PROD = pentoxy-ROD; BROD = benzyloxy-ROD.

Snout vent length and final body weight were included as covariates in the ANCOVA, relating dose levels and genders with monooxygenase activities. In a separate ANCOVA, time course was added as an independent variable and gender was excluded because of an insufficient number of females. Backward elimination followed multivariate analyses to obtain appropriate models. The activities of ethoxyresorufin-*O*-dealkylase (EROD), methoxy-ROD (MROD), benzyloxy-ROD (BROD), and pentoxy-ROD (PROD) were $\log_{10}$ transformed, if necessary, to obtain homogeneity of variance. Multiple comparisons of enzyme activity among treatment groups was performed by Fisher's least significant difference test. Power tests were performed for the dose–response experiment to test the power of the ANCOVA model to detect a tripling in enzyme level. The relationships among monooxygenase activities as well as the relationships among tissue PCB 126 residue levels and monooxygenase activities were examined using Pearson's correlation matrix and the Bonferroni probability matrix. All statistical analyses were performed using SYSTAT® for Windows®, version 5 (SYSTAT, Evanston, IL, USA). Statistical significance in all analyses was set at $\alpha = 0.05$.

RESULTS

Body weight, liver weight, gross liver and kidney appearance, and behavior

Throughout the study, frogs responded normally to human disturbance and exhibited no anorexia, skin or organ lesions, or mortality. The initial body weights of leopard frogs did not differ significantly among treatment groups ($p = 0.133$, $n = 59$) (Table 1). A reduced repeated-measures ANOVA applied for the time course experiment showed that body weight increases between initial body weight and final body weight were significant over time ($p = 0.008$, $n = 42$) but not for dose ($p = 0.845$, $n = 42$). At the time of killing, all frogs had food residues in their stomachs. Backward elimination analyses following ANCOVA showed that dose and time were not significant factors in explaining the variation in liver weights but that body weight was significant ($p < 0.0001$, $n = 42$).

In the time course experiment, the incidence of transparent or yellow kidneys was significantly higher in frogs injected with 7.8 mg/kg PCB 126 (7 of 20 individuals) than in control individuals (2 of 22 individuals) ($\chi^2 = 4.18$, $p < 0.05$). The discolored kidneys in the PCB-exposed group occurred in frogs killed 2 and 4 weeks after injection. No significant difference in the incidence of pale livers between the PCB-exposed group (5 of 20) and the control group (4 of 22) ($\chi^2 = 0.3$, $p > 0.5$) was observed.

Dose–response experiment

In the dose–response experiment relating PCB 126 dose level and gender with four monooxygenase activities, backward elimination following ANCOVA suggested that only the dose effect was significant ($p < 0.0001$, $n = 27$). The $\log_{10}$ specific activities of EROD, MROD, PROD, and BROD were not elevated 1 week after PCB administration in groups receiving 0.2- and 0.7-mg/kg doses ($p > 0.05$, $n = 27$) but were significantly increased in those that had received the 2.3- and 7.8-mg/kg doses ($p < 0.05$, $n = 27$) (Fig. 1). The increase in activities of these four enzymes ranged from 3- to 6.4-fold relative to control groups. We calculated the statistical power to detect an increase in each enzyme activity of three times assuming an $\alpha = 0.05$ and using the estimated within-group variance from our experiment. The powers for the EROD, MROD, and PROD ANCOVA models were >99%. The power for the BROD ANCOVA model was 80%. Correlation analysis of week 1 data indicated a significant association among EROD, MROD, BROD, and PROD ($r = 0.903$–0.973, $p < 0.05$, $n = 27$) (Fig. 2).

Time course experiment

For the time course experiment, backward elimination following ANCOVA showed that dose, time, and dose × time were significant ($p < 0.0001$). Although enzyme activities in the treatment group were already elevated above the control group 1 week after treatment (above), the EROD and MROD activities of treatment frogs were subsequently increased fur-

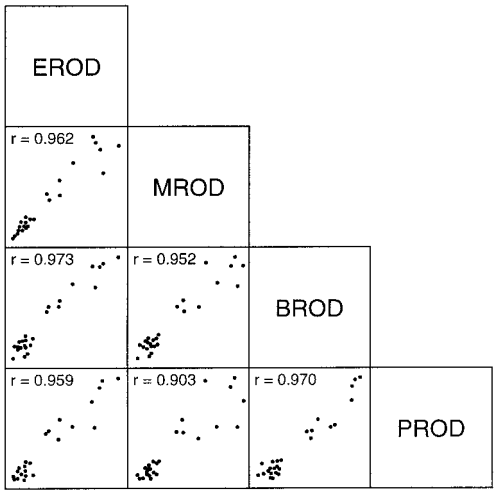

Fig. 2. Correlation analyses of monooxygenase activities 1 week after administration of polychlorinated biphenyl 126 administration. Each column heading defines the x axis for the plots below it. Each row heading defines the y axis for the plots on that line. In each case, the enzyme activities are in units of picomoles per minute per milligram of microsomal protein. (See the legend of Fig. 1 for definitions of enzyme abbreviations.)

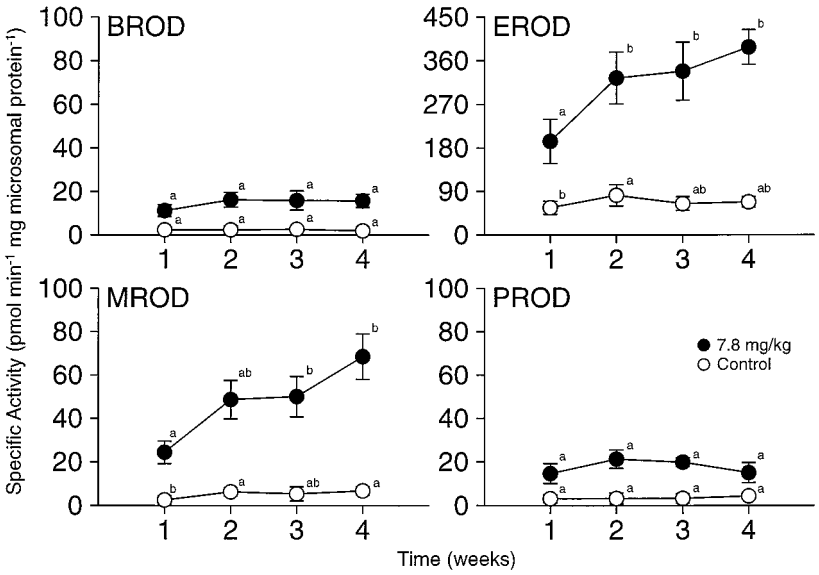

Fig. 3. Time course of monooxygenase activity following administration of polychlorinated biphenyl 126. Within each treatment group (n = 4–6/group), those values with the same lowercase letter are not significantly different. (See the legend of Fig. 1 for definitions of enzyme abbreviations.)

ther by a factor of 2 by the second week and stabilized thereafter (Fig. 3). No time effects on BROD or PROD activities were observed in either the treatment ($p > 0.05$, n = 4–6) or control groups ($p > 0.05$, n = 4–6). The EROD and MROD activities in control groups did increase slightly (≤20%; $p < 0.05$, n = 4–6) after the first week, but the differences did not persist (Fig. 3). Correlations among EROD, MROD, BROD, and PROD of the 7.8-mg/kg dosage over time were significant (r = 0.714–0.950, $p < 0.05$, n = 20), except for EROD and PROD (r = 0.346, p = 0.813, n = 20) and MROD and PROD (r = 0.173, p = 1.000, n = 20) (Fig. 4).

Tissue residue levels

Carcasses pooled for each treatment group at week 1 were analyzed for PCB residues (Table 2). The lipid contents in the five treatment groups at week 1 ranged from 2.51 to 3.11%.

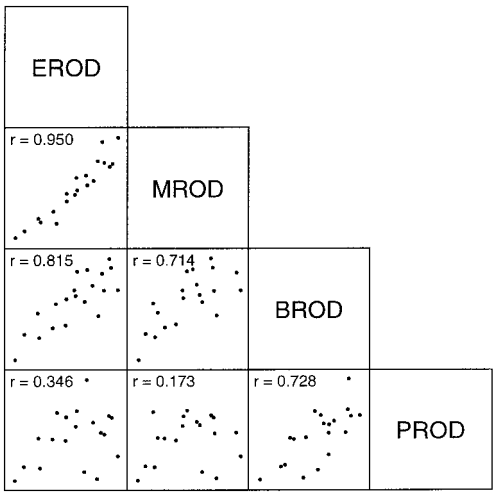

Fig. 4. Correlation analyses of monooxygenase activities in the time course experiment. Each row heading defines the y axis for the plots on that line. In each case, the enzyme activities are in units of picomoles per minute per milligram of microsomal protein. (See the legend of Fig. 1 for definitions of enzyme abbreviations.)

The total PCB residues, excluding PCB 126, in experimental frogs ranged from 14.66 to 20.28 ng/g based on wet body weight. No coplanar PCBs were found in any of the pooled carcasses. The mean activities of BROD, EROD, and MROD were significantly correlated with the tissue PCB 126 residue levels (r = 0.912–0.955, $p < 0.05$, n = 5; data not shown). A weak correlation was found between the PROD activity and the tissue PCB 126 residue (r = 0.865, p = 0.058, n = 5).

The mean activities of MROD, EROD, PROD, and BROD in the experimental frogs were not significantly correlated with the total tissue PCB residue levels, excluding PCB 126, in each treatment group (r = −0.269–−0.235, $p > 0.60$, n = 5). Based on the backward elimination following an ANCOVA model relating PCB 126 dose levels with four monooxygenase activities, as well as correlation between tissue PCB residue levels and enzyme activities, we concluded that the elevated enzyme activities were due to the administration of PCB 126.

DISCUSSION

Induction of hepatic cytochrome P450-associated monooxygenases and the potential as biomarkers

No gender difference was observed in enzyme level or induction according to our ANCOVA model relating dose lev-

Table 2. Mean percentage of lipids and concentrations of polychlorinated biphenyl (PCB) 126 and total PCB in leopard frogs

Treatment pool[a]	Lipid content (%)	PCB concn. (ng/g body weight)	
		PCB 126	Other PCBs[b]
Control	2.66	1.4	14.66
0.2 mg/kg	3.11	106.1	7.10
0.7 mg/kg	2.51	280.9	11.16
2.3 mg/kg	2.62	774.6	10.04
7.8 mg/kg	3.00	1,648.3	20.28

[a] Individuals in the same treatment group 1 week after injection were proportionally pooled on the basis of body weight. Each analysis was run in duplicate.

[b] Total PCB levels excluding PCB 126. Polychlorinated biphenyl 126 was not found in the control group.

els and genders with monooxygenase activities. Only the dose effect was significant. Our dose–response study showed that a single intraperitoneal injection of PCB 126 significantly increased the activities of EROD, MROD, BROD, and PROD in leopard frogs at and above doses of 2.3 mg/kg. In previous studies, the estimated 50% effective dose for induction of EROD by PCB 126 in rainbow trout (*Oncorhynchus mykiss*) was 329 μg/kg [20], and rats had a threshold level of 0.47 μg/kg for PCB 126 [21]. The northern leopard frogs used in our study had a threshold for monooxygenase induction between 0.7 and 2.3 mg/kg PCB 126, which is much higher than those of rats and rainbow trout.

Correlation analyses among the activities of EROD, MROD, BROD, and PROD indicated that the induction could be via the same mechanism, namely the aryl hydrocarbon receptor-mediated mechanism. We also suspect that EROD and MROD activities could result from the same enzyme because they were correlated significantly in the dose–response and time course experiments. The possible explanation might be that ethoxyresorufin ($C_{14}H_{11}NO_3$) and methoxyresorufin ($C_{13}H_9NO_3$) have very similar chemical structures, so both could fit well into the active site of the same enzyme, which could catalyze many different substrates, like cytochrome 1A1 enzymes (CYP1A1), in other species. As for assessing candidate enzyme activity as biomarkers, we preferred EROD activity for three reasons. First, although EROD and MROD activities might be from the same enzyme, EROD activity was much higher than MROD activity and thus easier to detect accurately. Second, in our studies, the statistical power for detecting an increase in enzyme activity was higher for EROD than for MROD. Third, the substrate of EROD, ethoxyresorufin, is reported to be much less toxic than benzo[*a*]pyrene, which is considered carcinogenic to humans.

Enzyme activities were maximally induced by 2 weeks after exposure to PCB 126 and remained elevated for at least 4 weeks in this study. Also, in northern leopard frogs acclimated to 20°C, benzo[*a*]pyrene hydroxylase activity peaked rapidly, 4 d after injection with a single 40-mg/kg dose of 3-MC [22]. In contrast, in lake trout acclimated to 12.5°C [23], maximal induction of EROD in groups receiving a single injection of 6.3 or 25 mg/kg of PCB 126 was attained at 6 weeks, not 3 weeks, and was maintained for at least 30 weeks. The earlier induction in leopard frogs might be related to the higher acclimation temperature.

Although the induction of monooxygenase activities might serve as a biomarker for environmental PCB exposure of frogs, a sensitive biomarker should respond to environmental levels of the contaminant of interest. The geometric means of total PCB residues in a field-collected salamander species [24] and a frog species, *R. perezi,* in Spain [25] ranged from 0.05 to 1.2 mg/kg wet body weight. The total PCB levels of a *Rana* spp. in three polluted Louisiana (USA) watersheds were not detectable [26]. Northern leopard frogs collected by our laboratory from seven sites along Green Bay and the Lower Fox River, Wisconsin, had total PCB residues that ranged from 2.8 to 151.9 μg/kg wet body weight (Y. Huang et al., in preparation). Because of a lack of induction in our experiment at low doses of PCB 126, which were relatively high compared to the environmental concentrations from the above reports, we conclude that EROD, MROD, BROD, and PROD activities are likely not sensitive biomarkers for coplanar PCBs exposure in leopard frogs. Additionally, the activities of several presumptive cytochrome 1A and 2B family enzymes of hepatic microsomes from northern leopard frogs were not elevated by phenobarbital [22] as well as a single dose of 5 ppm DDT to *R. temporaria* [27]. Apparently, monooxygenases in *Rana* sp. are not responsive to phenobarbital-type inducers and insensitive to 3-MC-type inducers.

Toxicity of polyhalogenated hydrocarbons in Rana *spp.*

Body weight of the experimental frogs was not affected by PCB 126 dosage but varied significantly with time. Instead of suffering from weight loss (wasting syndrome), which has been shown in fish species [28] and mammal species [29] treated with 2,3,7,8-TCDD, frogs in both our control and treated groups, except for the control group at week 2, ate and gained weight. Liver weight was not affected by dose but varied significantly with body weight. The observations of gross kidney appearance, behavior, skin and other organ conditions, and mortality indicated that frogs treated with up to 7.8 mg/kg PCB 126 maintain growth but may, to some extent, suffer from nephronic toxicity. Future research should try to describe this toxicity.

The few other studies on amphibian responses to polyhalogenated hydrocarbons also indicate relatively low toxicity compared with other animals. Adult bullfrogs (*R. catesbeiana*) collected along the 2,3,7,8-TCDD-contaminated Rock Branch Creek in Arkansas, USA, had 2,3,7,8-TCDD levels ranging from 87 ng/kg in a muscle sample to 68,000 ng/kg in a fat sample but were alive and appeared to be healthy [30]. Beatty et al. [31] observed no significant mortality up to 50 d after intraperitoneal injection of 1,000 μg/kg of 2,3,7,8-TCDD into bullfrog tadpoles or up to 35 d after intraperitoneal injection of up to 500 μg/kg into adult bullfrogs, and histopathological examination of various tissues, such as liver, lung, and kidney, of the metamorphosed tadpoles and adult frogs failed to show any abnormalities. Studies on other animal species showed that median lethal dose values (for a single oral dose) for 2,3,7,8-TCDD ranged from 2 μg/kg for the guinea pig to 50 μg/kg for the monkey to 1,157 μg/kg for the hamster [32]. Based on our and others' results, we conclude that, compared with fish and mammals, bullfrogs and leopard frogs can tolerate a substantial body burden of PCBs and TCDD by acute exposures without experiencing physiological, physiochemical, or histopathological alterations.

Suitability of experimental frogs

We used wild-caught frogs in our experiment, and even control frogs had some PCBs in their tissue. However, it is not possible at this time to study induction in laboratory-reared, contaminant-free *Rana* sp. because they are not easily bred in captivity. Furthermore, the levels in our sampled population were low, and coplanar PCBs were below detection level. It may not be possible to find contaminant-free wild frogs anywhere. In any event, the elevated enzyme activities were statistically correlated to the administered PCB 126, not the low levels of other PCBs.

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