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EFFECTS OF IN OVO EXPOSURE OF WHITE LEGHORN CHICKEN, COMMON PHEASANT, AND JAPANESE QUAIL TO 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN AND TWO CHLORINATED DIBENZOFURANS ON CYP1A INDUCTION

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Abstract—In birds, activation of the aryl hydrocarbon receptor (AhR) by some polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) results in induction of cytochrome P4501A (*CYP1A*) expression. This response has been useful for predicting relative sensitivity of birds to dioxin-like compounds. To further investigate species-sensitivity to dioxins and dioxin-like compounds induction of cytochrome P450 1A4 and 1A5 (*CYP1A4* and *CYP1A5*) mRNA and ethoxyresorufin *O*-deethylase (EROD) activity were quantified in liver of posthatch white leghorn chicken, common pheasant, and Japanese quail exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF) via air cell injection. The rank-order of sensitivity of TCDD- and TCDF-exposed birds, based on *CYP1A*, was chicken > pheasant > quail. Based on *CYP1A5* mRNA expression and EROD induction, the order of sensitivity of PeCDF-exposed birds was identical to that for TCDD and TCDF. However, based on *CYP1A4* mRNA expression the rank-order was pheasant > chicken > quail. When comparing the potency of the three compounds in each species, based on *CYP1A4* mRNA expression, TCDD was the most potent compound in chicken. However, PeCDF was equally potent to TCDD in quail and was more potent than TCDD in pheasant. These results suggest that quantitative real-time polymerase chain reaction (Q-PCR) analysis of *CYP1A* expression, particularly *CYP1A4* mRNA expression, may be a more sensitive biomarker of exposure than analysis of EROD induction, especially in less responsive avian species. Based on these findings future risk assessments should consider the sensitivity of the species inhabiting a site and the congeners of concern that are present. Environ. Toxicol. Chem. 2010;29:1490–1502. © 2010 SETAC

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INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and structurally related polychlorinated dibenzofurans (PCDFs) as well as some polychlorinated biphenyls (PCBs) and other structurally similar compounds, are a group of environmental contaminants referred to as dioxin-like compounds. Exposure to these chemicals has been shown to cause a range of effects, including endocrine disruption, immunological effects, developmental abnormalities, reduced egg production and hatchability, and lethality in avian species [1–8].

Toxic and adaptive responses to TCDD and structurally related dioxin-like compounds are largely mediated by the aryl hydrocarbon receptor (AhR) [9–11]. AhR is a ligand-activated transcription factor that regulates expression of a suite of biotransformation enzymes, one group of which is the mixed function monooxygenase (MFO) enzymes, including the cytochrome P4501A (*CYP1A*) genes [12,13]. Two *CYP1A* genes, *CYP1A4* and *CYP1A5*, inducible by TCDD, are constitutively expressed in avian liver [14–17]. The CYP1A4 enzyme exhibits ethoxyresorufin *O*-deethylase (EROD) activity while the CYP1A5 isoform preferentially catalyzes arachadonic acid metabolism and uroporphyrinogen oxidation (UROX) [18,19].

The AhR signaling pathway, including induction of CYP1A activity, is conserved in vertebrates [11,20]. Despite this conservation, there are differences in both sensitivity and efficacy of responses to TCDD and dioxin-like compounds among vertebrates [15,21,22]. Differences in sensitivity and efficacy of responses to dioxin-like compounds at the whole organism, biochemical, and molecular levels have been observed in birds, and both relative sensitivity and efficacy of responses could contribute to differential toxicity of dioxin-like compounds among species of birds. Here we will distinguish between sensitivity and efficacy. Sensitivity of a species is defined as the threshold concentration of a chemical to cause a statistically significant response in a species. A species exhibiting a lesser threshold concentration would be more sensitive. For instance, when exposed via egg injection, the ring-necked pheasant (*Phasianus colchicus torquatus*) is tenfold less sensitive to the embryotoxic effects of TCDD than the domestic chicken (*Gallus gallus domesticus*) [23–25]. The efficacy of a response refers to the magnitude of responsiveness and is measured by the maximum response observed. A species exhibiting a greater

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magnitude of induction of an enzyme or upregulation of gene expression when exposed to a compound of interest would be more responsive and the compound would have greater efficacy in that species. The greater responsiveness of the chicken to TCDD compared to the pheasant is demonstrated by the observation that EROD activity is tenfold greater in chicken hepatocytes compared to pheasant hepatocytes [6]. Thus, the chicken is more sensitive and more responsive. A further example of differential responsiveness among species is the fact that *CYP1A4* and *CYP1A5* mRNA expression are 27.7- and 5.8-fold greater in chicken than in herring gull (*Larus argentatus*) hepatocytes exposed to 100 nM TCDD [15], respectively.

Although the mechanisms responsible for differential sensitivity and responsiveness among avian species are not completely understood, evidence of a molecular basis has been suggested [26]. Specifically, amino acid substitutions in the ligand binding domain (LBD) of the AhR appear to account for differences in affinities of ligands for the AhR. The greater occupancy rates of the dioxin-like compounds on the AhR lead to differences in TCDD-dependent transactivation [26]. Based on these findings and existing toxicity data, a sensitivity classification scheme has been developed for members of the Order Galliformes. Specifically, based on embryotoxicity data the white leghorn chicken (*Gallus gallus domesticus*) is classified as type 1 (very sensitive), the ring-necked pheasant (*Phasianus colchicus*) is classified as type 2 (moderately sensitive), and Japanese quail (*Coturnix japonica*) is classified as type 3 (insensitive) [24].

Differences in sensitivity and responsiveness among species present a significant challenge in avian ecological risk assessments. Currently, many assessments are based on responses of the chicken. An implicit assumption in these assessments is that the chicken is a sensitive surrogate that would be equally or more sensitive/responsive to dioxin-like compounds. Thus, risk assessments based on the chicken would be protective of other species. However, due to its sensitivity to TCDD and dioxin-like compounds this species may not be representative of any wild avian species and therefore its use may lead to inaccurate assessments and unnecessary remediation and subsequent loss of habitat. However, risk assessors need to consider hundreds of species that might occupy a site being assessed. Because comprehensive toxicity profiles are not available for all of these species, the ability to accurately predict avian sensitivities and responsiveness to TCDD and structurally related dioxin-like compounds would be advantageous. Such a predictive classification scheme, based on the amino acid sequence of the AhR LBD [26,24], could greatly enhance avian risk assessment. However, there is a need to validate this classification scheme for a range of endpoints, such as molecular and biochemical responses as well as lethality. The present study was conducted to quantify responses of commonly used functional indicators of exposure relative to the classification of species based on AhR LBD. Specifically, *CYP1A4* and *CYP1A5* mRNA expression and EROD activity were determined in liver of posthatch chicken, pheasant, and quail exposed to TCDD, PeCDF, or TCDF injected into the air cell of fertilized eggs.

MATERIALS AND METHODS

Source of eggs

Eggs were injected at the Avian Research Center of the Department of Animal Science, Michigan State University (East Lansing, MI). Fertilized eggs of quail, pheasant, and chicken were obtained from the Michigan State University Poultry Research and Teaching Center and stored in a cooler at 13.5 to 15.0°C until 24 h prior to injection. Eggs were weighed and grouped so that each treatment group received an equal distribution of eggs weighing from 52.0 to 64.0 g for chicken, 25.0 to 34.0 g for pheasant, and 6.8 to 13.8 g for quail. For each species there were no significant differences in egg weights among different treatment groups (data not shown).

Egg injection and incubation conditions

Each of the compounds TCDD, PeCDF, and TCDF (Wellington Laboratories) were dissolved and diluted in cold-filtered sterile triolein (Sigma). Injection volume per egg was calculated based on 5.8 μl/58 g egg for chicken, 3.0 μl/30 g egg for pheasant, and 2.0 μl/10 g egg for quail. The following species-specific dosing solutions of each compound were prepared: chicken (0.0494, 0.0963, 0.195, 0.416, 0.767, 1.57, 3.07 pmol/g egg for TCDD, 0.0438, 0.0867, 0.142, 0.335, 0.693, 1.38, 2.49 pmol/g egg for PeCDF, and 0.0742, 0.148, 0.245, 0.516, 1.05, 1.83, 4.02 pmol/g egg for TCDF); pheasant (0.0745, 0.0994, 0.224, 0.311, 0.820, 3.17, 6.68 pmol/g egg for TCDD, 0.141, 0.235, 0.388, 0.599, 1.07, 4.08, 6.76 pmol/g egg for PeCDF, and 0.131, 0.170, 0.288, 0.654, 1.12, 4.77, 14.2 pmol/g egg for TCDF), and quail (0.223, 0.497, 0.745, 1.24, 2.86 pmol/g egg for TCDD, 0.411, 0.911, 1.82, 2.61, 5.31, 11.16 pmol/g egg for PeCDF, and 0.418, 0.628, 1.59, 2.90, 4.81, 8.56 pmol/g egg for TCDF). Following dose preparation, injection vials were flooded with argon to preserve the triolein, capped, and sterilized in an autoclave. Concentrations in triolein were confirmed by high-resolution mass spectrometry (described below).

Egg injection was done in a laminar flow hood (NuAire). Eggs were candled to mark the center of the air cell and the injection site was sterilized with 70% ethanol and then a single hole was drilled using a Dremel tool (Robert Bosch Tool). Triolein as a vehicle control or TCDD, PeCDF, or TCDF stock solutions was injected into the air cell using a positive displacement pipettor (Gilson) with sterile pipette tips changed after each injection. The injection site was then sealed with liquid paraffin wax (Royal Oak Sales) and a sterilized wooden applicator.

Eggs were incubated with the sealed injection site up at 37.5 to 37.7°C with 50 to 60% humidity in a Petersime Rotary Incubator (Petersime Incubator) and rotated automatically every 2 h. Eggs were transferred to the hatching trays of a Surepip hatcher (Agro Environmental Systems) 3 d prior to the expected hatching date with only one treatment group per hatching tray. The hatcher was maintained at 37.5 to 37.7°C with 70 to 75% humidity and each hatching tray was divided into individual compartments for each egg.

Hatching eggs were monitored 1 d prior to and 2 d after the expected hatching date. Once sufficiently dry, the hatchlings were moved into a Petersime Brood Unit (Petersime Incubator) maintained at 30.0°C and identified with a Swiftack (Heartland Animal Health) identification tag bearing their unique egg number. Chicks were weighed and examined for abnormalities, and then raised for two weeks after the hatching date. Chicks were introduced to clean feed and water by dipping their beaks in both, which were then provided ad libitum. After the two-week grow-out period, 10 chicks were randomly selected from each treatment group, euthanized by cervical dislocation, and necropsied. The liver was removed, weighed, and divided into four portions; the first portion was placed in an I-Chem jar on ice for contaminant analysis, the second placed in a microtube

containing RNAlater® for mRNA analysis, the third portion was placed in a microtube that was frozen in liquid nitrogen for analysis of enzyme activity, and the fourth was placed in 10% formalin for assessment of histopathology. The liver tissue for mRNA and EROD analysis was then delivered to the Environmental Toxicology Laboratory, University of Saskatchewan (Saskatoon, SK, Canada).

Quantification of TCDD, PeCDF, and TCDF injection solutions

Concentrations of injection solutions were confirmed by isotope dilution following U.S. Environmental Protection Agency (U.S. EPA) method 1613 [27] with ^{13}C surrogate standards (DF-CS-C100, Wellington Laboratories). Identification and quantification of TCDD, PeCDF, and TCDF was performed using a Hewlett-Packard 5890 series high-resolution gas chromatograph interfaced with a Micromass® Autospec® high-resolution mass spectrometer (HRGC-HRMS) (Micromass). The mass spectrometer was operated in a selected ion-monitoring (SIM) mode and the resolution for all reference gas peaks in all time windows was greater than 10,000. Concentrations of TCDD, PeCDF, and TCDF were quantified by the internal standard isotope dilution method using mean relative response factors determined from standard calibration runs. Recoveries of ^{13}C-labeled PCDD/Fs internal standards and all other quality assurance/quality control criteria were within ranges specified by the U.S. EPA methods [27].

Total RNA isolation and cDNA synthesis

Total RNA was extracted from approximately 30 mg of liver tissue with the RNeasy® Mini Kit (Qiagen) using a QIAshredder (Qiagen) according to the manufacturer's protocol with one slight modification: a 50% ethanol solution was used instead of a 70% ethanol solution because it provided greater RNA yields [15]. Purified RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Samples were checked for RNA integrity on a 1% denaturing formaldehyde-agarose gel and visualized by staining the gel with ethidium bromide and visualizing the bands under ultraviolet (UV) light using a VersaDoc 4000 MP imaging system (Bio-Rad). The purified RNA samples were stored at −80°C until analysis.

First-strand cDNA synthesis was performed using an iScript™ cDNA Synthesis Kit (Bio-Rad). A volume of 1 μg total RNA was combined with 4 μl of 5 × iScript Reaction Mix, 1 μl of iScript Reverse Transcriptase, and RNase-free water to a final volume of 20 μl. Reaction mixes were incubated at 25°C for 25 min, 42°C for 20 min, and, on completion, were inactivated at 85°C for 5 min. The cDNA samples were stored at −80°C until further analysis.

Sequencing of Japanese quail CYP1A5 and CYP1A5

A fragment of Japanese quail *CYP1A4* and *CYP1A5* cDNA was amplified using primers (Table 1) designed against conserved regions identified by aligning available homologous sequences from other avian species. The polymerase chain reaction (PCR) reactions were performed using a Bio-Rad MyCycler Thermal Cycler in a volume of 20 μl, consisting of 10 × Taq buffer with $(NH_4)_2SO_4$, 0.25 mM of each dNTP, 1.25 mM of $MgCl_2$, 0.125 μM of each primer, 1 μl of liver cDNA template, and 2.5 U Taq polymerase (Fermentas). The reaction mixture was initially denatured at 95°C for 5 min followed by 40 cycles of amplification with the reaction profile of denaturing at 95°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s. Following amplification a final extension was performed at 72°C for 10 min. A small volume of the amplified PCR fragments was visualized on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light on a VersaDoc 4000 MP imaging system (Bio-Rad). The remaining volume of PCR products was purified using a QIAQuick PCR purification system (Qiagen) according to the manufacturer's protocol. Purified PCR products were cloned into the pGEM-T easy vector using a DNA ligation kit (Invitrogen), and transformation into competent JM109 *E. coli* cells (Promega). Plasmids were isolated with a Qiagen plasmid purification kit and the products were sequenced at the National Research Council of Canada's Plant Biotechnology Institute (University of Saskatchewan).

Table 1. Nucleotide sequences of primers used for cloning and sequencing of Japanese quail cytochrome P450 1A4 (*CYP1A4*) and cytochrome p4501A5 (*CYP1A5*) by polymerase chain reaction (PCR)

Primer		Sequence (5' - 3')
CYP1A4	Forward	5'-ATGTACGCTGCCTTGTACCC-3'
	Reverse	5'-CCGTACTGAGGGGTGATGTC-3'
CYP1A5	Forward	5'-ACCTGGTCACCAAATTCCTG-3'
	Reverse	5'-CTCCAGGATGAAGGCTTCTG-3'
RACE primers		
CYP1A4	5' RACE	5'-CGTCCCGAATGTGCTCCTTAT-CAAAAG-3'
	3' RACE	5'-AATGTTCGCGTCCAACCTTCT-GATA-3'
CYP1A5	5' RACE	5'-CGTCTCGGATGCTGTTCTTGTCA-TAGG-3'
	3' RACE	5'-AGTGGTGCCCTTCAGATCC-CAAATG-3'

3′ and 5′ rapid amplification of cDNA ends

Gene-specific primers (Table 1) were designed based on the partial cDNA sequences determined for quail CYP1A4 and CYP1A5. The 3′ and 5′ rapid amplification of cDNA ends (RACE) reactions were performed using the SMART™ RACE cDNA Amplification Kit (ClonTech) according to the manufacturer's protocol. A small volume of the amplified PCR fragments was visualized on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light on a VersaDoc 4000 MP imaging system (Bio-Rad). The remaining volume of PCR products was purified using a QIAQuick PCR purification system (Qiagen) according to the manufacturer's protocol. Purified PCR products were cloned into the pGEM-T Easy Vector using a DNA ligation kit (Invitrogen), followed by transformation into competent JM109 *E. coli* cells. Plasmids were isolated with a Qiagen plasmid kit and the products were then sequenced at the National Research Council of Canada's Plant Biotechnology Institute (University of Saskatchewan). Full-length cDNA sequences were assembled by aligning sequenced PCR products.

Real-time PCR

Gene-specific primers against *CYP1A4* and *CYP1A5* from the chicken were from Head and Kennedy [16] and the pheasant primers were from Hervé et al. [28]. Gene-specific primers against quail *CYP1A4* and *CYP1A5* were designed based on the full-length sequences determined in the present study. To ensure amplification of desired transcripts the PCR products for each primer pair were sequenced as outlined above.

Quantitative real-time PCR was performed in 96-well PCR plates using an ABI 7300 Real-Time PCR System (Applied Biosystems). A separate 80 μl PCR reaction mixture consisting

Table 2. Nucleotide sequences of primers used for real-time polymerase chain reaction (Q-PCR) quantification of cytochrome P4501A4 (*CYP1A4*) and cytochrome p4501A5 (*CYP1A5*) mRNA expression in Japanese quail, common pheasant, and white leghorn chicken

Transcript		Sequence (5′ - 3′)	Final Concn. (nM)
β-Actin	Forward	AAATTGTGCGTGACATCAAGGA	(325)
	Reverse	GAGGCAGCTGTGGCCATCT	(325)
Japanese quail			
CYP1A4	Forward	ATGTACGCTGCCTTGTACCC	(325)
CYP1A5	Reverse	CTCCAGGATGAAGGCTTCTG	(325)
	Forward	TACAGGCAGCTGTGGATGAG	(325)
	Reverse	GATCTGAAGGGCACCACTG	(325)
Common pheasant			
CYP1A4	Forward	GAGCACATTCGGGATGTCA	(250)
CYP1A5	Reverse	CAGAGAGTTGGACACGGACA	(250)
	Forward	CATCCGAGATGTCACCGACT	(750)
	Reverse	TTGGGATCTGTGTGGCACTA	(750)
White leghorn chicken			
CYP1A4	Forward	TAAGGACGTCAATGCTCGTTTC	(300)
CYP1A5	Reverse	CGTCCCGAATGTGCTCCTTAT	(300)
	Forward	ACAGCTGTGGAAGAGCACTACCA	(300)
	Reverse	TCTCCACGCACTGCTCGAT	(300)

of gene-specific primers (Table 2), 40 μl of 2× SYBR Green master mix (Applied Biosystems), 3 μl cDNA, and nuclease-free water to the final volume was prepared for each cDNA sample of interest and for each primer pair. The gene-specific Q-PCR primers for each species as well as the volumes and concentration of each component are shown (Table 2). A final reaction volume of 25 μl was transferred to each well and reactions were performed in triplicate. The PCR reaction mixture was denatured at 95°C for 10 min before the first PCR cycle. The thermal cycle profile was denatured for 10 s at 95°C and extension for 1 min at 60°C. A total of 40 PCR cycles was performed.

To quantify Q-PCR results, the cycle at which the fluorescence signal was first significantly different from background (Ct) was determined for each reaction. The expression levels of *CYP1A4* and *CYP1A5* were normalized with reference to β-actin to derive the mean normalized expression (MNE) value as described by Simon [29]:

$$\mathrm{MNE} = [(\mathrm{E}_{\mathrm{reference}})]^{\wedge}(\mathrm{Ct}^{\wedge}(\mathrm{reference, mean}))/ [(E_{\mathrm{target}})]^{\wedge}(\mathrm{Ct}^{\wedge}(\mathrm{target, mean})) \quad (1)$$

where $\mathrm{E}_{\mathrm{reference}}$ and $\mathrm{E}_{\mathrm{target}}$ represent the PCR efficiencies ($=10^{-1}$/slope) of the target gene (*CYP1A4* and *CYP1A5*) and β-actin, respectively, as determined from the slope of a standard curve constructed using serial dilutions of a cDNA sample prepared by pooling random cDNA samples [29]. Levels of expression relative to control were calculated:

$$\mathrm{N-fold\,change} = \mathrm{MNE(experimental)/MNE(control)} \quad (2)$$

Ethoxyresorufin O-*deethylase*

Microsome preparation and EROD assays were performed according to the methods of Kennedy and Jones [30]. Wherever possible, all procedures were performed on ice using chilled equipment and reagents. Briefly, approximately 100 to 200 mg of liver tissue (frozen in liquid nitrogen) was rinsed in ice-cold phosphate buffer (0.08 M sodium phosphate, 0.02 M potassium phosphate, pH 7.4). Tissue was minced into small pieces with cold scissors and quantitatively transferred into a 2-ml microcentrifuge tube. Tissue was homogenized with 10 strokes using a Fisher Scientific Powergen 125 (FTH-115) blade-type homogenizer. The mixture was kept on ice during the whole procedure. The homogenate was centrifuged at 9,000*g* in a Sorvall® Legend RT+ Centrifuge (Thermo Fisher Scientific) for 15 min at 4°C, and the supernatant from each sample, representing the S9 fraction, was transferred into separate ultracentrifuge tubes (Seton, Los Gatos), and centrifuged at 100,000*g* in a Sorvall Ultraspeed Centrifuge (Thermo Fisher Scientific) for 60 min at 4°C. The supernatant was discarded, the pellet was resuspended in 0.6 ml of ice-cold phosphate buffer, and aliquots were stored at −80°C until further use.

The EROD activities and protein concentrations in each microsome preparation were assayed in 96-well plates. Dilutions of resorufin (Sigma) and bovine serum albumin (BSA; Sigma) were used to establish resorufin and protein standard curves according to Kennedy et al. [31]. Each microsome sample was analyzed in triplicate together with a blank control. All wells contained 15 μl of microsomes, 50 μl of 7-ethoxyresorufin (7-ER, Sigma) working solution (final concentration 2 μM), and sodium phosphate buffer to a final volume of 235 μl for blank controls or 185 μl for wells containing microsomes. Following a 5-min incubation at 37°C, the enzymatic reaction was initiated by adding 50 μl of nicotinamide adenine dinucleotide phosphate (NADPH) to make a final concentration of 0.5 mM. Reactions were allowed to proceed for exactly 10 min at 37°C, after which time the reactions were terminated by adding 100 μl cold acetonitrile containing fluorescamine (2.16 mM, Sigma). Plates were immediately scanned using a fluorescence plate reader (POLARstar OPTIMA, BMG LABTECH) according to Kennedy et al. [31] in order to quantify both resorufin formation and protein concentrations. Resorufin was quantified at 530 nm excitation and 590 nm emission wavelengths. Protein concentration was determined with a 400 nm excitation filter and a 460 nm emission filter.

Relative sensitivity and relative potency determination

Relative sensitivity (ReS) and relative potency (ReP) values were calculated in order to determine relative sensitivity of each species exposed to each compound and to compare the relative potency of the three compounds within each species. The first step in the calculations was to determine the lowest-observed-effect concentration (LOEC) for each response. The threshold for effect (LOEC) was determined differently depending on whether the data met the assumptions of parametric statistics.

The LOEC values for parametric data (EROD) were obtained by calculating the geometric mean of the least dose that stimulated a significant increase in EROD activity and the preceding dose. For nonparametric data (mRNA expression) the LOEC was estimated as the point of intersection on the x axis of the lower 95% confidence interval of the linear regression line. The ReS and ReP were calculated:

$$\text{ReS} = (\text{LOEC chicken exposed to A})/(\text{LOEC species of interest exposed to A}) \quad (3)$$

$$\text{ReP} = (\text{LOEC TCDD species A})/(\text{LOEC compound of interest species A}) \quad (4)$$

Relative responsiveness was not calculated; however, a discussion of responsiveness is included here.

Statistical analyses

Values for all measurements were summarized as mean $\pm$ standard error of the mean (SEM). Statistical analyses were conducted using SPSS 16. The normality of each dataset was assessed using the Kolomogrov–Smirnov one-sample test and homogeneity of variance was determined using Levene's test, and both untransformed and log transformed data were evaluated. The *CYP1A4* and *CYP1A5* mRNA expression data were nonparametric so the Kruskal–Wallis (KW) test, with post-hoc Mann–Whitney U test, was used for comparisons between treatment groups and the vehicle control. We analyzed EROD data by one-way analysis of variance with a Dunnett's post-hoc test to make comparisons between treatment groups and the vehicle control. Differences were considered statistically significant at $p < 0.1$ in order to minimize type II error.

RESULTS

Japanese quail CYP1A4 and CYP1A5

A partial cDNA sequence for an MFO enzyme designated as Japanese quail *CYP1A1* is available in GenBank (Access. no. AB359052.1). However, to ensure accurate quantification of *CYP1A4* and *CYP1A5* mRNA expression by real-time PCR, as part of the present study, these transcripts were cloned from quail hepatic tissue and sequenced. The nucleotide sequences for quail *CYP1A4* and *CYP1A5* have been submitted to GenBank under Accession numbers GQ906939 and GQ906938, respectively. The cloned full-length quail *CYP1A4* cDNA consists of a 1593-bp open reading frame (ORF) encoding 530 amino acids. The quail *CYP1A5* cDNA consists of a 1587-bp ORF encoding 528 amino acids.

The deduced amino acid sequence for quail *CYP1A4* had a 90% overall amino acid identity with the chicken (*G. gallus*) (Access. no. NP990478). The quail CYP1A5 protein sequence had 91% and 77% overall amino acid identities with the turkey (*Meleagris gallopavo*) (Access. no. AY964644) and the great cormorant (*Phalacrocorax carbo*) (Access. no. AB239445), respectively. The quail *CYP1A* sequences also display great sequence homology with *CYP1A*s of other avian species, including the pheasant and jungle crow (*Corvus macrorhynchos*).

Chemical-induced effects on hepatic CYP1A4 mRNA expression

Chicken hepatic *CYP1A4* mRNA expression was significantly greater than constitutive levels at all doses of each chemical. Maximum upregulation of approximately 53.6-fold was observed at 3.07 pmol TCDD/g egg (Fig. 1A), while a 30.2-fold upregulation was observed at 2.49 pmol PeCDF/g egg (Fig. 1B) and a 13.4-fold upregulation was observed at 1.83 pmol TCDF/g egg (Fig. 1C).

Exposure to each of the three chemicals had much less of an effect on pheasant chicks than on white leghorn chicks. The *CYP1A4* mRNA expression was significantly upregulated in chicks exposed to 0.311 and 6.68 pmol TCDD/g egg. The maximum response was approximately 6.4-fold at 6.68 pmol/g egg (Fig. 2A). The *CYP1A4* mRNA expression was significantly upregulated at 0.141, 0.388, 0.599, 1.07, 4.08, and 6.76 pmol PeCDF/g egg, to a maximum of approximately tenfold at 6.76 pmol PeCDF/g egg (Fig. 2B). The *CYP1A4* mRNA expression was significantly upregulated at 0.654, 4.77, and 14.2 pmol TCDF/g egg, to a maximum of approximately 3.5-fold at 4.77 pmol TCDF/g egg (Fig. 2C).

Changes in quail hepatic *CYP1A4* mRNA expression were observed. Both upregulation and downregulation were observed. The *CYP1A4* mRNA expression was not significantly upregulated in response to TCDD. Significant downregulation was observed at 1.24 and 2.86 pmol TCDD/g egg (Fig. 3A). Exposure to 5.31 or 11.16 pmol/g egg PeCDF significantly upregulated *CYP1A4* mRNA expression to a maximum of approximately 7.3-fold at 11.16 pmol TCDD/g egg (Fig. 3B). No significant upregulation in *CYP1A4* mRNA expression was observed in either of the TCDF exposure groups. However, a significant downregulation of mRNA expression was observed at 1.59 and 2.90 pmol TCDF/g egg (Fig. 3C).

Chemical-induced effects on hepatic CYP1A5 mRNA expression

Chicken hepatic *CYP1A5* mRNA expression was significantly upregulated by 0.0963, 0.416, 1.57 and 3.07 pmol TCDD/g egg, to a maximum of approximately 9.2-fold at 0.416 pmol TCDD/g egg (Fig. 1D). The *CYP1A5* mRNA expression was significantly upregulated at 0.142, 0.693, and 1.38 pmol PeCDF/g egg, to a maximum of approximately 4.7-fold at 0.142 pmol PeCDF/g wet weight egg (Fig. 1E). The *CYP1A5* mRNA expression was significantly upregulated at 0.148, 0.245, 0.516, and 1.83 pmol TCDF/g wet weight egg, to a maximum of approximately 8.2-fold at 0.245 pmol TCDF/g wet weight egg (Fig. 1F).

Expression of *CYP1A5* mRNA was less in pheasant and quail exposed to TCDD, PeCDF, and TCDF. No significant changes in pheasant hepatic *CYP1A5* mRNA expression were observed for either TCDD (Fig. 2D) or PeCDF (Fig. 2E). The CYP1A5 mRNA expression was significantly downregulated at 0.170, 0.288, and 4.77 pmol TCDF/g egg (Fig. 2F). No significant changes in quail hepatic *CYP1A5* mRNA were caused by TCDD (Fig. 3D), PeCDF (Fig. 3E), or TCDF (Fig. 3F).

Chemical-induced effects on hepatic EROD activity

Hepatic EROD activity in the chicken was significantly greater for all doses of TCDD (Fig. 4A), PeCDF (Fig. 4B), and TCDF (Fig. 4C). The EROD activity was induced to a maximum of approximately 12-fold by 0.416 pmol TCDD/g egg, 9.3-fold by 1.38 pmol PeCDF/g egg, and 12.7-fold by 0.516 pmol TCDF/g egg.

Hepatic EROD activity was not significantly greater in pheasant exposed to TCDD (Fig. 5A). The EROD activity was significantly greater in the 6.76 pmol PeCDF /g egg exposure group, with a maximum response of approximately 1.5-fold (Fig. 5B). The EROD activity was significantly greater in the 0.654 pmol TCDF/g egg group, with the increase being approximately 1.4-fold (Fig. 5C).

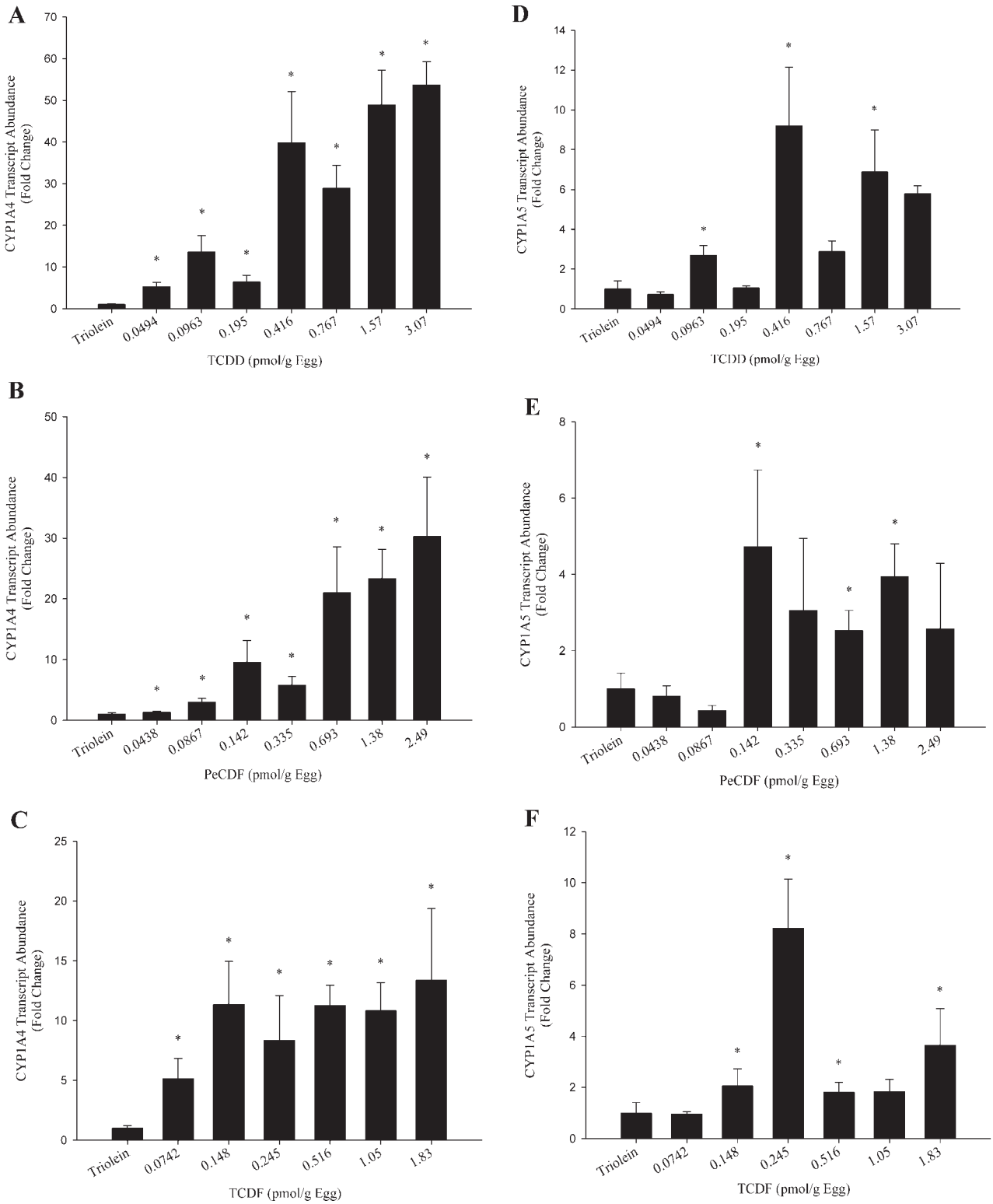

Fig. 1. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (**A,D**), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (**B,E**), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (**C,F**) on cytochrome P4501A4 (*CYP1A4*) (A–C) and cytochrome P4501A5 (*CYP1A5*) (D–F) mRNA expression in the liver of the white leghorn chicken. Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance followed by Mann–Whitney *U* post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of *CYP1A4* mRNA expression in treatment groups relative to control group and error bars represent standard error ($n = 4$–7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

Hepatic EROD activity of quail was not significantly greater in any of the exposure groups. No significant changes in quail hepatic EROD activity were caused by TCDD (Fig. 6A), PeCDF (Fig. 6B), or TCDF (Fig. 6C).

LOEC, relative sensitivity, and relative potency

The LOEC values based on *CYP1A4* and *CYP1A5* mRNA abundance and EROD activity are shown (Table 3). Since either

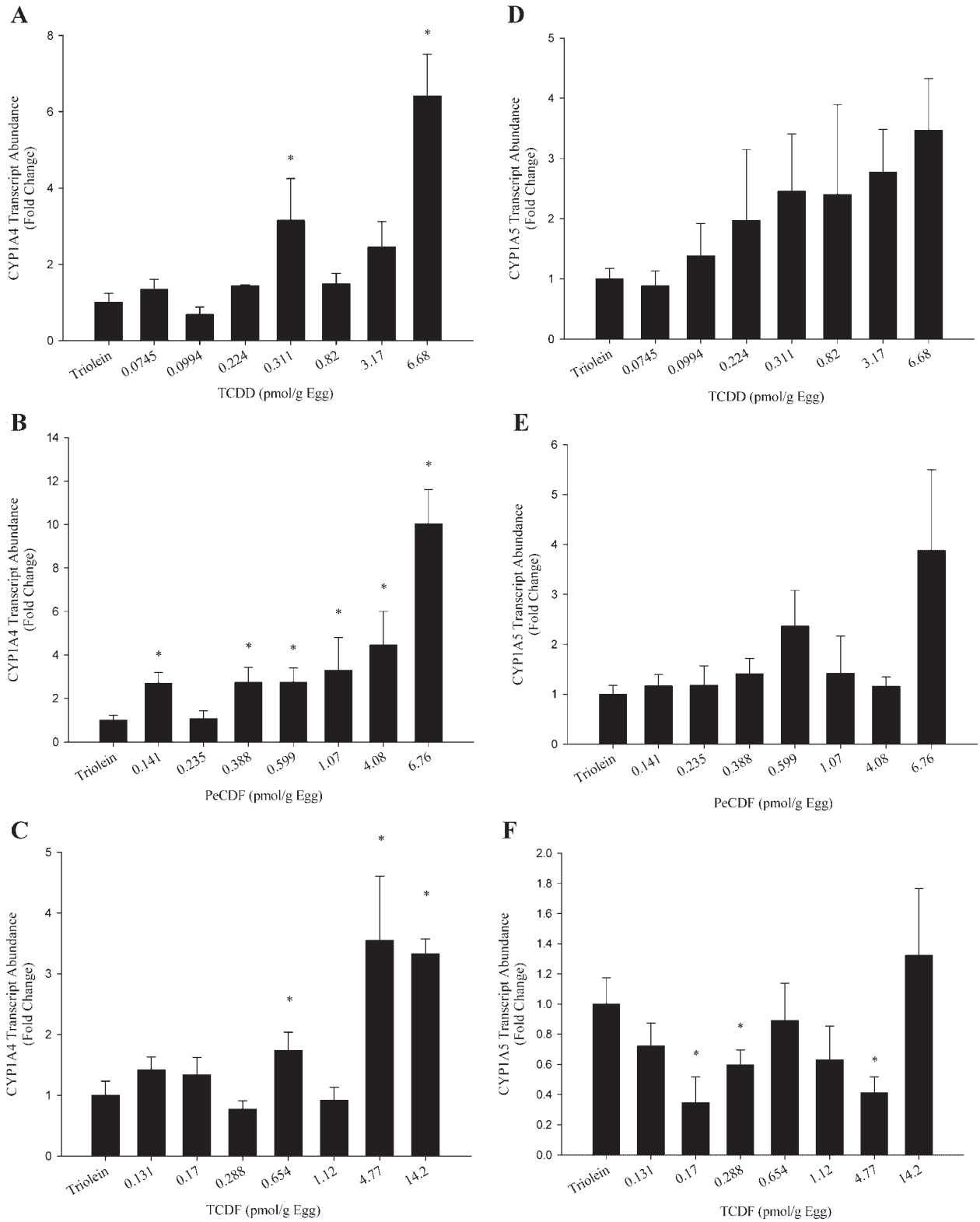

Fig. 2. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (**A,D**), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (**B,E**), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (**C,F**) on cytochrome P4501A4 (*CYP1A4*) (A–C) and cytochrome P4501A5 (*CYP1A5*) (D–F) mRNA expression in the liver of the common pheasant. Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance followed by Mann–Whitney *U* post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of *CYP1A4* mRNA expression in treatment groups relative to control group and error bars represent standard error ($n = 4$–7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

significantly greater magnitudes of responses of some endpoints were stimulated at the least dose injected, or no significant increase was observed, LOEC values could not be determined for all exposures. Therefore, the LOEC values for these situations are considered to be less than the least doses or greater than the greatest doses, respectively. Due to the inability to calculate LOEC values for all responses, relative sensitivity values were not attainable. However, it is possible to determine the rank order

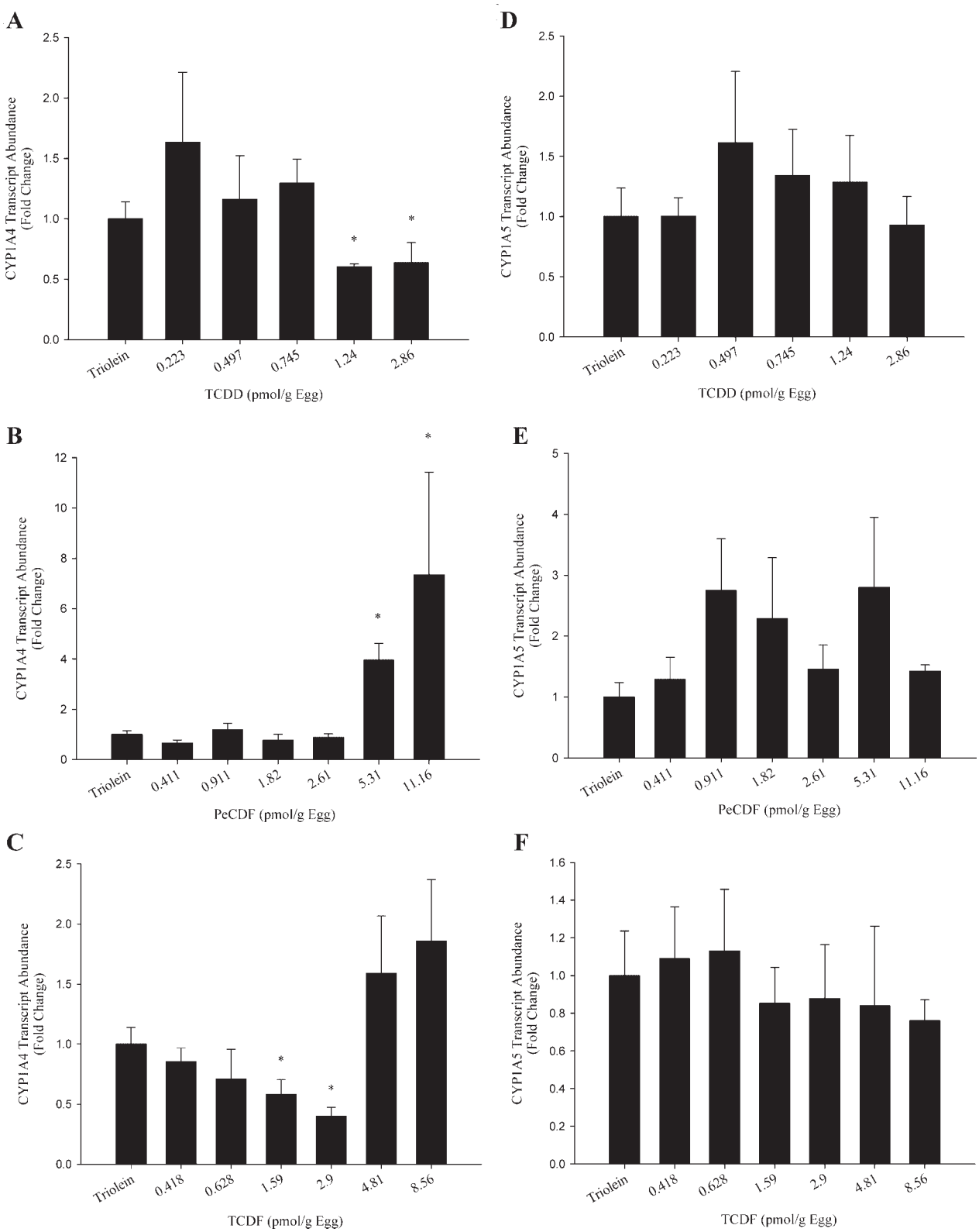

Fig. 3. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (**A,D**), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (**B,E**), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (**C,F**) on cytochrome P4501A4 (*CYP1A4*) (A–C) and cytochrome P4501A5 (*CYP1A5*) (D–F) mRNA expression in liver of Japanese quail. Statistical analyses were performed using Kruskal–Wallis one-way analysis of variance followed by Mann–Whitney *U* post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of *CYP1A4* mRNA expression in treatment groups relative to control group and error bars represent standard error ($n = 4$–7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

of relative sensitivity of each species (Table 4). The relative potencies values of each chemical (Table 5) were estimated based on the LOEC values. However, where LOEC values could not be established it was not possible to determine these values.

DISCUSSION

Differential sensitivities and responsiveness based on upregulation of *CYP1A4* and *CYP1A5* mRNA expression and

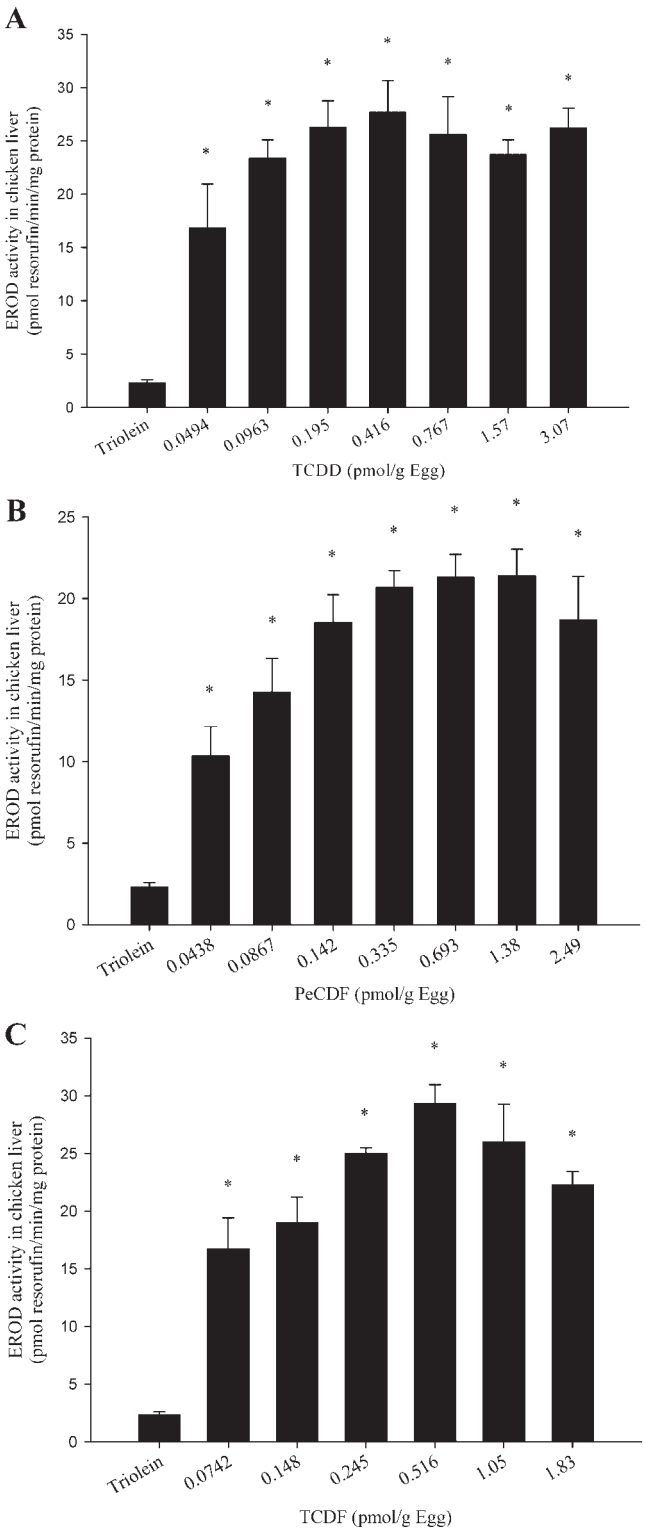

Fig. 4. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (**A**), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (**B**), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (**C**) on ethoxyresorufin *O*-deethylase (EROD) activity in the liver of the white leghorn chicken. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's post-hoc test. Bars represent the percentage of EROD activity in treatment groups relative to the control group and error bars represent standard error ($n = 4$–5). Significant changes in EROD activity are indicated by an asterisk ($p \leq 0.1$).

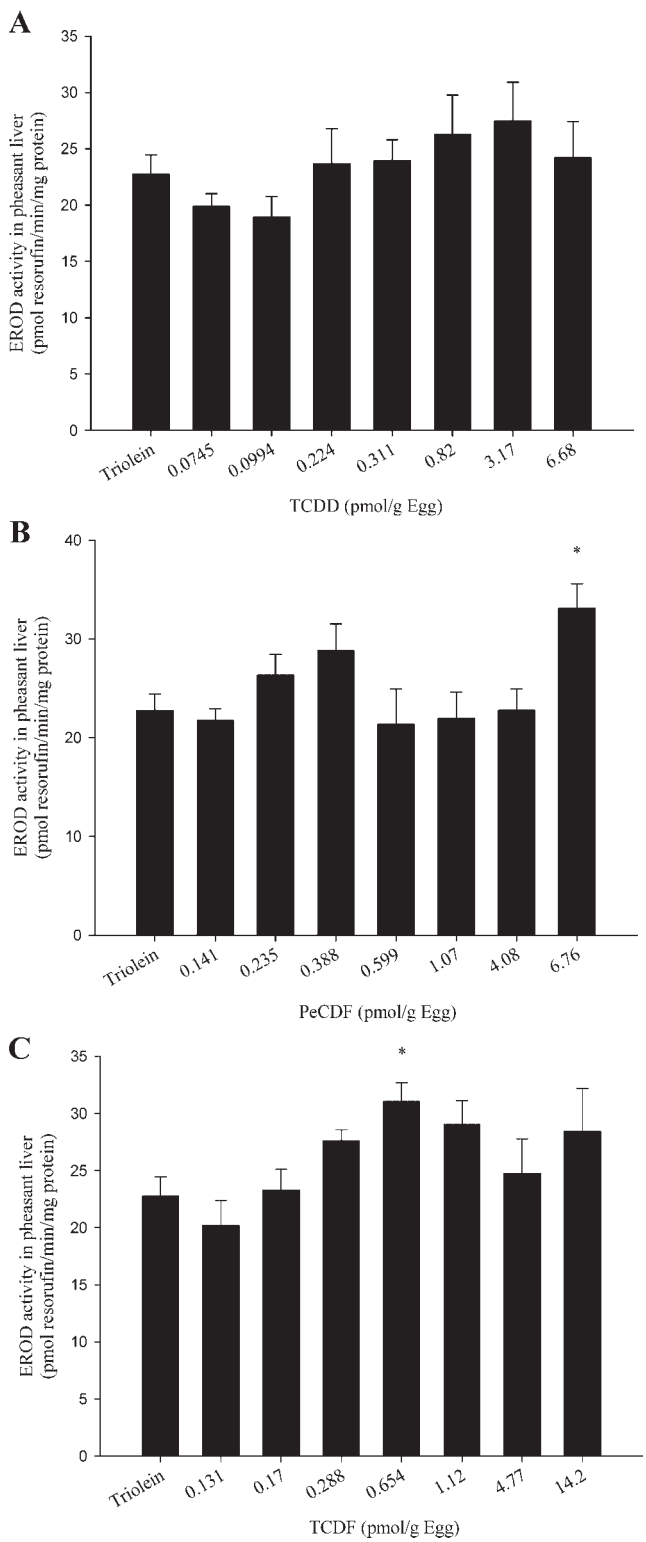

Fig. 5. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (**A**), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (**B**), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (**C**) on ethoxyresorufin *O*-deethylase (EROD) activity in the liver of the common pheasant. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's post-hoc test. Bars represent the percentage of EROD activity in treatment groups relative to the control group and error bars represent standard error ($n = 4$–5). Significant changes in EROD activity are indicated by an asterisk ($p \leq 0.1$).

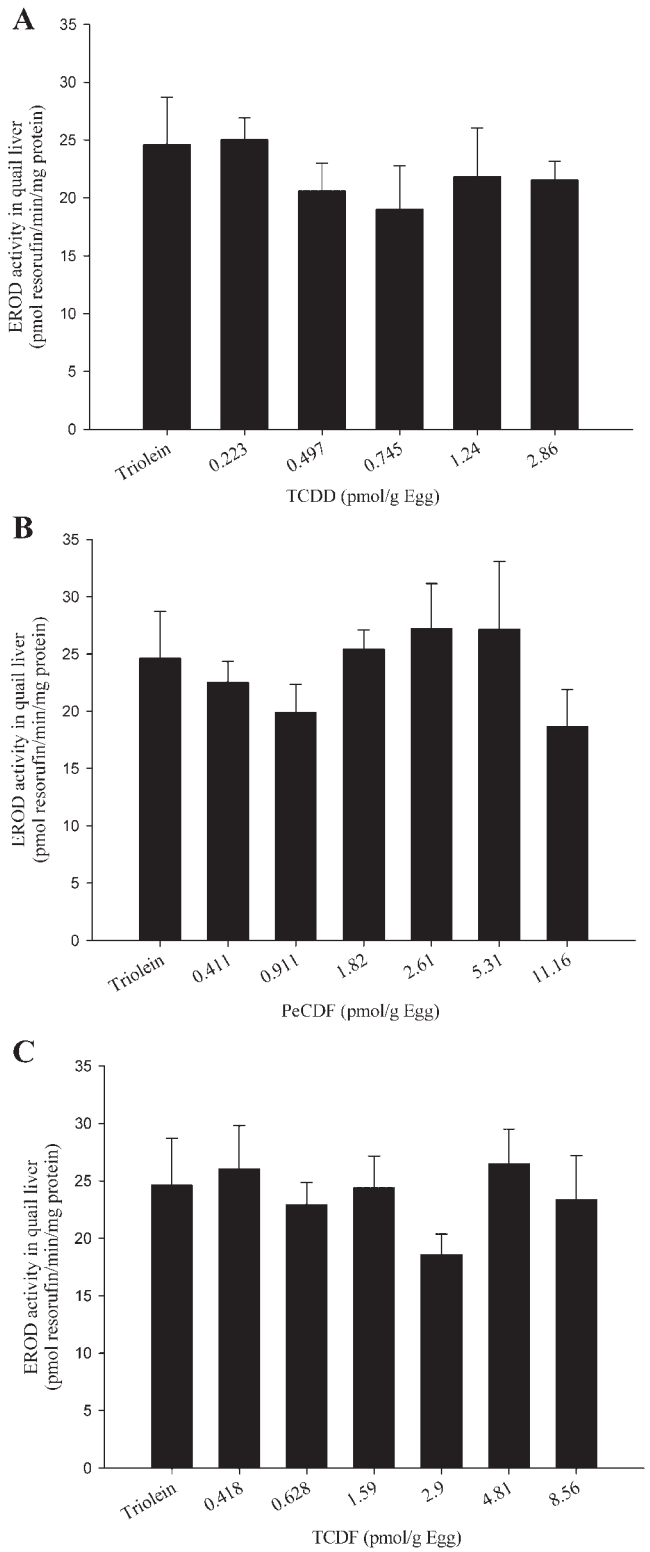

Fig. 6. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (**A**), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) (**B**), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) (**C**) on ethoxyresorufin *O*-deethylase (EROD) activity in the liver of the Japanese quail. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's post-hoc test. Bars represent the percentage of EROD activity in treatment groups relative to the control group and error bars represent standard error ($n = 4$–5). Significant changes in EROD activity are indicated by an asterisk ($p \leq 0.1$).

Table 3. Estimated lowest-observed-effect concentration (LOEC) values (pmol/g egg) for significant induction of cytochrome P4501A4 (*CYP1A4*) and cytochrome P4501A5 (*CYP1A5*) mRNA expression and ethoxyresorufin *O*-deethylase (EROD) activity in white leghorn chicken, common pheasant, and Japanese quail exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF)

Biomarker	Chemical	White leghorn chicken	Common pheasant	Japanese quail
CYP1A4	TCDD	≤0.0494[a]	0.466	>2.86[b]
	PeCDF	≤0.0438[a]	0.0294	3.08
	TCDF	≤0.0742[a]	0.654	4.90
CYP1A5	TCDD	0.0621	>6.68[b]	>2.86[b]
	PeCDF	0.117	>6.76[b]	>11.16[b]
	TCDF	0.0980	>14.2[b]	>8.56[b]
EROD	TCDD	≤0.0494[a]	>6.68[b]	>2.86[b]
	PeCDF	≤0.0438[a]	5.26	>11.16[b]
	TCDF	≤0.0742[a]	0.425	>8.56[b]

[a] LOEC values were not determined because the lowest dose injected caused a significant increase in the biomarker response. The LOEC was assumed to be less than the lowest injected dose.
[b] LOEC values were not determined because no significant increase in the biomarker response was observed at any of the doses injected. The LOEC was assumed to be greater than the highest injected dose.

induction of EROD activity were observed in posthatch chicken, pheasant, and quail chicks exposed to TCDD, PeCDF, or TCDF via injection into the air cell. The sensitivities of each of these members of the order Galliformes to the effects of dioxin-like compounds were classified based on the amino acid sequences of their AhR LBD. Specifically, the chicken is a member of the most sensitive group (type 1), the pheasant is classified as moderately sensitive (type 2), and the quail is classified as insensitive (type 3) [24]. Numerous studies have demonstrated *CYP1A* responsiveness to TCDD, PeCDF, and TCDF in a variety of avian species [6,14,15,17,28,32]. Therefore, the relative sensitivities of the three species exposed to each compound and the relative potencies for the three dioxin-like compounds within each species were determined based on *CYP1A* responses.

Interspecies comparisons: relative sensitivities

The rank-order of sensitivity to TCDD- and TCDF-stimulated induction of *CYP1A4* and *CYP1A5* based on mRNA

Table 4. Rank-order of relative sensitivity (ReS) of white leghorn chicken (chicken), common pheasant (pheasant), and Japanese quail (quail) to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF) based on cytochrome P4501A4 (CYP1A4) and cytochrome P4501A5 (CYP1A5) mRNA expression and ethoxyresorufin *O*-deethylase (EROD) activity

Response	Compound	Rank order (ReS)
CYP1A4	TCDD	chicken > pheasant > quail
	PeCDF	pheasant ≥ chicken > quail[a]
	TCDF	chicken > pheasant > quail
CYP1A5	TCDD	chicken > pheasant ≥ quail[a]
	PeCDF	chicken > pheasant ≥ quail[a]
	TCDF	chicken > pheasant ≥ quail[a]
EROD	TCDD	chicken > pheasant ≥ quail[a]
	PeCDF	chicken > pheasant > quail
	TCDF	chicken > pheasant > quail

[a] Where lowest-observed-effect concentration (LOEC) values were not calculated because the lack of a significant increase in the response (see Table 3 for rationale) the ReS of one species was reported to be greater than or equal to that of another species.

Table 5. Rank-order of relative potencies (ReP) and ReP values (in parentheses) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or 2,3,7,8-tetrachlorodibenzofuran (TCDF) in white leghorn chicken, common pheasant and Japanese quail based on cytochrome P4501A4 (*CYP1A4*) and cytochrome P4501A5 (*CYP1A5*) mRNA expression and ethoxyresorufin *O*-deethylase (EROD) activity

Response	Species	Rank order (ReP)
CYP1A4	White leghorn chicken	Undetermined
	Common pheasant	PeCDF (15) > TCDD (1) > TCDF (0.7)
	Japanese quail	PeCDF (NV) > TCDF (NV) ≥ TCDD (NV)
CYP1A5	White leghorn chicken	TCDD (1) > TCDF (0.6) > PeCDF (0.5)
	Common pheasant	Undetermined
	Japanese quail	Undetermined
EROD	White leghorn chicken	Undetermined
	Common pheasant	TCDF (NV) > PeCDF (NV) > TCDD (NV)
	Japanese quail	Undetermined

Values in parentheses are the ReP values determined based on lowest-observed-effect concentration (LOEC) values reported in Table 3. NV means no ReP value was calculated because no LOEC value was established.

expression and EROD activity was: chicken > pheasant > quail. This rank order is consistent with the sensitivity ranking predicted by the classification based on the amino acid sequence of the AhR LBD [24]. The rank-order of relative sensitivities to TCDD and TCDF reported here is identical to the rank-order based on lethal dose 50% (LD50) values derived from embryolethality data (A.M. Cohen-Barnhouse, Michigan State University, East Lansing, MI, pers. commun.). In addition, Hervé et al. [28] demonstrated the same rank-order of species sensitivity to TCDD and TCDF based on induction of *CYP1A4* and *CYP1A5* mRNA expression and EROD activity in primary hepatocyte cultures from each of the species studied here. Although actual relative potency values could not be determined for *CYP1A5* mRNA expression in pheasant and quail, it is clear that based on this endpoint the chicken is more sensitive to the effects of TCDD and TCDF than either of the other two species.

The rank-order of relative sensitivities of the three species to PeCDF were less clear, because it varied according to the endpoint used. As seen for TCDD and TCDF, when the species are exposed to PeCDF the rank-order of relative sensitivity to EROD induction is: chicken > pheasant > quail, which is consistent with the predicted order of Head et al. [24]. The rank-order for relative sensitivities, based on *CYP1A5* mRNA expression, of chicken > pheasant ≥ quail, suggests that pheasant and quail are approximately equally sensitive to the effects of PeCDF. In contrast, the rank-order for relative sensitivities, based on induction of *CYP1A4* mRNA expression, of pheasant ≥ chicken > quail, suggests that the pheasant may be as sensitive as the chicken to the effects of PeCDF. Based on embryolethality LD50 values (A.M. Cohen-Barnhouse, personal communication), pheasant is also more sensitive than chicken to PeCDF. The rank order based on embryolethality LD50 values (A.M. Cohen-Barnhouse, personal communication) was most similar to that based on *CYP1A4* mRNA expression. This contrasts with the greater sensitivity of chicken compared to pheasant when they are exposed to TCDD or TCDF. Based on in vitro *CYP1A4* and *CYP1A5* mRNA expression and EROD induction in primary hepatocyte cultures, it has recently been demonstrated that the chicken, pheasant, and quail are equally sensitivity to PeCDF [28].

Intercompound comparisons: relative potencies

Current World Health Organization (WHO) avian toxic equivalency factors (TEF) for TCDD, PeCDF, and TCDF are based on a limited number of studies that have been performed in the chicken. Based on these studies, each compound has been assigned a TEF of 1.0 [33]. Although TCDD is generally accepted as being the most potent AhR agonist the biomarker inducing potency of PeCDF and TCDF relative to TCDD were determined in differentially sensitive avian species. Unfortunately, due to limitations in the volume of carrier that could be injected and the solubility of the compounds of interest in triolein, it was not possible to accurately determine relative potency values for all chemicals and all endpoints in the present study. However, the results of the present study do suggest that PeCDF and TCDF may be as potent, if not more potent, than TCDD in some avian species. Specifically, in the chicken it was observed that each compound is an equipotent inducer of *CYP1A5* mRNA expression. Based on induction of EROD activity in the liver of the pheasant, both TCDF and PeCDF are more potent than TCDD. However, induction of EROD activity in TCDF- and PeCDF-exposed pheasants was weak, and was observed in only one exposure group. Based on CYP1A4 mRNA expression, PeCDF may be as much as 15-fold more potent than TCDD and 20-fold more potent than TCDF in the pheasant. Based on LD50 values, the rank-order of relative potency values observed for each of these species (chicken: TCDF > TCDD > PeCDF; pheasant and quail: PeCDF > TCDF > TCDD) (A.M. Cohen-Barnhouse, personal communication) is consistent with the observation that TCDD may not be the most potent AhR agonist in these species. In addition, greater potency of PeCDF relative to TCDD in primary hepatocytes of the pheasant has been reported [28]. While those results were based on induction of *CYP1A4* and *CYP1A5* mRNA expression in vitro as well as EROD activity, clearly there is merit for continued study of the effects of PeCDF on avian species. While it is unclear why the results from these two studies are not completely consistent, differences in the nature of the in vitro system used by Hervé et al. [28] versus the egg injection protocol used might be potential explanations. The observation that PeCDF might be more potent than TCDD is not without precedence. It has been reported that PeCDF was more potent as an inducer of EROD activity than TCDD in primary hepatocytes from the double-crested cormorant (*Phalacrocorax auritus*) and Forster's tern (*Sterna forsteri*) [34]. A similar observation has also been reported in green frog (*Rana esculenta*) hepatocytes [35].

General observations

Induction of EROD activity is a routine marker of exposure to dioxins and dioxin-like compounds, including the chlorinated furans TCDF and PeCDF. Hervé et al. [28] reported greater EROD activity in hepatocytes of the chicken, pheasant, and quail exposed to TCDD, PeCDF, and TCDF than the basal activity in unexposed hepatocytes. In the current study, the chicken was the only species in which EROD activity was induced by each chemical. It is possible that the lack of induction of EROD activity is related to low AhR activation, because both *CYP1A4* and *CYP1A5* mRNA expression was low in the pheasant and quail. Alternatively, basal hepatic EROD activity in the pheasant and quail was tenfold greater than in the chicken. A similar observation was made in the Pekin duck

(*Anas platyrhynchos domestica*), where basal hepatic EROD activity in 26-d-old embryos was 3-fold greater than in 19-d-old chicken embryos [36]. Based on analysis of the AhR LBD, the Pekin duck would be classified as an insensitive (type 3) species [37]. Future studies should include greater doses of TCDD, PeCDF, and TCDF in order to determine the relative potency of these compounds in ovo. However, due to solubility of the chemicals in triolein and the potential for greater background mortality due to increased volumes of carrier solvents, this will be difficult in the type of in ovo exposures conducted here.

Differences in the magnitude of the *CYP1A4* and *CYP1A5* mRNA expression in each exposure group were observed. Thus, it is important to consider the relative responsiveness as well as relative sensitivities of species. In each species and in response to each compound the maximum fold-change in mRNA expression was greater for *CYP1A4* than *CYP1A5*. This is consistent with results from Hervé et al. [28], who also demonstrated that *CYP1A4* mRNA expression was greater in hepatocytes from chicken, pheasant, and quail exposed to TCDD, PeCDF, and TCDF. It has also been reported that *CYP1A4* mRNA expression was greater than *CYP1A5* mRNA expression in chicken hepatocytes exposed to TCDD [15,16]. Although transcription of both genes results from activation of the AhR it is possible that transactivation of *CYP1A4* is greater than transactivation of *CYP1A5*. Alternatively, differences in mRNA stability may account for the observed differences in transcript abundance in vivo, although no evidence for such an in vitro mechanism was reported by Head and Kennedy [15].

In addition to differences between *CYP1A4* and *CYP1A5* mRNA expression in the same species exposed to the same chemical, differences in the magnitude of mRNA expression were also observed among species exposed to the same compound. Specifically, *CYP1A4* mRNA expression was the greatest in the chicken, followed by the pheasant and then the quail. This observation is consistent with the predicted rank-order of sensitivity proposed by Head et al. [24]. Magnitudes of *CYP1A5* mRNA expression were also the greatest in the chicken compared to the pheasant and quail, but no difference between maximal mRNA expressions in the pheasant and quail was observed.

Within each species the magnitude of *CYP1A4* mRNA expression in response to each chemical also presents interesting findings. In the chicken, *CYP1A4* mRNA levels were greatest in the TCDD-exposed organisms, followed by PeCDF and TCDF. However, in the pheasant and quail, *CYP1A4* mRNA levels were greatest in the PeCDF-exposed birds. The observation that PeCDF stimulates greater expression of *CYP1A4* mRNA than TCDD in the pheasant is also consistent with the observation that PeCDF is at least as potent as an activator of *CYP1A4* mRNA expression as TCDD. These results suggest that responsiveness, as it is related to the magnitude of *CYP1A* expression, may be related to the sensitivity of the species. If indeed changes in the AhR LBD amino acid sequence of the pheasant and quail [26,24] decrease binding affinity, then this may also explain decreased *CYP1A* responsiveness in less sensitive species, such as the pheasant and quail.

Results demonstrating the utility of biomarkers of AhR activation in avian species predicted to be differentially sensitive to the effects of dioxins and dioxin-like compounds were presented. Although each of the compounds studied is known to activate AhR, each biomarker shows a unique response pattern and the results suggest that Q-PCR analysis of *CYP1A* expression, in particular *CYP1A4* mRNA expression, may be a more sensitive biomarker of exposure than analysis of EROD induction. In particular, analysis of *CYP1A4* mRNA may be particularly beneficial for the analysis of exposure in less sensitive (i.e., type 2 and type 3) species; where determination of relative sensitivity values was possible, the general rank order of sensitivity in the TCDD and TCDF exposed groups was chicken $>$ pheasant $>$ quail. However, based on *CYP1A4* mRNA expression in the PeCDF-exposed groups the rank order of sensitivity was pheasant $\geq$ chicken $>$ quail. Of particular interest was the observation that PeCDF is more potent than TCDD in pheasant. Based on this observation it appears that further studies are required to address the current TEF values assigned to TCDD, PeCDF, and TCDF in avian species.

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