2,3,4,7,8-PENTACHLORODIBENZOFURAN IS A MORE POTENT CYTOCHROME P4501A INDUCER THAN 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN IN HERRING GULL HEPATOCYTE CULTURES

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Abstract—Concentration-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) on cytochrome P4501A (CYP1A) induction were determined in primary cultures of embryonic herring gull (*Larus argentatus*) hepatocytes exposed for 24 h. Based on the concentration that induced 50% of the maximal response (EC50), the relative potencies of TCDD and TCDF did not differ by more than 3.5-fold. However, also based on the EC50, PeCDF was 40-fold, 21-fold, and 9.8-fold more potent for inducing ethoxyresorufin-*O*-deethylase (EROD) activity, CYP1A4 mRNA expression, and CYP1A5 mRNA expression than TCDD, respectively. The relative CYP1A-inducing potencies of PeCDF and of other dioxin-like chemicals (DLCs) in herring gull hepatocytes (HEH RePs), along with data on concentrations of DLCs in Great Lakes herring gull eggs, were used to calculate World Health Organization toxic equivalent (WHO-TEQ) concentrations and herring gull embryonic hepatocyte toxic equivalent (HEH-TEQ) concentrations. The analysis indicated that, when using avian toxic equivalency factors (TEFs) recommended by the WHO, the relative contribution of TCDD (1.1–10.2%) to total WHO-TEQ concentration was higher than that of PeCDF (1.7–2.9%). These results differ from the relative contribution of TCDD and PeCDF when HEH RePs were used; PeCDF was a major contributor (36.5–52.9%) to total HEH-TEQ concentrations, whereas the contribution by TCDD (1.2–10.3%) was less than that of PeCDF. The WHO TEFs for avian species were largely derived from studies with the domestic chicken (*Gallus gallus domesticus*). The findings of the present study suggest that it is necessary to determine the relative potencies of DLCs in wild birds and to re-evaluate their relative contributions to the biochemical and toxic effects previously reported in herring gulls and other avian species. Environ. Toxicol. Chem. 2010;29:2088–2095. © 2010 SETAC

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INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and some polychlorinated biphenyls (PCBs) are referred to as dioxin-like chemicals (DLCs) because 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and DLCs elicit similar toxic and biochemical effects [1–3]. Adverse effects of DLCs in birds have been observed in both the laboratory [2,4–6] and the environment. During the early 1970s, signs of toxicity in colonial fish-eating birds of the Great Lakes of North America, including the herring gull, *Larus argentatus*, were associated with high levels of DLCs and other organochlorine contaminants [7]. Adverse effects included embryonic and chick mortality, developmental abnormalities, liver impairment, edema, and porphyria [7,8].

The molecular mechanisms underlying the toxic effects of DLCs are not fully understood, but various studies indicate that most effects occur subsequent to activation of the aryl hydrocarbon receptor (AHR), which is a ligand-activated transcription factor [2,9,10]. It is worth noting that the numbers and functions of AHRs vary among taxa. Although mammalian species appear to possess a single AHR, at least two forms of the AHR occur in birds and fishes, AHR1 and AHR2 [11]. In birds, AHR1 is the most transcriptionally active form, but in fishes AHR2 is the dominant form [11,12]. In the present study, the term AHR1 is used for details about birds, but the term AHR is used for other taxa and for general comments. After they are bound to the AHR, DLCs cause several biochemical responses, including the induction of cytochrome P4501A (CYP1A). The relationship between CYP1A induction and the occurrence of toxic effects is not entirely clear [13,14], but the relative potency of DLCs to up-regulate CYP1A expression is predictive of the relative toxicity of DLCs in avian embryos [4,15–17]. Dioxin-like chemicals differ in toxicity and biochemical potency, with TCDD generally considered to be the most potent [3,15,16,18,19]. In addition to the wide range of potencies observed among DLCs, avian species also differ in sensitivity. For example, the herring gull is over 200 times less sensitive than the chicken to the embryotoxic effects of 3,3′,4,4′-tetrachlorobiphenyl polychlorinated biphenyl (PCB 77) [20].

Differences in both potencies of DLCs and species sensitivity complicate hazard and risk assessments. These complexities are compounded by the fact that DLCs exist as mixtures in the environment. Furthermore, the relative concentrations of individual congeners change with time and among trophic levels [21]. To address these complexities, the World

Health Organization (WHO) suggested the formal application of toxic equivalency factors (TEFs) for risk and hazard assessments, wherein TEFs are applied to individual congeners to calculate the overall toxic equivalent (TEQ) concentrations in environmental matrices [3]. The TEF is a value that represents the relative toxicity of individual DLC congeners compared with TCDD. The total TEQ concentration is the sum of the product of concentrations of individual congeners multiplied by their TEF values. The WHO TEFs are derived by consensus and are based on a number of both in vivo and in vitro responses to DLCs. The most recent WHO assessment that included avian species concluded that TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) are equipotent DLCs in birds. As such, each compound was assigned a TEF of 1.0 [3]. The term WHO-TEQ is used in the present study when referring to TEQ concentrations calculated with the TEF values that were derived in the WHO assessment.

It was recently discovered that PeCDF is three to 27 times more potent than TCDD as an inducer of CYP1A in primary hepatocyte cultures prepared from ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) embryos [22,23] and that PeCDF is also approximately 5.5 times more potent than TCDD in causing lethality in ring-necked pheasant and Japanese quail embryos (Cohen-Barnhouse et al., unpublished data). These findings, along with studies on the molecular mechanisms that underlie the reasons for species differences in sensitivity to DLCs [20,24], stimulated us to conduct studies to determine the relative potencies of TCDD, PeCDF, and TCDF in wild species of birds. Some of this work is conducted with primary hepatocyte cultures because CYP1A data obtained from cultured hepatocytes are predictive of toxic effects of DLCs that occur in ovo [15,17].

In the present study, primary cultures of embryonic herring gulls were exposed to graded concentrations of TCDD, PeCDF, and TCDF to determine the potencies of these compounds as CYP1A inducers. An earlier report [25] indicated that PeCDF was a more potent ethoxyresorufin-*O*-deethylase (EROD) inducer than TCDD in herring gull hepatocytes. A followup study was needed to determine the relative potencies of PeCDF on both EROD activity (a measure of CYP1A catalytic activity) and CYP1A4/5 mRNA expression. The present study confirms the conclusions of the earlier report: PeCDF is indeed more potent than TCDD as an EROD inducer. In addition, PeCDF is also more potent than TCDD at inducing CYP1A4/5 mRNA expression in herring gull hepatocytes. The present study differs from the earlier study in the following respects. First, the earlier study examined effects on EROD activity in hepatocytes prepared from individual birds; the present study was conducted with hepatocytes prepared from a pool of sixty birds. Second, in the present study, compounds were administered as dimethyl sulfoxide (DMSO) solutions rather than as isooctane solutions, thus allowing for reliable comparison of the present work with numerous other studies, including the recent study with chicken, ring-necked pheasant and Japanese quail hepatocytes [22]. Third, the present study includes, for the first time, a discussion on the possible implications to environmental risk assessments of the finding that PeCDF is more potent than TCDD as a CYP1A inducer in herring gulls and other avian species with a similar AHR genotype. Because the herring gull has been used for approximately 40 years to monitor the levels and effects of DLCs in the Great Lakes [26], data on the relative sensitivity of this species to PeCDF are needed for current and retrospective analyses of the causality of effects of DLCs in birds [27].

MATERIALS AND METHODS

Source of eggs and incubation conditions

Unincubated herring gull eggs were collected from nests containing one egg on April 28, 2009, from Chantry Island, Lake Huron (44°29′22″N, 81°24′7″W), which is a Great Lakes reference colony. Eggs were transported from the field in insulated coolers to the laboratory at the National Wildlife Research Center and artificially incubated at 37.5°C and 60% relative humidity for 26 d (1–2 d prehatch). Eggs were candled periodically; infertile eggs and eggs that contained dead embryos were discarded. All field and laboratory procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Center. It should be noted that exposure of wild bird eggs to contaminants during their embryonic development (through maternal transfer) could theoretically affect CYP1A responses measured in vitro. However, based on previous measurements of concentrations of residues in eggs from Chantry Island, these residues are not expected to influence the half-maximal (EC50) CYP1A responses [28].

Preparation and dosing of cultured hepatocytes

Primary hepatocyte cultures were prepared from 26-d-old herring gull embryos using previously described methods [29]. Reagents were from Sigma, unless another supplier is indicated. In brief, 60 embryos were decapitated, and their livers were removed, pooled, and digested with collagenase. Percoll (Amersham Bioscience) was used to separate hepatocytes from erythrocytes and DNAse (Roche) was used to prevent the clumping of cells [30]. Hepatocytes were plated in 48-well culture plates that contained 500 μl of cell culture medium 199 supplemented with insulin (1 μg/ml) and thyroxine (1 μg/ml). Hepatocytes were then incubated for 24 h at 37°C in a humidified incubator with 5% CO_2 prior to the addition of TCDD, PeCDF, or TCDF. For each compound, EROD assays were carried out in triplicate plates (three plates per compound), and mRNA expression was measured in a single plate (one plate per compound). In each plate, the hepatocytes were treated with triplicate concentrations, with in-well concentrations ranging from 0.003 to 100 nM (TCDD and TCDF) or from 0.0003 to 100 nM (PeCDF; 2.5 μl chemical/well in DMSO) and incubated for 24 h. Cell culture medium was removed, and plates were flash frozen on dry ice and stored at −80°C until they were analyzed for CYP1A4/5 mRNA expression.

Preparation of TCDD, PeCDF, and TCDF solutions

A detailed description of the preparation of TCDD, PeCDF, and TCDF solutions has been provided by Herve et al. [22]. In brief, serial dilutions of TCDD, PeCDF, and TCDF were prepared from DMSO stock solutions with nominal concentrations ranging from 650 to 1,635 nM. Identification and quantification of TCDD, PeCDF, and TCDF in stock solutions were determined by isotope dilution following U.S. Environmental Protection Agency method 1613 [31] by use of high-resolution gas chromatography-mass spectrometry.

EROD assays

The EROD assays were conducted as described previously [28,32]. All reagents were from Sigma. In brief, hepatocytes in 48-well plates were incubated at 37.5°C in the presence of nicotinamide adenine dinucleotide phosphate (NADPH, reduced) and 7-ethyoxyresorufin for 7 min. The EROD reaction was stopped with the addition of cold acetonitrile that contained

fluorescamine (0.15 mg/ml). Resorufin and protein standard curves were prepared for each assay [32]. Plates were analyzed for EROD activity and total protein content using a fluorescence plate reader (Cytofluor 2350; Millipore).

RNA isolation and cDNA synthesis

RNeasy 96 kits (Qiagen) were used to extract total RNA from 48-well plates using protocols provided by the manufacturer. On-column DNAse treatment was performed by use of 50% ethanol solution for RNA isolation instead of the suggested 70%, because earlier studies in our laboratory indicated that greater yields of RNA are obtained with 50% ethanol. Total RNA was treated a second time with DNAse from the Ambion DNA-free kit, according to manufacturer's instructions to ensure maximal removal of genomic DNA. Total RNA (11.5 µl from each well) was reverse transcribed to complementary DNA (cDNA) with Superscript II and random hexamers (Invitrogen) according to the manufacturer's instructions. For each 48-well 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-polymerase chain reaction

A multiplex quantitative reverse transcription-polymerase chain reaction (QPCR) assay with dual-labeled fluorescent hydrolysis probes was used to quantify herring gull CYP1A4, CYP1A5, and β-actin (normalizer gene) mRNA abundance using the Stratagene Mx3000P instrument. All primers (Invitrogen) and probes (Biosearch Technologies, Novato) were developed and validated as previously described [33]. Brilliant QPCR Core Reagent kits (Stratagene) were used to conduct each assay. Each 25-µl reaction contained forward and reverse primers and probes of the genes of interest (CYP1A4 and CYP1A5) and the normalizer gene (see Head and Kennedy [33] for concentrations), $1\times$ PCR buffer, 5 mM $MgCl_2$, 0.8 mM deoxynucleoside triphosphate (dNTPs), 0.08 v/v glycerol, 0.05 U SureStart Taq polymerase, and 25 nM reference dye (ROX). The thermal profile for all reactions was as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Data collection was at the end of each 60°C phase. Standard curves were produced from a 1:2 dilution series of concentrated cDNA, covering two orders of magnitude. Quantification of CYP1A4/5 mRNA -fold induction by TCDD, PeCDF, and TCDF was assessed using the $2^{-\Delta\Delta Ct}$ method [34].

Cell viability

Cell viability was determined by using the calcein-AM assay (Invitrogen-Molecular Probes). 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 calcein-AM (1 mg/ml) to 10 ml phosphate-buffered saline–ethylene diaminetetraacetic acid (PBS-EDTA). The culture medium was removed, and 200 µl of the calcein-acetoxymethyl ester (calcein-AM) solution was added to each well. Plates were incubated in the dark for 45 min, and fluorescence was measured using a Cytofluor 2350 fluorescence plate-reader with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

EROD and CYP1A4/5 mRNA data analysis

Fluorescence data from the EROD assay were imported into GraphPad Prism 5.0. The EROD concentration–response data were fit to a modified Gaussian curve as described elsewhere [35]. For each compound, three EROD curves were generated from data originating from separate cell culture plates. The EC50 and maximal response values were derived from the curve fits and are presented as the mean value of the three replicate plates ± standard error. Statistical differences for EC50 and maximal response values for EROD activity were determined by using a one-way analysis of variance with a Bonferroni correction. Significance was set at $p < 0.05$ for all tests. CYP1A4/5 mRNA expression data were fitted to a four-parameter logistic model as described previously [29]. One plate was used for each compound, and one curve fit was generated for each plate using data from the average of three wells, assessed in duplicate. The EC50 and maximal response values are the values calculated from the curve fit ± standard error. No statistical test could be performed for mRNA data, because only one EC50 value per compound was obtained.

Calculation of relative potencies and TEQ concentrations

The concept of using relative potency (ReP) values to compare the potencies of DLCs is widely known [3]. The ReP is the comparison of CYP1A-inducing potency of a compound relative to the CYP1A-inducing potency of TCDD in the same species (herring gull in this case). In the present study, RePs are defined as [EC50$_{TCDD}$]/[EC50$_{PeCDF}$] for PeCDF and [EC50$_{TCDD}$]/[EC50$_{TCDF}$] for TCDF. It is important to note that total WHO-TEQ concentration, as defined by the WHO, has a meaning different ftom total herring gull embryonic hepatocyte toxic equivalent (HEH-TEQ). Specifically, WHO-TEQ is the total contribution of DLCs to the potency of a mixture based on the consensus TEF values established and agreed to by the WHO [3]; HEH-TEQ is the total contribution of DLCs to the potency of a mixture based on herring gull embryonic hepatocyte (HEH) ReP values. We used CYP1A4 mRNA RePs rather than the EROD RePs to analyze the data in the present study, because PeCDF elicited an unusually high EROD maximal response that might have affected the EROD EC50 for this compound (see Discussion for details). Total concentrations of WHO-TEQ and HEH-TEQ were calculated as the sum of the products of the concentration of each compound measured in herring gull eggs collected in 2001 [36] by WHO TEF values reported by Van den Berg et al. [3] for WHO-TEQ concentrations or HEH ReP values reported in the present study for PeCDF and TCDF in combination with HEH ReP values for PCBs reported by Kennedy et al. [15] for HEH-TEQ concentrations.

RESULTS

Characteristics of the concentration–response curves

EROD activity. TCDD, PeCDF, and TCDF caused concentration-dependent induction of EROD activity. After reaching a maximum EROD activity, greater concentrations resulted in lesser EROD activity (Fig. 1). The decrease in EROD activity at greater concentrations of TCDD, PeCDF, and TCDF was not due to overt toxicity (determined by the calcein-AM assay; data not shown). This decrease in activity at the greatest concentrations of DLC is due to the competitive inhibition of the reaction by the DLC itself [37]. Maximal EROD induction by PeCDF was significantly greater than the maximal induction elicited by TCDD and TCDF (Fig. 1 and Table 1). The EROD activity was normalized on a per well basis rather than per total protein concentration (the usual protocol) for reasons discussed below.

CYP1A4/5 mRNA induction. CYP1A4/5 mRNA expression was up-regulated by TCDD, PeCDF, and TCDF in a
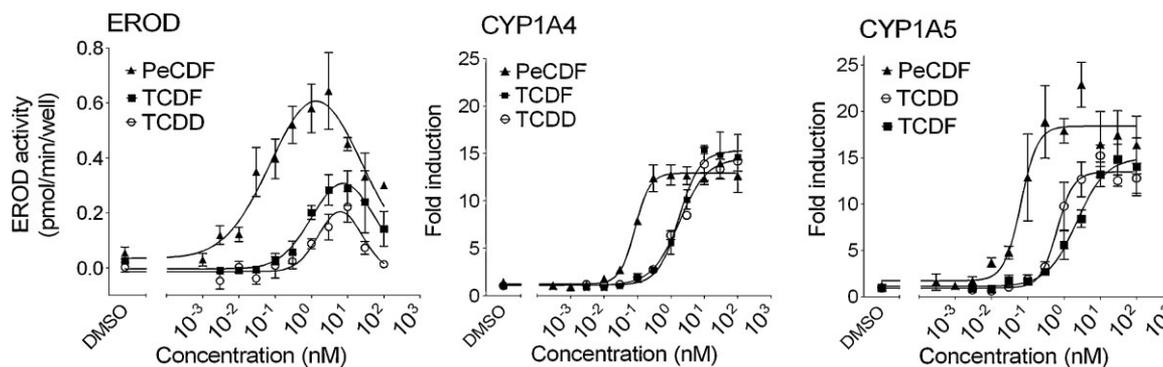
Fig. 1. Concentration-dependent effects of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF; triangles), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; circles), and 2,3,7,8-tetrachlorodibenzofuran (TCDF; squares) on ethoxyresorufin-*O*-deethylase (EROD) activity, cytochrome P450 1A (CYP1A4), and CYP1A5 messenger RNA (mRNA) expression in herring gull hepatocyte cultures exposed for 24 h. For EROD activity, triplicates of cell culture plates were exposed to each compound, and each point represents the mean value obtained from three different cell culture plates. For mRNA expression, one cell culture plate was exposed for each compound, and each point represents the mean value obtained from three different wells from the same cell culture plate. Values before the axis break indicate response observed for control (dimethyl sulfoxide [DMSO]-treated hepatocytes). Bars represent standard errors.

concentration-dependent manner. Expression of mRNA reached a plateau at the greatest concentrations of each compound (Fig. 1). CYP1A4 mRNA and CYP1A5 mRNA expression were both induced to similar maximal levels by TCDD, PeCDF, and TCDF (<1.2-fold and 1.4-fold differences, respectively; Table 1). No amplification of β-actin, CYP1A4, or CYP1A5 was observed in no-RT controls or no-template controls, which confirmed the absence of contamination. Therefore, changes in mRNA expression were a result of changes in CYP1A4 and CYP1A5 mRNA expression and were not due to changes in β-actin mRNA (the normalizer gene) expression.

Relative potencies

For all three indicators of AHR activation (EROD activity, CYP1A4 and CYP1A5 mRNA expression), the EC50s for PeCDF were less than the EC50s for TCDD, whereas the EC50s for TCDD and TCDF were similar (Table 1). Thus, the ReP values for PeCDF were 40, 21, and 9.8, based on EROD activity and CYP1A4 mRNA and CYP1A5 mRNA expression, respectively. The ReP values for TCDF were 2.0, 1.0, and 0.3, based on EROD activity and CYP1A4 mRNA and CYP1A5 mRNA expression, respectively (Table 1). Overall, the rank order of potency of the compounds was PeCDF > TCDD ~ TCDF.

DISCUSSION

The results of the present study indicate that the CYP1A mRNA induction potencies of TCDD and TCDF were similar in herring gull hepatocytes (less than 3.5-fold different). This finding is similar to findings of an earlier study that determined the EROD induction potencies of TCDD and TCDF [15]. The discovery that the CYP1A induction potency of PeCDF was greater than the potency of TCDD is also consistent with the results of an earlier study by Sanderson et al. [25] that measured EROD induction in herring gull embryo hepatocytes. The study by Sanderson and colleagues determined the relative potencies of PeCDF and TCDD in hepatocyte cultures prepared from single embryos (*n* = 2), whereas, in the present study, a pool of hepatocytes prepared from 60 embryos was used. The ReP values in the Sanderson et al. [25] study (1.4–4) were smaller than the ones reported here (21- and 9.8-fold) for CYP1A4 mRNA and CYP1A5 mRNA, respectively. Differences in details of the methods could explain the differences in the actual RePs obtained in the two studies; these differences might reflect differences among individual herring gulls but could also be due to technical differences between the two studies. For example, the present study used DMSO as the carrier solvent, whereas Sanderson et al. used isooctane. A discussion of the potential effects of different solvents is provided by Sanderson et al. [25]. It should be noted that a greater potency of PeCDF

Table 1. Effective concentration 50 (EC50), relative potency (ReP), and maximal response for ethoxyresorufin-*O*-deethylase (EROD) activity, cytochrome P4501A4 (CYP1A4), and CYP1A5 messenger RNA expression determined in herring gull hepatocyte cultures exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and 2,3,7,8-tetrachlorodibenzofuran (TCDF) for 24 h[a]

Compound	Endpoint	EC50 (nM)	ReP	Maximal response (EROD: pmol/min/well, CYP1A4/5: -fold change)
TCDD	EROD	1.61 ± 0.6 A	1.0	0.20 ± 0.03 A
PeCDF	EROD	0.04 ± 0.01 B	40.0	0.61 ± 0.04 B
TCDF	EROD	0.79 ± 0.3 A	2.0	0.31 ± 0.03 A
TCDD	CYP1A4	1.80 ± 0.37	1.0	15 ± 0.7
PeCDF	CYP1A4	0.084 ± 0.012	21.0	13 ± 0.3
TCDF	CYP1A4	1.74 ± 0.37	1.0	15 ± 0.8
TCDD	CYP1A5	0.64 ± 0.1	1.0	13 ± 0.6
PeCDF	CYP1A5	0.065 ± 0.02	9.8	18 ± 0.8
TCDF	CYP1A5	2.23 ± 0.7	0.3	15 ± 1.1

[a] Data for each compound are the average values derived from three replicate cell culture plates ± standard error (EROD data) or values derived from one cell culture plate ± standard error of the curve fit (CYP1A4/5 mRNA data). EROD data were fit to a modified Gaussian curve, and CYP1A4/5 mRNA expression data were fit to a four-parameter logistic curve. Capital letters indicate significant differences (*p* < 0.05). Statistical tests could not be performed on the mRNA data, because only one curve fit was generated, so only one EC50 value was derived for each dioxin-like chemical.

relative to TCDD was also reported in hepatocytes prepared from double-crested cormorant (*Phalacrocorax auritus*; 10–13-fold*)*, Forster's tern (*Sterna forsteri*; 10–15-fold), ring-necked pheasant (3–4.5-fold), Japanese quail (13–27-fold), green frog (*Rana esculenta*; 10–11-fold), and zebrafish (*Danio renio*; 6–10-fold) [22,25,38].

The EROD activity in avian hepatocyte cultures is typically expressed relative to the total protein concentration within cell culture wells [15,25,29], but this was not possible because of a minor difficulty that occurred with the herring gull hepatocytes. The hepatocytes could not be rinsed with PBS prior to freezing (the normal protocol), because examination under the microscope suggested that the cells may not have been firmly attached to the bottoms of the wells of the 48-well plates. This is a phenomenon that we have observed with hepatocyte cultures prepared from a few other species of wild birds (e.g., osprey [*Pandion haliaetus*; unpublished results]), and we did not want to risk losing the cells with the PBS wash. Therefore, unequal volumes of medium remained in each well. Amino acids present in the residual medium would alter apparent total protein measurements, because fluorescamine reacts with the NH_2 groups of free amino acids present in cell culture medium [32,39]. This being the case, the higher maximal EROD activity elicited by PeCDF, compared with TCDD and TCDF, likely is because concentrations of rather loosely attached hepatocytes were slightly higher in the plates treated with PeCDF than in the plates treated with TCDD and TCDF. This hypothesis is supported insofar as the three compounds induced CYP1A4 and CYP1A5 mRNA to similar maximal levels (the issue regarding the PBS rinse has no impact on mRNA analysis). Moreover, in chicken, ring-necked pheasant, and Japanese quail hepatocytes, TCDD, PeCDF, and TCDF induced similar maximal level of EROD activity and CYP1A4 and CYP1A5 mRNA within each species [22], and maximal EROD induction in the livers of chicken embryos injected with PeCDF and TCDD were similar [40].

Because avian species differ in sensitivity to DLCs [20], it was of interest to compare results of the present study with previous findings in other species. The difference in sensitivity among avian species has been attributed, in part, to the identity of specific amino acids within the AHR1 ligand binding domain [20,24]. The amino acid residues corresponding to Ile324/Ser380 in the chicken were compared among several avian species, and it was found that their identity was predictive of broad categories of DLC sensitivity among species [20]. The herring gull and Japanese quail have the Val324/Ala380 genotype, which is associated with their lower sensitivity to embryotoxic and biochemical responses to most DLCs. The present study, with herring gull hepatocytes, and a previous study, with Japanese quail hepatocytes [22], found a lower sensitivity of these species to CYP1A induction by TCDD and TCDF compared with the chicken. However, when hepatocytes were exposed to PeCDF, the sensitivity of the herring gull and Japanese quail to CYP1A induction was similar to that of chicken [22]. A similar change in rank order of sensitivity to CYP1A5 mRNA and EROD induction was observed in livers of posthatched chicken and Japanese quail exposed in ovo to TCDD, PeCDF, and TCDF [41]. The reason that chicken, Japanese quail, and herring gull hepatocytes have a similar sensitivity to CYP1A induction when exposed to PeCDF is not known. It is important to note that a similar situation was observed in embryotoxicity studies with Japanese quail. The dose of TCDD that caused 50% mortality (LD50) in Japanese quail embryos was approximately 43 times higher

than the TCDD LD50 of the chicken. However, the PeCDF LD50 in Japanese quail embryos was approximately seven times higher than the PeCDF LD50 chicken embryos (Cohen-Barnhouse et al., unpublished data). To our knowledge, no embryotoxicity study has been made of herring gull embryos exposed to PeCDF in the laboratory; such a study is warranted.

The present study and previous studies showed that PeCDF is a more potent CYP1A inducer than TCDD in hepatocytes of at least three avian species with the Val/Ala genotype (the least sensitive genotype): the double-crested cormorant, the herring gull, and the Japanese quail [22,25]. It is intriguing that, in fish species, a similar situation appears to exist; PeCDF is more potent than TCDD in zebrafish. Zebrafish is the least sensitive piscine species to TCDD toxicity based on egg mortality [42], and PeCDF is a more potent CYP1A-inducer than TCDD in zebrafish hepatocytes [38]. Further studies to determine the mechanisms associated with differences in piscine species to DLCs should be considered. However, when studying species differences in sensitivity to DLCs, it is important also to consider factors other than the 324/380 amino acid residues to address the differences in sensitivity to CYP1A induction and toxicity. For example, differences occur in the rates of DLC metabolism [20], and four additional amino acid of interest in avian AHR1 [20] may have effects on the binding properties of DLCs.

This recent contribution along with the work by Sanderson et al. [25] on the relative potencies of TCDD, PeCDF, and TCDF in herring gull hepatocytes brings into question both current and previous assessments of the relative contributions of DLCs in herring gull eggs. Environment Canada has been measuring the levels of contaminants in herring gull eggs for the past 40 years [26,36]. Here we compare the relative contribution of TCDD, PeCDF, TCDF, PCB 77, 2,3,3',4,4'-pentachlorobiphenyl (PCB 105), 2,3',4,'4,5-pentachlorobiphenyl (PCB 118), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169) in a subset of herring gull eggs collected from 13 colonies on the Great Lakes in 2001. The year 2001 was

Table 2. World Health Organization toxic equivalency factor (WHO TEF) and herring gull embryonic hepatocyte relative potency (HEH ReP) of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF), 3,3',4,4'-tetrachlorobiphenyl (PCB 77), 2,3,3',4,4'-pentachlorobiphenyl (PCB 105), 2,3',4,4',5-pentachlorobiphenyl (PCB 118), 3,3',4,4',5-pentachlorobiphenyl (PCB 126), and 3,3',4,4',5,5'-hexachlorobiphenyl (PCB 169)

Compound	WHO TEF[a]	HEH ReP
TCDD	1	1[b]
PeCDF	1	21[b]
TCDF	1	1[b]
PCB 77	0.05	0[c,d]
PCB 105	0.0001	0[c,d]
PCB 118	0.00001	0[c,d]
PCB126	0.1	0.06[c]
PCB 169	0.001	0.07[c]

[a] WHO TEFs are from Van den Berg et al. [3].
[b] The HeH ReP values for TCDD, PeCDF, and TCDF are from the present study (Table 1), and are based on CYP1A4 mRNA induction. CYP1A4 is the gene that is associated with EROD activity, and the CYP1A4 mRNA was used for the HEH RePs for the reason explained in the Discussion.
[c] The HEH RePs for the PCB congeners are from Kennedy et al. [15].
[d] Neither PCB 77, nor PCB 105, nor PCB 118 caused EROD induction at the highest concentrations (3×10^3 to 1×10^4) administered to the cells, so they were assigned HEH RePs of 0 rather than <0.0003 (PCB 77) or <0.00009 (PCB 105 and PCB 118) as indicated by Kennedy et al. [15], because we now believe that RePs of 0 more correctly represent the findings of that study.

chosen because it is the most recent year for which data are available for all the DLCs listed in Table 2. The WHO TEF and the HEH ReP values are consistent for some compounds (TCDD, TCDF, and PCB 126), but they vary greatly (ninefold and over) for other compounds (PeCDF, PCB 77, PCB 105, PCB 118, and PCB 169; Table 2). Therefore, we decided to compare the relative contribution of these compounds to the total WHO-TEQ concentration and total HEH-TEQ concentrations. For total WHO-TEQ concentrations, PCB 126 was the major contributor in all colonies (70.1–82.3%), whereas TCDD (1.1–10.2%), PeCDF (1.7–2.9%), and TCDF (0.3–0.7%) were smaller contributors (Table 3). However, for total HEH-TEQ concentrations, the contribution of PCB 126 decreased (36.7–48.2%), whereas the contribution of PeCDF increased substantially (36.5–52.9%). The contributions of TCDD (1.2–10.3%) and TCDF (0.2–0.7%) to HEH-TEQ and WHO-TEQ concentrations were similar.

The observed differences in percentage contribution to total TEQs in herring gull eggs when using HEH RePs rather than

WHO TEFs may suggest alteration of the rank order of the DLCs that were most important for causing previously reported embryotoxic effects in herring gulls and other colonial fish-eating birds in the Great Lakes [27,43]. It would be instructive to apply the HEH ReP values to concentrations of DLCs measured in herring gull eggs in the 1970s to re-evaluate the possible contribution of PeCDF to the HEH-TEQ in the period when signs of overt toxicity such as chick mortality and developmental abnormalities were observed. It should also be noted that Sanderson et al. [25] found 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD) to be a more potent EROD inducer than TCDD in herring gull hepatocytes; therefore, further studies with PeCDD and other DLCs are also warranted in herring gulls.

In summary, the present study indicated that PeCDF was a more potent CYP1A inducer than TCDD in herring gull hepatocytes. It also demonstrated that, based on HEH RePs, PeCDF and PCB 126 were the two main contributors to the HEH-TEQ concentrations in herring gull eggs of the Great Lakes in 2001.

Table 3. Concentrations (pg/g wet weight) of eight dioxin-like chemical (DLCs) in herring gull eggs collected from five colonies in the Great Lakes in 2001 (top) compared with World Health Organization (WHO) toxic equivalent (TEQ) concentrations and herring gull embryonic hepatocytes (HEH) TEQ concentrations (center) and the relative concentrations of the eight DLCs to total WHO-TEQs and HEH-TEQs (bottom; see Table 2 for definition of compound acronyms)

	Concentration (pg/g wet weight)[a]				
Compound	Granite	Channel Shelter	Gull	Fighting	Snake
TCDD	8.1	36.6	3.6	8.7	25.6
PeCDF	4.1	24.6	5.4	5.6	4.3
TCDF	0.4	2.9	1.1	1.6	NA
PCB 77	92	1,205	782	405	159
PCB 105	86,000	1,041,000	259,000	173,000	172,000
PCB 118	195,000	2,547,000	584,000	463,000	420,000
PCB 126	1,304	5,967	2,334	1,654	1,926
PCB 169	261	889	465	218	232

	WHO-TEQ or HEH-TEQ[b] (pg/g wet weight)									
	Granite		Channel Shelter		Gull		Fighting		Snake	
	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ
TCDD	8.1	8.1	36.6	36.6	3.6	3.6	8.7	8.7	25.6	25.6
PeCDF	4.1	86.1	24.6	516.6	5.4	113.4	5.6	117.6	4.3	90.3
TCDF	0.4	0.4	2.9	2.9	1.1	1.1	1.6	1.6	NA	NA
PCB 77	4.60	0[c]	60.3	0[c]	39.1	0[c]	20.3	0.0[c]	8.0	0[c]
PCB 105	8.6	0[c]	104.1	0[c]	25.9	0[c]	17.3	0.0[c]	17.2	0[c]
PCB 118	2.0	0[c]	25.5	0[c]	5.8	0[c]	4.6	0.0[c]	4.2	0[c]
PCB 126	130.4	78.2	596.7	358.0	233.4	140.0	165.4	99.2	192.6	115.6
PCB 169	0.3	18.3	0.9	62.2	0.5	32.6	0.2	15.3	0.2	16.2
Total TEQ	158.4	191.1	851.5	976.4	314.8	290.7	223.7	242.4	252.1	247.7

	Percent contribution to total WHO-TEQ or HEH-TEQ (%)									
	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ	WHO-TEQ	HEH-TEQ
TCDD	5.1	4.2	4.3	3.7	1.1	1.2	3.9	3.6	10.2	10.3
PeCDF	2.6	45.1	2.9	52.9	1.7	39.0	2.5	48.5	1.7	36.5
TCDF	0.3	0.2	0.3	0.3	0.3	0.4	0.7	0.7	NA	NA
PCB 77	2.9	0[c]	7.1	0[c]	12.4	0[c]	9.1	0[c]	3.2	0[c]
PCB 105	5.4	0[c]	12.2	0[c]	8.2	0[c]	7.7	0[c]	6.8	0[c]
PCB 118	1.2	0[c]	3.0	0[c]	1.9	0[c]	2.1	0[c]	1.7	0[c]
PCB 126	82.3	40.9	70.1	36.7	74.1	48.2	73.9	40.9	76.4	46.7
PCB 169	0.2	9.6	0.1	6.4	0.1	11.2	0.1	6.3	0.1	6.6

[a] Concentrations are from Jermyn-Gee et al. [36]. NA = not available.
[b] Total concentrations of WHO-TEQ and HEH-TEQ were calculated as the sum of the products of the concentration of each compound measured in herring gull eggs in 2001 [1] by 1) WHO TEF values reported by Van den Berg et al. [3] for WHO-TEQ concentrations or 2) herring gull embryonic hepatocyte (HEH) ReP values reported in this paper (based on CYP1A4 mRNA induction in combination with HEH ReP values for PCBs reported in Kennedy et al. [15] for HEH-TEQ concentrations.
[c] HEH RePs for PCB 77, PCB 105 and PCB 118 are 0, so their HEH-TEQ and percentage contribution to HEH-TEQ are 0. See footnotes of Table 2 for further explanation.

These findings highlight the importance of assessing the relative potencies of DLCs in species other than the chicken in order to address the risk and hazard to wild avian species exposed to these classes of contaminants.

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