

## Cytochrome P4501A Induction by 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin and Two Chlorinated Dibenzofurans in Primary Hepatocyte Cultures of Three Avian Species

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Relative potencies of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were determined *in vitro* in primary hepatocyte cultures of chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*), and Japanese quail (*Coturnix japonica*) embryos. Concentration-dependent effects on ethoxyresorufin *O*-deethylase (EROD) activity and expression of cytochrome P4501A4 and cytochrome P4501A5 (*CYP1A4* and *CYP1A5*) messenger RNA (mRNA) were determined in hepatocytes exposed to serial dilutions of TCDD, PeCDF, or TCDF for 24 h. In chicken hepatocytes, the three compounds were equipotent inducers of EROD activity and *CYP1A4/CYP1A5* mRNA expression. However, in ring-necked pheasant and Japanese quail hepatocytes, PeCDF was more potent than TCDD (3- to 5-fold in ring-necked pheasant and 13- to 30-fold in Japanese quail). Among species, the rank order of sensitivity (most to least) to EROD and *CYP1A4/CYP1A5* mRNA induction for TCDD and TCDF was chicken > ring-necked pheasant > Japanese quail. In contrast, the three species were approximately equisensitive to EROD and *CYP1A4/CYP1A5* mRNA induction by PeCDF. It has generally been assumed that TCDD is the most potent “dioxin-like compound” (DLC) and that the chicken is the most sensitive avian species to CYP1A induction by all DLCs. This study indicates that PeCDF is more potent than TCDD in ring-necked pheasant and Japanese quail hepatocytes and that ring-necked pheasant, Japanese quail, and chicken hepatocytes are equally sensitive to CYP1A induction by PeCDF.

**Key Words:** PCDD; PCDF; EROD; bird; hepatocytes; AHR.

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are toxic and persistent contaminants generated unintentionally during the incineration of wastes and during the production of chlorine, polychlorinated biphenyls (PCBs), and some pesticides (Hutzinger *et al.*, 1985;

Rappe, 1992). PCDDs/PCDFs and some PCB congeners are referred to as “dioxin-like compounds” (DLCs) because they induce similar toxic and biochemical effects to those caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). It is generally accepted that TCDD is the most biologically potent DLC. In avian species, effects of DLC exposure include reduced egg production and hatchability, teratogenicity, edema, liver toxicity, porphyria, and enzyme induction (Brunström and Darnerud, 1983; Casini *et al.*, 2003; Kennedy *et al.*, 1996; Nosek *et al.*, 1992; Poland and Knutson, 1982).

The molecular mechanisms of DLC toxicity are not fully understood, but the involvement of the aryl hydrocarbon receptor (AHR) (Mammalian species possess a single AHR but there are at least two forms of the AHR in birds and fishes: AHR1 and AHR2 (Hahn *et al.*, 2006). In birds, AHR1 is the most transcriptionally active form, but in fishes, AHR2 is the dominant form (Hahn *et al.*, 2006; Yasui *et al.*, 2007). In this paper, we refer to AHR1 when discussing details regarding avian species, but for simplicity, we will refer only to “AHR” for other taxa and general comments.), a ligand-activated transcription factor, has been demonstrated in various studies (Bohonowych and Denison, 2007; Mimura and Fujii-Kuriyama, 2003; Poland and Knutson, 1982). When DLCs bind to the AHR, the DLC-AHR complex enters the nucleus, binds to the xenobiotic response element on DNA, and interacts with transcriptional cofactors to alter the transcription of numerous genes, including increased transcription of cytochrome P4501A (*CYP1A*) genes (Mimura and Fujii-Kuriyama, 2003). *CYP1A* induction is not a toxic response *per se* because the relationship between *CYP1A* gene expression and the occurrence of toxic effects is not clear (Nukaya *et al.*, 2009; Uno *et al.*, 2004). While *CYP1A* induction may be an adaptive response, induction may also lead to adverse effects through generation of free radicals and reactive oxygen species (Hilscherova *et al.*,

2003a; Jin *et al.*, 2001). Nonetheless, CYP1A induction has been shown to be predictive of the relative toxicity of DLCs in avian embryos (Brunström, 1991; Head and Kennedy, 2009; Kennedy *et al.*, 1996) and other species (Poland and Knutson, 1982).

Two major CYP1A isoforms induced by TCDD in chicken embryo liver were characterized several years ago by Rifkind *et al.* (Gilday *et al.*, 1996; Rifkind *et al.*, 1994; Sinclair *et al.*, 1997). CYP1A4 exhibits catalytic specificity for aryl hydrocarbon hydroxylase and ethoxyresorufin *O*-deethylase (EROD). CYP1A5 is principally responsible for the epoxygenation of the endogenous fatty acid, arachidonic acid, and also exhibits catalytic specificity for uroporphyrinogen oxidase. Measurement of EROD activity (Kennedy *et al.*, 1993, 1996; Sanderson *et al.*, 1998) and the characterization of CYP1A4 and CYP1A5 messenger RNA (mRNA) expression (Head and Kennedy, 2007a,b; Jones and Kennedy, 2009; Mahajan and Rifkind, 1999) are practical methods to quantify CYP1A induction in birds.

Despite the fact that all DLCs cause a similar pattern of effects and that they act through the same mechanism of action, there is a wide range of potency among compounds. For example, in chicken, the potency of compounds to induce embryotoxic or biochemical responses ranges over three orders of magnitude (Head *et al.*, 2008; Kennedy *et al.*, 1996; Van den Berg *et al.*, 1998); TCDD is generally considered to have the greatest potency to elicit these responses (Brunström, 1991; Kennedy *et al.*, 1996; Poland and Knutson, 1982; Van den Berg *et al.*, 1998). Furthermore, there is a wide range of sensitivity to DLCs among species. For example, the chicken (*Gallus gallus*), which is considered the most sensitive avian species, is over 1000 times more sensitive than the mallard (*Anas platyrhynchos*) to the embryo-lethal effects of some DLCs (Head *et al.*, 2008). A range of sensitivity is also observed among species within the order of Galliformes; for the DLCs selected for review by Head *et al.* (2008), the rank order of sensitivity was chicken > ring-necked pheasant (*Phasianus colchicus*) > Japanese quail (*Coturnix japonica*).

In this study, CYP1A induction by TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was measured in chicken, ring-necked pheasant, and Japanese quail embryonic hepatocytes. These species were chosen because they are representative of the broad range of sensitivity to DLCs among birds. PeCDF and TCDF were the DLCs selected for this study because little is known regarding their potency to induce CYP1A in birds and because their possible effects on birds could be of concern in certain parts of North America (Hilscherova *et al.*, 2003b) and elsewhere (Kiguchi *et al.*, 2007). The objectives of the study were to use embryonic primary hepatocyte cultures to (1) compare the relative potencies (RePs) of TCDD, PeCDF, and TCDF as CYP1A inducers in each species; (2) compare the relative sensitivities (ReSs) of chicken, ring-necked pheasant, and Japanese quail hepatocytes when exposed to TCDD,

PeCDF, or TCDF; and (3) characterize the relative expression of CYP1A4 mRNA and CYP1A5 mRNA in the three species when exposed to TCDD, PeCDF, or TCDF.

## MATERIALS AND METHODS

**Source of eggs and incubation conditions.** Chicken eggs were obtained from the Canadian Food Inspection Agency (Ottawa, Ontario, Canada); ring-necked pheasant and Japanese quail eggs were obtained from Couvoir Simetin (Mirabel, Quebec, Canada). Eggs from each species were incubated at 37.5°C and 60% relative humidity until 1- to 3-day pre-hatch. Chicken eggs were incubated for 19 days, ring-necked pheasant eggs for 23 days, and Japanese quail eggs for 16 days. Eggs were candled periodically, and infertile eggs and eggs containing dead embryos were discarded.

**Preparation and dosing of cultured hepatocytes.** Primary cultures of hepatocytes were prepared from avian embryos using methods described elsewhere (Kennedy *et al.*, 1995) and including subsequent modifications (Head and Kennedy, 2007b; Kennedy *et al.*, 2003). All procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre. Reagents were obtained from Sigma (St Louis, MO) unless another supplier is indicated. In brief, embryos were decapitated, and livers were removed, pooled, and digested with collagenase. Pools of chicken, ring-necked pheasant, or Japanese quail livers were prepared from 50, 63, and 50 embryos, respectively. Percoll (Amersham Bioscience, Uppsala, Sweden) density gradient centrifugation was used to separate erythrocytes from hepatocytes, and DNase (Roche, Laval, Quebec, Canada) treatment was carried out to prevent cell clumping. Cells were plated in 48-well culture plates containing 500 µl of cell culture medium 199, supplemented with insulin (1 µg/ml) and thyroxine (1 µg/ml), and incubated for 24 h at 37°C in a humidified incubator with 5% CO<sub>2</sub>, allowing them to form a monolayer attached to the bottom of the wells. Cells were treated in triplicate with in-well concentrations of TCDD, PeCDF, or TCDF ranging from 0.0003 to 10nM (2.5 µl of chemical per well in dimethyl sulfoxide [DMSO]). Cells were incubated for 24 h, medium was removed, plates were flash frozen on dry ice, and stored at -80°C until they were analyzed. Plates used for the EROD assays were rinsed with PBS-EDTA (200 µl/well) before they were flash frozen on dry ice.

**Preparation of TCDD, PeCDF, and TCDF solutions.** Serial dilutions of PCDDs/PCDFs were prepared in DMSO from stock solutions prepared in DMSO with concentrations that ranged between 40 and 100 µg/ml. Concentrations of PCDDs/PCDFs in the DMSO stock solutions were determined by isotope dilution following EPA Method 1613 (U.S. Environmental Protection Agency [U.S. EPA], 1994) with <sup>13</sup>C surrogate standards (DF-CS-C100; Wellington Laboratories, Guelph, Ontario, Canada). The actual concentrations of the stock solutions were 72.9, 82.8, and 104.4 µg/ml for TCDD, PeCDF, and TCDF, respectively. 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 (Micromass, Beverly, MD). The mass spectrometer was operated in a selected ion monitoring mode; resolution for all reference gas peaks in all time windows was more 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 <sup>13</sup>C-labeled PCDDs/PCDFs internal standards and all other Quality Assurance and Quality Control criteria were within ranges specified by the EPA methods (U.S. EPA, 1994).

**EROD assays.** EROD assays were conducted as described previously (Head *et al.*, 2006; Kennedy *et al.*, 1995). Reaction conditions for the EROD assays for chicken, ring-necked pheasant, and Japanese quail hepatocytes were validated as described in detail by Kennedy *et al.* (Kennedy and Jones, 1994; Kennedy *et al.*, 1993). Reagents were obtained from Sigma unless another

supplier is indicated. Briefly, hepatocytes were incubated at 37.5°C in the presence of nicotinamide adenine dinucleotide phosphate (reduced) and 7-ethoxyresorufin for 7 min. The reaction was stopped by the addition of cold acetonitrile containing fluorecamine. Resorufin and protein standard curves were prepared for each run. Plates were analyzed for both EROD activity (excitation wavelength: 530 and emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm and emission wavelength 460 nm) using a fluorescence plate reader (Cytofluor 2350; Millipore, Bedford, MA).

**RNA isolation and complementary DNA synthesis.** Total RNA was extracted from 48-well plates using RNeasy 96 kits (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. An on-column DNase treatment was performed; the only modification was the use of 50% ethanol solution for RNA isolation instead of the suggested 70% because previous tests in our laboratory showed that greater RNA yields were obtained with 50% ethanol. To ensure the maximal removal of genomic DNA, total RNA was treated a second time with DNase from the Ambion DNA-free kit (Ambion, Austin, TX) according to manufacturer's instructions. Total RNA (11.5 µl from each well) was reverse transcribed to complementary DNA (cDNA) with Superscript II and random hexamers (Invitrogen, Burlington, Ontario, Canada) as per manufacturer's instructions. From each plate, a control without reverse transcriptase enzyme (no-RT control) was included to verify the absence of genomic DNA in the RNA template.

**Quantitative reverse transcription-PCR.** A multiplex quantitative reverse transcription-polymerase chain reaction (QPCR) assay, using dual-labeled fluorescent hydrolysis probes (Head and Kennedy, 2007b), was used to quantify chicken *CYP1A4*, *CYP1A5*, and beta-actin mRNA abundance. Assays similar to the chicken assay were optimized for ring-necked pheasant and Japanese quail gene targets. Primers and probes were designed with Primer3 (Rozen and Skaletsky, 2000) based on gene sequences from Genbank: ring-necked pheasant *CYP1A4* (accession no. FJ872527) and *CYP1A5* (accession no. FJ872528) and Japanese quail *CYP1A4* (accession no. GQ906939) and *CYP1A5* (accession no. GQ906938). Beta-actin is highly conserved among chicken, ring-necked pheasant, and Japanese quail, and therefore, primers and probes were the same for all the three species. Primers were obtained from Invitrogen, and probes were obtained from Biosearch Technologies (Novato, CA). The amplicons of ring-necked pheasant and Japanese quail *CYP1A4*, *CYP1A5*, and beta-actin were cloned and sequenced to demonstrate the specificity of the primers. Brilliant QPCR Core Reagent kits (Stratagene, La Jolla, CA) were used to carry out each assay. Each 25 µl reaction contained 1× PCR buffer, 5mM MgCl<sub>2</sub>, 0.8mM deoxynucleoside triphosphate, 0.08 vol/vol glycerol, 0.05 U SureStart Taq polymerase, and 25nM reference dye (ROX). Validation studies for all QPCR assays were conducted as indicated elsewhere (Head and Kennedy, 2007b). In brief, each gene target was amplified alone and multiplexed with the other targets to determine the optimal primer concentrations (Table 1). The lowest concentration of each *CYP1A4* and *CYP1A5* primer that generated a relatively small and consistent cycle threshold (Ct) value with an elevated fluorescence value was selected for the assay. Based on previous assay optimization studies for chicken hepatocytes (Head and Kennedy, 2007b), the concentrations of beta-actin primers were limited to 50nM in the master mix. Standard curves for ring-necked pheasant and Japanese quail target genes, alone and multiplexed, were produced from a 1:2 dilution series of concentrated cDNA, covering two orders of magnitude. Quantification of *CYP1A4/CYP1A5* mRNA fold induction by TCDD, PeCDF, and TCDF was assessed using the 2<sup>-ddCt</sup> method (Livak and Schmittgen, 2001).

**Cell viability.** Cell viability was determined by use of the Calcein-AM assay (Invitrogen-Molecular Probes, Eugene, Oregon). Vehicle (DMSO)-treated cells were included as a positive control, and 99% ethanol-killed cells were used as a negative control. A working solution was prepared by adding 3 µl of Calcein-AM to 10 ml PBS-EDTA. The culture medium was removed, and 200 µl of the Calcein-AM solution was added to each well. The plates were incubated in the dark for 45 min, and fluorescence was measured using a Cytofluor 2350 fluorescence plate reader (Millipore, Billerica, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

**TABLE 1**  
Nucleotide Sequence of Primers and Probes, Fluorescent Dye/Quencher and Amplicon Size in Base Pairs (bp) for Ring-Necked Pheasant and Japanese Quail *CYP1A4*, *CYP1A5*, and Beta-Actin. Final Primer and Probe Concentrations Used for QPCR are Shown in Brackets

Ring-necked pheasant	
<i>CYP1A4</i>	
Dye/quencher	FAM/BHQ-1
Forward	5'-GAGCACATTCGGGATGTCA-3' (300nM)
Reverse	5'-CAGAGAGTTGGACACGGACA-3' (300nM)
Probe <sup>a</sup>	5'-CCTGGCAGTGCCCAATCAACG-3' (200nM)
Amplicon size	85 bp
<i>CYP1A5</i>	
Dye/quencher	Quasar 670/BHQ-3
Forward	5'-CATCCGAGATGTCACCGACT-3' (300nM)
Reverse	5'-TTGGGATCTGTGTGGCACTA-3' (300nM)
Probe <sup>b</sup>	5'-CCCTCATTGAGCAGTGCATGGA-3' (200nM)
Amplicon size	80 bp
Japanese quail	
<i>CYP1A4</i>	
Dye/quencher	FAM/BHQ-1
Forward	5'-GGATGTCAATACCCGTTTCG-3' (300nM)
Reverse	5'-CTGCCCAATCAATGAGTCTG-3' (300nM)
Probe <sup>a</sup>	5'-TGACGTCCCGAATGTGCTCCT-3' (200nM)
Amplicon size	109 bp
<i>CYP1A5</i>	
Dye/quencher	Quasar 670/BHQ-3
Forward	5'-TACAGGCAGCTGTGGATGAG-3' (300nM)
Reverse	5'-ACTGCTCAATGAGGGAGTTCG-3' (300nM)
Probe <sup>b</sup>	5'-GACAAGAACAGCATCCGAGACGTCA-3' (200nM)
Amplicon size	81 bp
Ring-necked pheasant and Japanese quail	
Beta-actin	
Dye/quencher	HEX/BHQ-1
Forward primer	5'-AAATTGTGCGTGACATCAAGGA-3' (50nM)
Reverse primer	5'-GAGGCAGCTGTGGCCATCT-3' (50nM)
Probe <sup>b</sup>	5'-TGCTACGTGCGACTGGATTTTCGAGC-3' (50nM)
Amplicon size	76 bp

*Note.* Primer and probes used for the chicken are presented elsewhere (Head and Kennedy, 2007b). Beta-actin primer, probe sequences, and concentrations are the same for both species.

<sup>a</sup>Probe anneals to the same strand as the reverse primer.

<sup>b</sup>Probe anneals to the same strand as the forward primer.

**EROD and *CYP1A4/CYP1A5* mRNA data analysis.** Fluorescence data were imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA) for curve fitting. EROD activity data were fit to a modified Gaussian curve as explained elsewhere (Kennedy *et al.*, 1993). For each treatment, three EROD curves were generated from data originating from separate cell culture plates. EC<sub>50</sub>, EC<sub>threshold</sub>, and maximal values are presented as the mean value of replicates obtained from three 48-well plate replicates ± SE. The EC<sub>threshold</sub> represents the concentration of a compound producing a response equivalent to 10% of the maximal response produced by TCDD in the same species

(see "Discussion" section for more details). EROD activity data were also fitted to a four-parameter equation (see below) in order to compare hillslopes (describes the steepness of the curve). The hillslopes of EROD activity are presented as the mean value obtained from three plates  $\pm$  SE.

*CYP1A* mRNA expression data were fitted to a four-parameter logistic model as described previously (Head and Kennedy, 2007b). The equation integrates the hillslope,  $EC_{50}$ , baseline response, and maximal response as parameters. A single curve fit was generated for mRNA induction, using data from the average of three wells from the same cell culture plate, assessed in duplicate.  $EC_{50}$ , maximal values, and hillslopes are presented as the values calculated from the curve fit  $\pm$  SE.

Statistical differences among  $EC_{50}$ ,  $EC_{\text{threshold}}$ , and hillslope values were tested using a one-way ANOVA with a Bonferroni correction. Significance was set at  $p < 0.05$  for all tests.

**Calculation of RePs and ReSs.** The concept of using ReP values to compare the potencies of DLCs is well established (Van den Berg *et al.*, 1998). In this study, RePs are defined as  $[EC_{50}$  (or  $EC_{\text{threshold}}$ ) of TCDD in species A] /  $[EC_{50}$  (or  $EC_{\text{threshold}}$ ) of the compound of interest in species A]. There is also value in comparing the sensitivities of avian species to a particular compound (e.g., the comparison of the  $EC_{50}$  of chicken, ring-necked pheasant, and Japanese quail when exposed to TCDD). Because the chicken is the most sensitive species tested to date, comparisons are based on this species. For such comparisons, the ReS is defined as  $[EC_{50}$  (or  $EC_{\text{threshold}}$ ) of compound A in chicken] /  $[EC_{50}$  (or  $EC_{\text{threshold}}$ ) of compound A in the species of interest].

## RESULTS

### Concentration-Dependent Effects of TCDD, PeCDF, and TCDF on EROD Activity

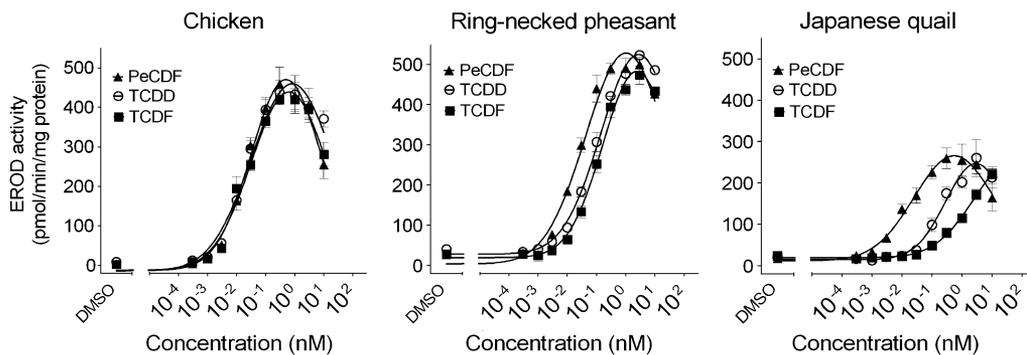
**General observations.** TCDD, PeCDF, and TCDF induced EROD activity in a concentration-dependent manner in chicken, ring-necked pheasant, and Japanese quail hepatocytes (Fig. 1). In most cases, maximal EROD activity was followed by a decrease in activity at greater concentrations of the inducer. The decrease in EROD activity at high concentrations of TCDD, PeCDF, or TCDF was not due to overt toxicity as measured by the Calcein-AM assay (results not shown). Maximal EROD activities for TCDD, PeCDF, and TCDF in chicken and ring-necked pheasant hepatocytes were similar (~440–550 pmol/min/mg protein), but maximal EROD activity was lower ( $p < 0.02$ ) in Japanese quail hepatocytes (~250–285 pmol/min/mg protein).

**Intercompound comparisons: RePs of TCDD, PeCDF, and TCDF.** In chicken hepatocytes, there was no statistical difference among  $EC_{50}$  or  $EC_{\text{threshold}}$  values among the three compounds (Table 2) and the EROD concentration-response curves for TCDD, PeCDF, and TCDF were essentially identical (Fig. 1). Therefore, RePs based on the  $EC_{50}$  for PeCDF and TCDF were 0.9 and RePs based on the  $EC_{\text{threshold}}$  for PeCDF and TCDF were 0.6 in chicken hepatocytes (Table 2). The three compounds were essentially equipotent (PeCDF = TCDD = TCDF) in chicken hepatocytes.

In ring-necked pheasant hepatocytes, the EROD concentration-response curve for PeCDF was shifted slightly to the left of the TCDD and TCDF curves (Fig. 1). The  $EC_{50}$  and  $EC_{\text{threshold}}$  values for PeCDF were statistically lower than that for TCDD, and the  $EC_{50}$  and  $EC_{\text{threshold}}$  values for TCDF were similar to the values for TCDD (Table 2). Consequently, the RePs for PeCDF based on the  $EC_{50}$  and  $EC_{\text{threshold}}$  were 3.4 and 4.6, respectively, and the RePs for TCDF based on the  $EC_{50}$  and  $EC_{\text{threshold}}$  were 0.8 and 0.6, respectively. In contrast with the chicken, the rank order of potency in ring-necked pheasant hepatocytes was PeCDF > TCDD = TCDF.

In Japanese quail hepatocytes, there were clear differences in the EROD concentration-response curves. From left to right, the order was PeCDF, TCDD, and TCDF (Fig. 1). The  $EC_{50}$  and  $EC_{\text{threshold}}$  values for PeCDF were statistically lower than that for TCDD. The  $EC_{50}$  and  $EC_{\text{threshold}}$  values for TCDF were statistically higher than that for TCDD (Table 2). Consequently, the RePs for PeCDF based on  $EC_{50}$  and  $EC_{\text{threshold}}$  were 13 and 27, respectively, and the RePs for TCDF based on the  $EC_{50}$  and  $EC_{\text{threshold}}$  were 0.1 and 0.3, respectively. In Japanese quail hepatocytes, the rank order of potency was PeCDF > TCDD > TCDF.

**Interspecies comparisons: ReSs of chicken, ring-necked pheasant, and Japanese quail.** The  $EC_{50}$  values for chicken, ring-necked pheasant, and Japanese quail hepatocytes exposed to TCDD were 0.018, 0.085, and 0.19 nM, respectively; these were statistically different among the three species (Table 3). The  $EC_{\text{threshold}}$  values for TCDD-exposed hepatocytes were also



**FIG. 1.** Concentration-dependent effects of PeCDF ( $\blacktriangle$ ), TCDD ( $\circ$ ), or TCDF ( $\blacksquare$ ) on EROD activity in chicken, ring-necked pheasant, and Japanese quail embryo hepatocyte cultures exposed for 24 h. Points represent mean EROD activity from three replicate cell culture plates; values before the axis break indicate EROD activity observed for control (DMSO-treated hepatocytes). Bars represent SEs.

**TABLE 2**  
**Maximal EROD Activity, EC<sub>50</sub>, EC<sub>thr</sub>, and ReP Values in Chicken, R-n. pheasant, and J. quail Hepatocyte Cultures Exposed to TCDD, PeCDF, or TCDF for 24 h**

Species	Chemical	EC <sub>50</sub> (nM)	EC <sub>50</sub> -based ReP	EC <sub>thr</sub> (nM)	EC <sub>thr</sub> -based ReP	Max EROD activity (pmol/min/mg protein)
Chicken	TCDD	0.018 ± 0.0007 <sup>a</sup>	1.0	0.00081 ± 0.00002 <sup>a</sup>	1.0	460 ± 36 <sup>a</sup>
Chicken	PeCDF	0.019 ± 0.0003 <sup>a</sup>	0.9	0.0013 ± 0.0002 <sup>a</sup>	0.6	471 ± 38 <sup>a</sup>
Chicken	TCDF	0.021 ± 0.001 <sup>a</sup>	0.9	0.0014 ± 0.0003 <sup>a</sup>	0.6	443 ± 17 <sup>a</sup>
R-n. pheasant	TCDD	0.085 ± 0.01 <sup>a</sup>	1.0	0.0051 ± 0.001 <sup>a</sup>	1.0	525 ± 6 <sup>a</sup>
R-n. pheasant	PeCDF	0.025 ± 0.002 <sup>b</sup>	3.4	0.0011 ± 0.0001 <sup>b</sup>	4.6	530 ± 15 <sup>a</sup>
R-n. pheasant	TCDF	0.11 ± 0.02 <sup>a</sup>	0.8	0.0090 ± 0.002 <sup>a</sup>	0.6	486 ± 12 <sup>a</sup>
J. quail	TCDD	0.19 ± 0.02 <sup>a</sup>	1.0	0.020 ± 0.001 <sup>a</sup>	1.0	248 ± 31 <sup>a</sup>
J. quail	PeCDF	0.015 ± 0.0009 <sup>b</sup>	13.0	0.00073 ± 0.0001 <sup>b</sup>	27.0	267 ± 27 <sup>a</sup>
J. quail	TCDF	1.57 ± 0.4 <sup>c,*</sup>	0.1*	0.077 ± 0.01 <sup>c</sup>	0.3	285 ± 24 <sup>a,†</sup>

Note. EC<sub>thr</sub>, EC<sub>threshold</sub>; J. quail, Japanese quail; R-n. pheasant, ring-necked pheasant.

Mean values were derived from three replicate cell culture plates ± SE. Superscript letters indicate significant differences among treatments ( $p < 0.05$ ) within each species. EROD data were fit to a modified Gaussian curve.

\*No maximal response was reached.

†Because no maximal value was reached when J. quail hepatocytes were exposed to TCDF (Fig. 1), the EC<sub>50</sub> for TCDF might be underestimated and EC<sub>50</sub>-based ReP might be overestimated.

different (0.00081, 0.0051, and 0.020nM, respectively). Consequently, the ReS values were 0.2 for ring-necked pheasant, and for Japanese quail, the ReS values based on EC<sub>50</sub> and EC<sub>threshold</sub> were 0.09 and 0.04, respectively. The rank order of sensitivity to EROD induction by TCDD (in the order of most to least sensitive) was chicken > ring-necked pheasant > Japanese quail.

The EC<sub>50</sub> values for chicken, ring-necked pheasant, and Japanese quail hepatocytes exposed to PeCDF were not statistically different from one another (Table 3), but there were small differences in EC<sub>threshold</sub> values among the species. The EC<sub>50</sub>- and EC<sub>threshold</sub>-based ReS values for ring-necked pheasant were 0.8 and 1.2, respectively. The ReS values based

on the EC<sub>50</sub> and EC<sub>threshold</sub> for Japanese quail were 1.3 and 1.8, respectively. These results generally indicate that the rank order of sensitivity to EROD induction by PeCDF was Japanese quail ≥ chicken = ring-necked pheasant.

The EC<sub>50</sub> and EC<sub>threshold</sub> values for ring-necked pheasant and Japanese quail hepatocytes exposed to TCDF were statistically greater than that for chicken hepatocytes (Table 3). Consequently, the ReS values based on the EC<sub>50</sub> and EC<sub>threshold</sub> for ring-necked pheasant were both 0.2, and for Japanese quail, they were 0.01 and 0.02, respectively. The rank order of sensitivity to EROD induction by TCDF was chicken > ring-necked pheasant > Japanese quail.

**TABLE 3**  
**Maximal EROD Activity, EC<sub>50</sub>, EC<sub>thr</sub>, and ReS Values in Chicken, R-n. pheasant, and J. quail Hepatocyte Cultures Exposed to TCDD, PeCDF, or TCDF for 24 h**

Chemical	Species	EC <sub>50</sub> (nM)	EC <sub>50</sub> -based ReS	EC <sub>thr</sub> (nM)	EC <sub>thr</sub> -based ReS
TCDD	Chicken	0.018 ± 0.0007 <sup>a</sup>	1.0	0.00081 ± 0.00002 <sup>a</sup>	1.0
TCDD	R-n. pheasant	0.085 ± 0.01 <sup>b</sup>	0.2	0.0051 ± 0.001 <sup>b</sup>	0.2
TCDD	J. quail	0.19 ± 0.02 <sup>c</sup>	0.09	0.020 ± 0.001 <sup>c</sup>	0.04
PeCDF	Chicken	0.019 ± 0.0003 <sup>a</sup>	1.0	0.0013 ± 0.0002 <sup>a</sup>	1.0
PeCDF	R-n. pheasant	0.025 ± 0.002 <sup>a</sup>	0.8	0.0011 ± 0.0001 <sup>a,b</sup>	1.2
PeCDF	J. quail	0.015 ± 0.0009 <sup>a</sup>	1.3	0.00073 ± 0.0001 <sup>b</sup>	1.8
TCDF	Chicken	0.021 ± 0.001 <sup>a</sup>	1.0	0.0014 ± 0.0003 <sup>a</sup>	1.0
TCDF	R-n. pheasant	0.11 ± 0.02 <sup>b</sup>	0.2	0.0090 ± 0.002 <sup>b</sup>	0.2
TCDF	J. quail	1.57 ± 0.4 <sup>c,*</sup>	0.01*	0.077 ± 0.01 <sup>c</sup>	0.02

Note. EC<sub>thr</sub>, EC<sub>threshold</sub>; J. quail, Japanese quail; R-n. pheasant, ring-necked pheasant.

Mean values were derived from three replicate cell culture plates ± SE. Superscript letters indicate significant differences among species ( $p < 0.05$ ) exposed to the same chemical. EROD data were fit to a modified Gaussian curve.

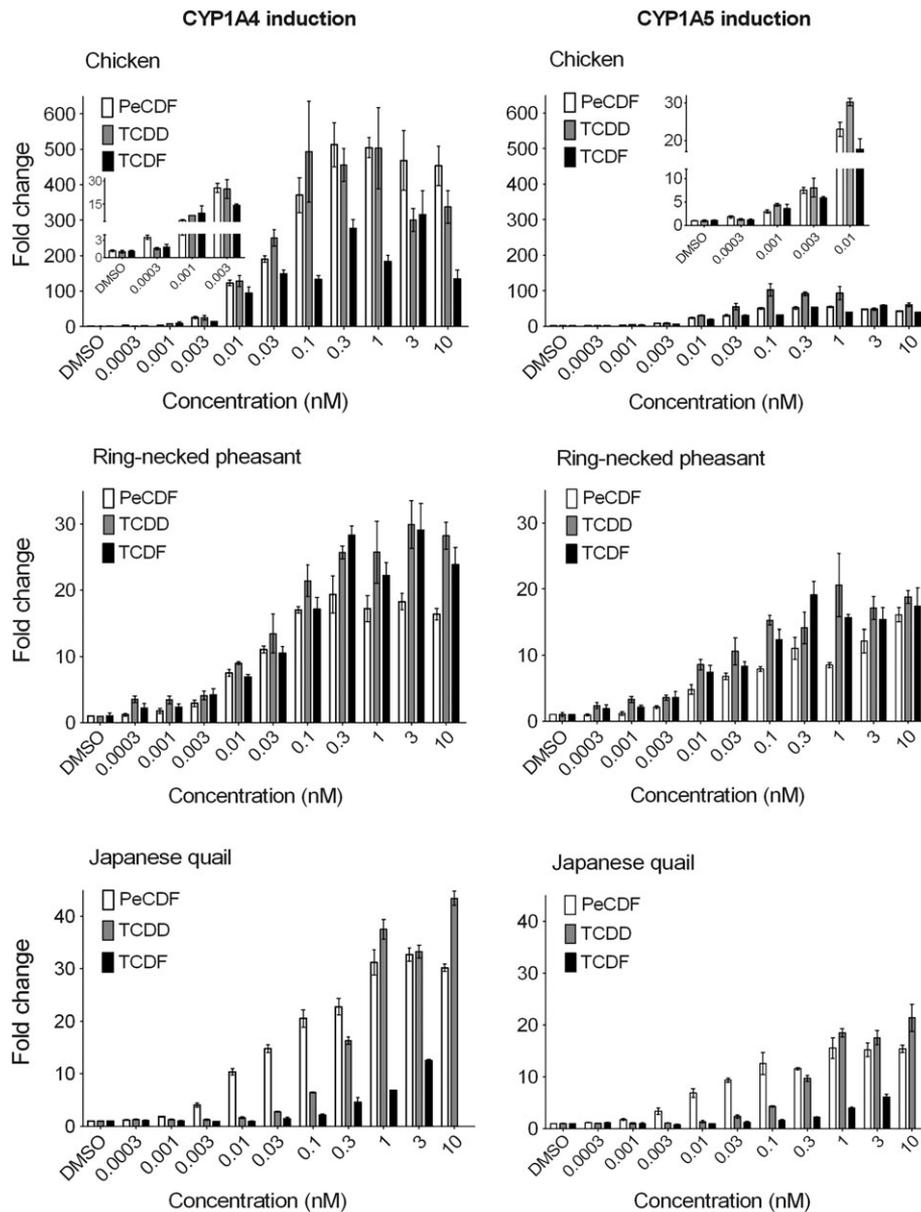
\*Because no maximal value was reached when J. quail hepatocytes were exposed to TCDF (Fig. 1), the EC<sub>50</sub> might be underestimated and ReS based on EC<sub>50</sub> might be overestimated.

*Concentration-Dependent Effects of TCDD, PeCDF, and TCDF on CYP1A4/CYP1A5 mRNA Expression*

**Assay optimization.** Standard curves for *CYP1A4*, *CYP1A5*, and beta-actin mRNA expression in chicken, ring-necked pheasant, and Japanese quail hepatocytes were obtained using triplex assays (i.e., measuring the mRNA expression of the three genes in the same tube). The Ct values were plotted against relative cDNA concentrations, and data were fit to a linear regression model. The efficiencies of the reactions for *CYP1A4*, *CYP1A5*, and beta-actin mRNA expression were similar within each species. In addition, standard curves for the three transcripts were nearly parallel (slopes were within 0.1 for

each gene for the three species), and  $r^2$  values for all curves were between 0.983 and 0.999. Beta-actin expression was not affected by TCDD, PeCDF, or TCDF in hepatocytes from any of the species. Therefore, changes in mRNA expression were a result of changes in *CYP1A4* and *CYP1A5* mRNA expression and not in changes in beta-actin mRNA expression, which validates the use of beta-actin as a housekeeping gene.

**General observations.** The mRNAs of both *CYP1A4* and *CYP1A5* isoforms were induced in a concentration-dependent manner by TCDD, PeCDF, and TCDF in chicken, ring-necked pheasant, and Japanese quail hepatocytes (Fig. 2). In the three species, the *CYP1A4* isoform had a greater maximal induction



**FIG. 2.** Concentration-dependent effects of PeCDF, TCDD, and TCDF on *CYP1A4* and *CYP1A5* mRNA induction in chicken, ring-necked pheasant, and Japanese quail hepatocyte cultures exposed for 24 h. Bars represent mean fold induction ( $\pm$  SEM) of three replicate cell culture wells from the same cell culture plate, assessed in duplicate.

**TABLE 4**  
**Curve fitting parameters for concentration-dependent effects of TCDD, PeCDF, and TCDF on EROD activity and CYP1A4/CYP1A5 mRNA induction in chicken, R-n. pheasant, and J. quail hepatocyte cultures exposed for 24 h**

Species	Compound	End point	EC <sub>50</sub> (nM)	Hillslope	Maximal response (EROD: pmol/min/mg protein, CYP1A4/CYP1A5: fold induction)
Chicken	TCDD	EROD	0.018 ± 0.0007 <sup>a</sup>	1.18 ± 0.2 <sup>a</sup>	460 ± 36
Chicken	TCDD	CYP1A4	0.026 ± 0.008 <sup>a</sup>	1.48 ± 0.7 <sup>a</sup>	502 ± 47
Chicken	TCDD	CYP1A5	0.021 ± 0.005 <sup>a</sup>	1.52 ± 0.5 <sup>a</sup>	78 ± 6
Chicken	PeCDF	EROD	0.019 ± 0.0003 <sup>a</sup>	1.09 ± 0.2 <sup>a</sup>	471 ± 38
Chicken	PeCDF	CYP1A4	0.037 ± 0.008 <sup>a</sup>	1.28 ± 0.3 <sup>a</sup>	490 ± 23
Chicken	PeCDF	CYP1A5	0.014 ± 0.003 <sup>a</sup>	1.22 ± 0.2 <sup>a</sup>	50 ± 2
Chicken	TCDF	EROD	0.021 ± 0.001 <sup>a</sup>	1.1 ± 0.1 <sup>a</sup>	443 ± 17
Chicken	TCDF	CYP1A4	0.017 ± 0.01 <sup>a</sup>	1.00 ± 0.5 <sup>a</sup>	222 ± 22
Chicken	TCDF	CYP1A5	0.020 ± 0.006 <sup>a</sup>	0.93 ± 0.2 <sup>a</sup>	47 ± 2
R-n. pheasant	TCDD	EROD	0.085 ± 0.01 <sup>a</sup>	1.00 ± 0.08 <sup>a</sup>	525 ± 6
R-n. pheasant	TCDD	CYP1A4	0.038 ± 0.01 <sup>a</sup>	0.87 ± 0.2 <sup>a</sup>	30 ± 6
R-n. pheasant	TCDD	CYP1A5	0.022 ± 0.01 <sup>a</sup>	0.69 ± 0.2 <sup>a</sup>	21 ± 8
R-n. pheasant	PeCDF	EROD	0.025 ± 0.002 <sup>a</sup>	1.32 ± 0.3 <sup>a</sup>	530 ± 15
R-n. pheasant	PeCDF	CYP1A4	0.017 ± 0.004 <sup>a</sup>	1.24 ± 0.2 <sup>a</sup>	18 ± 2
R-n. pheasant	PeCDF	CYP1A5	N/A*	N/A*	N/A*
R-n. pheasant	TCDF	EROD	0.11 ± 0.02 <sup>a</sup>	1.10 ± 0.01 <sup>a</sup>	486 ± 12
R-n. pheasant	TCDF	CYP1A4	0.048 ± 0.01 <sup>a</sup>	1.16 ± 0.03 <sup>a</sup>	29 ± 7
R-n. pheasant	TCDF	CYP1A5	0.026 ± 0.01 <sup>a</sup>	0.89 ± 0.1 <sup>a</sup>	17 ± 5
J. quail	TCDD	EROD	0.19 ± 0.02 <sup>a</sup>	1.02 ± 0.3 <sup>a</sup>	248 ± 31
J. quail	TCDD	CYP1A4	0.36 ± 0.03 <sup>a</sup>	1.83 ± 0.3 <sup>a</sup>	40 ± 1.3
J. quail	TCDD	CYP1A5	0.32 ± 0.04 <sup>a</sup>	1.44 ± 0.3 <sup>a</sup>	20 ± 0.9
J. quail	PeCDF	EROD	0.015 ± 0.0009 <sup>a</sup>	0.78 ± 0.2 <sup>a</sup>	267 ± 27
J. quail	PeCDF	CYP1A4	0.047 ± 0.01 <sup>a</sup>	0.65 ± 0.09 <sup>a</sup>	33 ± 1.4
J. quail	PeCDF	CYP1A5	0.019 ± 0.006 <sup>a</sup>	0.71 ± 0.2 <sup>a</sup>	16 ± 0.8
J. quail	TCDF	EROD	1.57 ± 0.4 <sup>a,†</sup>	0.97 ± 0.2 <sup>a</sup>	285 ± 24 <sup>‡</sup>
J. quail	TCDF	CYP1A4	0.69 ± 0.2 <sup>a,†</sup>	1.30 ± 0.3 <sup>a</sup>	12 ± 0.3 <sup>‡</sup>
J. quail	TCDF	CYP1A5	0.69 ± 0.1 <sup>a,†</sup>	1.53 ± 0.3 <sup>a</sup>	6 ± 0.5 <sup>‡</sup>

Note. J. quail, Japanese quail; N/A, not applicable; R-n. pheasant, ring-necked pheasant.

Mean values were derived from three replicate cell culture plates ± SE (EROD parameters) or from the average of three different wells of the same cell culture plate, assessed in duplicate (mRNA expression). Superscript letters indicate significant differences between end points ( $p < 0.05$ ). EROD data were fit to a modified Gaussian curve, and CYP1A4/CYP1A5 mRNA expression data were fit to a four-parameter logistic curve. EROD data were also fit to the four-parameter equation in order to compare the hillslope values of EROD activity and mRNA induction.

\*Despite the concentration-dependent increase in mRNA, the points could not be fitted to the four-parameter equation (Fig. 3).

<sup>†</sup>Because no maximal value was reached, EC<sub>50</sub> might be underestimated.

<sup>‡</sup>No maximal response was reached.

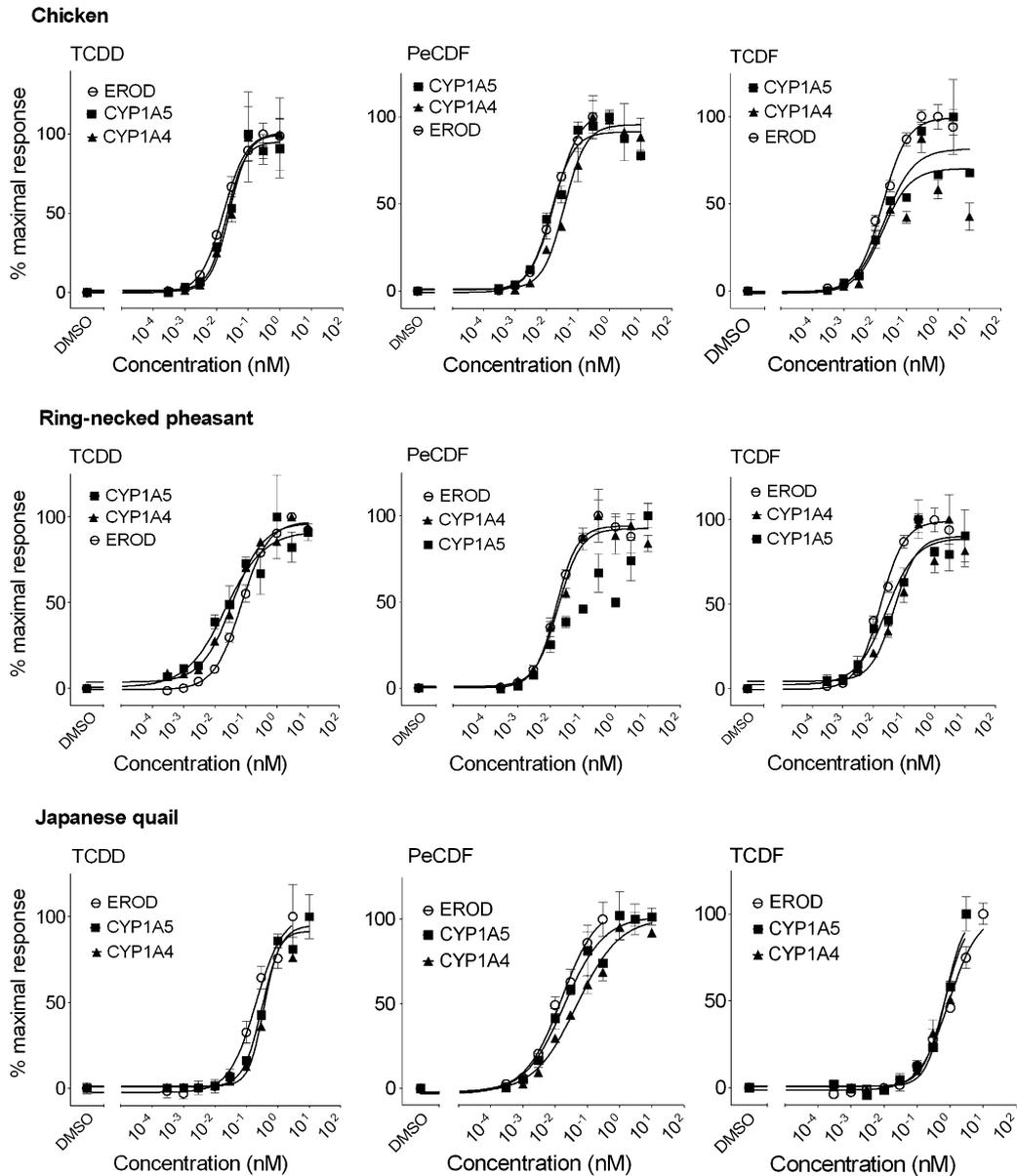
than the CYP1A5 isoform (Fig. 2, Table 4). The maximal induction of CYP1A4/CYP1A5 mRNA differed among species: Chicken hepatocytes showed a higher CYP1A4 and CYP1A5 mRNA induction (502-fold and 47-fold, respectively) than ring-necked pheasant (30-fold and 17-fold, respectively) and Japanese quail hepatocytes (40-fold and 6-fold, respectively; Fig. 2, Table 4).

*Comparisons between EROD activity and CYP1A4/CYP1A5 mRNA expression.* Concentration-dependent curves of EROD activity and CYP1A4/CYP1A5 mRNA expression were compared after normalizing the maximal responses of each end

point to 100% for each compound and in each species (Fig. 3). The shapes of the concentration-response curves of EROD activity and CYP1A4/CYP1A5 mRNA expression were similar, and there was general concordance in EC<sub>50</sub> and hillslope values among the three end points for each compound in each species (Table 4).

## DISCUSSION

The present experiments measured CYP1A induction by TCDD, PeCDF, and TCDF exposure in primary hepatocyte



**FIG. 3.** Concentration-dependent effects of TCDD, PeCDF, and TCDF on EROD activity (○), *CYP1A4* mRNA expression (▲), and *CYP1A5* mRNA expression (■) in chicken, ring-necked pheasant, and Japanese quail hepatocyte cultures exposed for 24 h. For comparison purposes, data are expressed as percent maximal response. For EROD activity, each point represents the mean of three different cell culture plates. For mRNA expression, each point represents the mean of three different wells from the same cell culture plate. Bars represent SEs of the means.

cultures, prepared from chicken, ring-necked pheasant, and Japanese quail embryos. These three species of Galliformes were chosen because they demonstrate differential overt and biochemical sensitivities to DLCs: The chicken is very sensitive, the ring-necked pheasant is moderately sensitive, and the Japanese quail is the least sensitive (Head *et al.*, 2008). Comparison of the potencies of compounds within each species (ReP) and comparison of sensitivities among species when exposed to each compound (ReS) were made. Concentration-dependent effects of TCDD, PeCDF, and TCDF on EROD activity and *CYP1A4/CYP1A5* mRNA expression were com-

pared in hepatocytes from the three species to (1) compare the concentration-response curves and (2) determine if there were differences in the ratios of *CYP1A4* and *CYP1A5* mRNA expression among the species.

TCDD and TCDF were approximately equipotent EROD inducers in both chicken and ring-necked pheasant hepatocytes, a finding that is consistent with an earlier study (Kennedy *et al.*, 1996). In contrast, TCDF was a less potent inducer of EROD activity (ReP = 0.1–0.3) than TCDD in Japanese quail hepatocytes. PeCDF was a more potent inducer of EROD activity and *CYP1A4/CYP1A5* mRNA than TCDD in

ring-necked pheasant (ReP = 3.4–4.6) and Japanese quail (ReP = 13–27) hepatocytes. This is the first report that PeCDF is a more potent inducer of EROD activity and *CYP1A4/CYP1A5* mRNA in ring-necked pheasant and Japanese quail hepatocytes. However, ~10 years ago, it was discovered that PeCDF was a more potent inducer of EROD activity than TCDD in double-crested cormorant (*Phalacrocorax auritus*) and Forster's tern (*Sterna forsteri*) hepatocytes (Sanderson *et al.*, 1998). A more recent study indicated that PeCDF was also a more potent EROD inducer than TCDD in green frog (*Rana esculenta*) hepatocytes (Rankouhi *et al.*, 2005).

Despite our previous knowledge that PeCDF was a more potent EROD inducer than TCDD in some species, our initial hypothesis was that the rank order of species sensitivity to EROD induction by all three compounds (TCDD, PeCDF, and TCDF) would be chicken (most sensitive), ring-necked pheasant (moderately sensitive), and Japanese quail (least sensitive). This hypothesis was based upon (1) earlier studies with TCDD- and TCDF-exposed chicken and ring-necked pheasant hepatocytes (Kennedy *et al.*, 1996) and (2) the identity of key amino acid residues within the ligand-binding domains of AHR1 for the three species. Amino acid residues corresponding to Ile324 and Ser380 in chicken were compared to amino acid residues in ring-necked pheasant, Japanese quail, and several other avian species, and it was found that the identity of the amino acid residues at sites 324 and 380 were predictive of broad categories of dioxin sensitivity among species (Head *et al.*, 2008). The chicken was unique in having the Ile/Ser genotype, the ring-necked pheasant has the Ile/Ala genotype and was classified as moderately sensitive, and the Japanese quail has the Val/Ala genotype, which places it in the least sensitive category. The results of the present study supported our hypothesis for TCDD and TCDF. Indeed, the EC<sub>50</sub>-based ReS values were 1.0, 0.2, and 0.09 for chicken, ring-necked pheasant, and Japanese quail exposed to TCDD, respectively, and EC<sub>50</sub>-based ReS values were 1.0, 0.2, and 0.01 for chicken, ring-necked pheasant, and Japanese quail exposed to TCDF, respectively (Table 3). In contrast, the ReS values for PeCDF were similar (within twofold) for all the three species, regardless of whether EC<sub>50</sub> or EC<sub>threshold</sub> values were used to compare EROD curves. Thinking retrospectively, it is probably not surprising that alterations of critical amino acids in the ligand-binding domain are able to affect ligand-binding specificity (e.g., PeCDF vs. TCDD) and general binding affinity. The amino acids within and near the ligand-binding domain determine the size and polarity of the domain as well as its shape and plasticity (Baldwin *et al.*, 1998; Pandini *et al.*, 2009).

The molecular mechanism(s) underlying the reason(s) why PeCDF is a more potent inducer of EROD activity and *CYP1A4/CYP1A5* mRNA expression than TCDD in some species and why hepatocytes from the three species were approximately equisensitive to EROD and *CYP1A4/CYP1A5* mRNA induction is not yet known, but the data presented here allow for the testing of several hypotheses. For example,

variation in potency among compounds might be partially explained by the binding affinity of the ligand to the AHR—the greater the binding affinity, the greater the potency (Hestermann *et al.*, 2000; Poland and Knutson, 1982; Safe, 1986). Therefore, in ring-necked pheasant and Japanese quail, PeCDF might have a greater binding affinity to AHR1 than TCDD. It would also be interesting to investigate the influence of four additional amino acids within the ligand-binding domain that are of interest (Head *et al.*, 2008) on AHR1-binding affinity and *in vitro* expression of AHR1-dependent responses for PeCDF, TCDD, and other DLCs in avian species. However, factors other than AHR binding should also be considered in future studies. For example, differences in the ReP of PCB 126 to induce or repress a number of genes in rat (ReP was 0.06) and human (ReP was 0.002) hepatocytes could not be explained by differences in their AHR-binding affinity (Carlson *et al.*, 2009). The authors suggested that intrinsic efficacy and events downstream of AHR binding (e.g., trans-activation) could be involved in the differences in ReP among rats and humans. Among other suggestions is the possibility of differences in ligand-specific AHR coactivator interactions among species (Zhang *et al.*, 2008). It is possible that in the ring-necked pheasant and Japanese quail, the interactions between the PeCDF-AHR1 complex and the coactivators could lead to greater CYP1A responses than the interactions with the TCDD-AHR1 complex. On the other hand, the interactions of PeCDF-AHR1 and TCDD-AHR1 with coactivators could lead to similar CYP1A responses in the chicken.

ReP and ReS values are often calculated by comparing the EC<sub>50</sub> values from EROD concentration-response curves. Because there is competition between the substrate (ethoxyresorufin) and the inducer (the DLC) for the CYP1A enzyme (Petrucci and Bunce, 1999), a decrease in EROD activity is observed at greater concentrations of DLCs. The inhibition of EROD activity can, in certain situations (mainly with weak AHR ligands), decrease the maximal activity. As such, there is a leftward shift of the EC<sub>50</sub>, creating an overestimation of the potency of a compound (Hahn *et al.*, 1993; Head and Kennedy, 2007b; Hestermann *et al.*, 2000). A less biased estimate can be derived from the threshold concentration for effect (EC<sub>threshold</sub>), which, as mentioned before, represents the concentration of a compound to produce a response equivalent to 10% of the maximal response produced by TCDD in the same species (Kennedy *et al.*, 1996). In this study, RePs and ReSs calculated with EC<sub>50</sub> and EC<sub>threshold</sub> were similar and the major conclusions are not changed by the measure of potency used. The results of using both approaches were included to allow careful examination of the data by others. Maximal EROD activity for the three compounds was lower in Japanese quail hepatocytes compared to chicken and ring-necked pheasant hepatocytes, but the reason for differences in maximal activity among species is not known. It should be noted that lower maximal EROD activity in Japanese quail hepatocyte cultures was also observed in other studies with this species (results not shown).

Both *CYP1A4* and *CYP1A5* mRNA were induced in a concentration-dependent manner by TCDD, PeCDF, and TCDF in the three species, but differences in maximal induction were observed within and among species. *CYP1A4* mRNA was upregulated more than *CYP1A5* mRNA in all species. It was previously shown that in chicken hepatocytes exposed to TCDD, *CYP1A4* mRNA was preferentially upregulated relative to *CYP1A5* mRNA; however, in herring gull hepatocytes, *CYP1A5* mRNA was more highly induced by TCDD than *CYP1A4* mRNA (Head and Kennedy, 2007a,b). The authors showed that the isoform that had the lower constitutive expression was preferentially induced (Head and Kennedy, 2007a). It is worth noting that, in the present study, the differential expression of *CYP1A4* mRNA and *CYP1A5* mRNA is greater in chicken hepatocytes (up to 10-fold) than in ring-necked pheasant (less than two fold) and Japanese quail hepatocytes (~two fold). The maximal fold induction of *CYP1A4/CYP1A5* mRNA was greater in chicken than in ring-necked pheasant and Japanese quail. Differences in the maximal responses of mRNA induction had been observed previously between chicken and herring gull (*Larus argentatus*) hepatocytes treated with TCDD (Head and Kennedy, 2007a). The investigators hypothesized that TCDD could regulate mRNA half-life differently among species. They tested this hypothesis by tracking the decay of relative transcript quantity at different time points 24 h after the addition of actinomycin D but did not find strong evidence for half-life regulation by TCDD in chicken or herring gull.

Finally, as shown in a previous study with chicken hepatocytes (Head and Kennedy, 2007b), EROD activity and *CYP1A4/CYP1A5* mRNA expression can be used interchangeably to predict potency of DLCs *in vitro*. Indeed, concentration-response curves of the three end points have similar curve slopes and EC<sub>50</sub> values for each compound in each species. It is therefore unlikely that the use of any of the three end points would lead to large differences in estimates of ReP or ReS among species. The EROD assay is cost- and time-effective and can be applied to virtually all species, unlike the *CYP1A4/CYP1A5* assay where experimental conditions must be optimized for each species. However, the *CYP1A4/CYP1A5* mRNA assay has lower detection limits and provides useful information when a limited amount of tissue is available.

In summary, this study demonstrated that TCDD, PeCDF, and TCDF were equipotent EROD and *CYP1A4/CYP1A5* mRNA inducers in chicken embryo hepatocyte cultures. However, PeCDF was a more potent inducer than TCDD in ring-necked pheasant and Japanese quail hepatocytes. The rank order of sensitivity among the three avian species when exposed to TCDD and TCDF was chicken > ring-necked pheasant > Japanese quail, but when exposed to PeCDF, the three species were essentially equisensitive to EROD and *CYP1A4/CYP1A5* mRNA induction. The results obtained in this study raise interesting questions about species-specific responses to DLCs.

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