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RELATIVE SENSITIVITIES AMONG AVIAN SPECIES TO INDIVIDUAL AND MIXTURES OF AHR-ACTIVE COMPOUNDS

Running title: Relative sensitivities of DLCs and combined effect

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Abstract: Dioxins and dioxin-like compounds (DLCs) are potent toxicants to most vertebrates. Sensitivities to DLCs vary among species. Here sensitivities of avian species (chicken \([\textit{Gallus gallus}]\), ring-necked pheasant \([\textit{Phasianus colchicus}]\), Japanese quail \([\textit{Coturnix japonica}]\)) to some polychlorinated dibenzo-\(p\)-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) were determined by use of species-specific, in vitro, transactivation assays based on a luciferase reporter gene (LRG) under control of species-specific aryl hydrocarbon receptors (AhRs).

2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin (TCDD) was not the most potent inducer of toxic effects in ring-necked pheasant or Japanese quail. Especially for Japanese quail, the relative potency (ReP) values of most of 9 PCDD/Fs tested were greater than TCDD. The rank order of avian species sensitivities to DLCs was chicken > ring-necked pheasant > Japanese quail. Effects of binary mixtures of TCDD, 2,3,7,8-tetrachlorodibenzo[\(p\)]furan (2,3,7,8-TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF) were strictly additive. Moreover, we also found that the primary DLCs that were responsible for most of the potency of DLCs mixtures can be deduced by use of ordination in a multi-dimensional space defined by the avian species sensitivities. Overall, the ReP and the species sensitivities of these chemicals could guide risk assessments to wild species when exposure to mixtures of DLCs in the environment. This article is protected by copyright. All rights reserved

Keywords: DLCs; Species sensitivity; Combined effect; Luciferase; In vitro
INTRODUCTION

Dioxins and dioxin-like compounds (DLCs), including polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs), polychlorinated napthalenes (PCNs) and polycyclic aromatic hydrocarbons (PAHs), cause toxicities in most vertebrates. In vertebrates, the critical mode of toxic actions of DLCs are mainly mediated by the aryl hydrocarbon receptor (AhR) [1]. A long evolutionary period of AhR gene replication and diversification has led to a variety of forms of the AhR, including AhR1, AhR2, AhR3 [2, 3].

In order to facilitate assessment of effects of DLCs, concepts of toxic equivalents (TEQs) based on in vivo effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and toxicity equivalency factors (TEFs) have been widely accepted [4, 5]. However, recent studies have found that TCDD is not the most potent compound in some species [6, 7]. For example, the potency of 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF) is 3- to 5-fold of TCDD in ring-necked pheasant and 30-fold of TCDD in Japanese quail [6]. The potency of 2,3,4,7,8-PeCDF is 21-fold, 9.8-fold, 40-fold greater than of TCDD for expression of RNA of CYP1A4, CYP1A5 and activity of the mixed function, monooxygenase enzyme, ethoxyresorufin-O-deethylase (EROD) in primary embryonic herring gull (Larus argentatus) hepatocytes, respectively [7]. Meanwhile, there are differences in sensitivity to DLCs both within and among classes of vertebrates [6, 8, 9]. The rank order of sensitivities to 2,3,7,8-tetrachlorodibenzoferan (2,3,7,8-TCDF) and TCDD in inducting CYP1A4/CYP1A5 mRNA and EROD is chicken > ring-necked pheasant > Japanese quail [6, 10].

Among birds, chicken is the most sensitive to DLCs [9]. For TCDD, sensitivity of Japanese quail is
100-fold less than that of chicken [11]. Although a complete understanding of reasons underling differences in sensitivities among species is not yet available [12], A factor is differential binding of DLCs to the ligand binding domain (LBD) of the AhR due to differences among sequences of amino acids of the LBD, especially at positions 324 and 380 of the AhR1-LBD of birds [9, 13]. Thus, it is necessary to assess the species sensitivities of individual DLCs in addition to TCDD.

Since organisms are usually simultaneously exposed to mixtures, there has been concern about combined effects, and most evidence suggests AhR-mediated potencies of mixtures of DLCs are additive and potential for synergisms or supra-additive effects is small [5, 14, 15]. But, components of mixtures are also probably antagonistic, if they are less potent agonists of the AhR and their presence in environmental samples is relatively great. Some studies have found that combined effects of 2,3,4,7,8-PeCDF and 1,2,3,4,7,8-hexachlorodibenzofuran (1,2,3,4,7,8-HxCDF) or 2,3,3′,4,4′,5-hexachlorobiphenyl (PCB 169) were additive [16, 17]. A recent study has found that effects of co-exposure to PCBs and TCDD varied as a function of dose and end points measured, with effects of mixtures being additive, synergistic, or antagonistic [18].

Information about species sensitivity and the relative potency (ReP) of DLCs was limited, especially for the AhR-LRG assays. AhR-LRG is an effective assay for monitoring AhR activity, which has been used to test AhR-mediated activity and inter-species sensitivities of DLCs among avian species [12, 19, 20]. Besides, effects of binary mixtures of DLCs needed to be verified. Therefore, 9 PCDD/Fs, which are known to be primary contributors to TEQs in environmental media [21-26], were selected as test substances by use of in vitro (cell-based), transactivation assays based on luciferase as the reporter gene (LRG) under control of species-specific AhR [13]. Specific objectives of the present
study were 4: 1) to compare ReP of 9 PCDD/Fs in avian species (chicken, ring-necked pheasant, and Japanese quail) by use of avian AhR1-LRG assay; 2) to determine sensitivities of avian species to 9 PCDD/Fs; 3) to determine effects of binary mixtures of PCDD/Fs; 4) to identify dominant substances based on contribution to TEQs in mixtures of DLCs.

MATERIALS AND METHODS

Chemicals and solutions

A detailed description of the preparation of PCDD/Fs (Table 1) (Sigma-Aldrich) solutions is provided elsewhere [27]. In brief, PCDD/Fs were diluted from stock solutions in dimethyl sulfoxide (DMSO; Sigma-Aldrich), by use of a gradient and concentrations were determined by high-resolution gas chromatography high-resolution mass spectrometry (HRGC-HRMS) by use of isotope dilution following EPA method 1613 [28].

For the AhR1-LRG assays of chicken, ring-necked pheasant, and Japanese quail, the concentration of the primary stock solution of TCDD, prepared in DMSO, was 60,000 nM [12]. Stock solutions of each PCDD/Fs in DMSO were prepared with different concentrations ranging from 60,000 nM to 600,000 nM. Test solutions of PCDD/Fs or TCDD were prepared by dissolving serially diluted solutions with cell culture medium. The final in-well concentration of DMSO added to 96-well plates was 0.5%.

Culture, transfection of COS-7 cells and avian AhR1-LRG assays

COS-7 cells, provided by Dr. R. Haché (University of Ottawa, Ottawa, ON, Canada), were cultured in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum in an incubator (37 °C, 5% CO₂, 99% humidity). Details for preparation of chicken, ring-necked pheasant, and Japanese quail AhR1 constructs are provided elsewhere [20]. A vector containing sequence coding for
the aromatic hydrocarbon transporter (ARNT1) and promoter region of CYP1A5 of the common cormorant (*Phalacrocorax carbo*) were generously provided by Dr. Hisato Iwata (Ehime University, Japan) [29, 30]. Details of the procedure for conducting the AhR1-LRG assay is provided elsewhere [20]. Briefly, transfection was carried out 18 h after plating of COS-7 cells at a concentration of 10,000 cells per well in 96-well plates. 50 ng of deoxyribonucleic acid (DNA) and 0.2 μL Fugene 6 transfection reagent (Promega) were mixed in Opti-MEM (Invitrogen). The 6 μL mixture was transfected into cells in each well 50 ng of DNA including 8 ng of chicken, ring-necked pheasant, or Japanese quail AhR1 expression construct, 7.5 ng of CYP1A5 reporter construct, 1.55 ng of cormorant ARNT1, 0.75 ng of Renilla luciferase vector, and 32.2 ng of salmon sperm DNA (Invitrogen). TCDD or PCDD/Fs or DMSO (solvent control) was added to cells 5 h after transfection. The AhR activity was quantified as luminescence by use of Dual-Glo luciferase assay kits (Promega) in a Microplate reader (BioTek Instruments) 20 h after dosing.

**Potencies of DLCs singly or in combination**

Potencies of 9 PCDD/Fs, including TCDD, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD), 1,2,3,7,8,9-hexachlorodibenzo-*p*-dioxin (1,2,3,7,8,9-HxCDD), 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin (1,2,3,4,6,7,8-HpCDD), 1,2,3,4,6,7,8,9-octachlorodibenzo-*p*-dioxin (OCDD), 2,3,7,8-TCDF, 1,2,3,7,8-pentachlorodibenzofuran (1,2,3,7,8-PeCDF), 2,3,4,7,8-PeCDF, and 1,2,3,4,6,7,8,9-octachlorodibenzofuran (OCDF), were determined by use of the AhR1-LRG assay.

Binary mixtures (B1-B7) among TCDD, 2,3,7,8-TCDF or 2,3,4,7,8-PeCDF were made up based on contribution rates to TEQs respectively (Table 2). Based on preliminary results, mixtures were diluted
serially by 2-fold to make a gradient of 8 concentrations, which were then used to determine
sensitivities of avian species by the AhR1-LRG assay procedure.

Avian AhR1-LRG assays data analysis

For the AhR1-LRG assays of chicken, ring-necked pheasant, Japanese quail,
concentration-response curves of each AhR1 construct and PCDD/Fs treatments were obtained from 3
independent assays. To order to minimize variability due to operation and transfection efficiency, a
ratio of firefly luciferase units to Renilla luciferase units was used to normalize luciferase activity [31].
Concentration-response curves were calculated with GraphPad (GraphPad Prism 5.0 software) using a
4 parameter logistic mode [32]. For each replicate concentration-response curve, maximal response
values were determined using a 4 parameter logistic mode, and were presented as the mean ± standard
error (SE). If the concentration-response curve did not reach a plateau, The 50% effective
concentration (EC50) could not be calculated. In this case, the greatest observed response was
reported instead of the maximal response.

Estimation of relative sensitivity(ReS) and ReP

Since the chicken is the most sensitive avian species to DLCs tested to date. The ReS values
based on EC values were calculated by dividing EC values of the chicken if it was available in the
AhR1-LRG assay. The ReS values were calculated (Equation 1).

\[
\text{ReS}_x = \frac{\text{EC}_{x, \text{chicken}}}{\text{EC}_{i, \text{species}}}
\]  

The ReP values were calculated using the framework proposed by Villeneuve et al. [33] with some
modifications. The ReP values based on EC derived from regression models were calculated. When
the concentration-response curve did not reach a plateau, in addition to ReP10 values, ReP50 and
ReP80 were also calculated. The average of ReP (RePavg) and the range of ReP (RePrange) were calculated from ReP10, ReP50 and ReP80 values. The ReP values were calculated (Equation 2).

\[ \text{ReP}_x = \frac{\text{EC}_{x, \text{TCDD}}}{\text{EC}_{x, \text{dioxin}}} \]  

(2)

**Combined toxicity assessment**

The concentration addition model (CA) is usually applied to estimate effects of mixtures. The equivalent curve diagram method is a visual application of this model. In the figure, 2 components of binary mixtures are expressed as toxic unit (TU), which were calculated (Equation 3).

\[ \text{TU} = \frac{C_i}{\text{EC50},i} \]  

(3)

Where TU = toxic unit (TU) of component \( i \); EC50,\( i \) = EC50 of component \( i \) in separate test; and \( C_i \) = concentration of component \( i \) equivalent to the EC50 in the mixture.

Based on TU with 2 components in the mixture, an axis coordinate system was established. When each compound causes effects through an independent joint action the value of TU is 1.0. Connecting TU of 2 compounds and applying 95% confidence intervals generates an additivity belt. When TU of mixtures overlap with the additivity belt, it shows that 2 compounds produce additive effects. Thus, a concentration addition model can be applied [34].

**RESULTS AND DISCUSSION**

*Induction of luciferase activity in COS-7 cells transfected with AhRs of birds*

Concentration-dependent effects of TCDD and PCDD/Fs on luciferase activity expressed by COS-7 cells transfected with chicken, ring-necked pheasant, or Japanese quail AhR1 constructs were shown (Figure 1). Luciferase activity induced by 300 nM of TCDD (a positive control for normalization of the luciferase activity data) reached a plateau in all 3 kinds of avian AhR1 constructs.
All PCDD/Fs of interest, except OCDD, induced significant luciferase activity and reached a plateau in cells transfected with AhR1 of chicken, ring-necked pheasant or Japanese quail. 1,2,3,7,8-PeCDD, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF and 2,3,7,8-TCDF induced the greatest responses in cells containing the 3 kinds of AhR1 constructs, while OCDD and OCDF generally caused less responses.

Although OCDF in cells transfected with constructs containing AhR1 of chicken, pheasant, or Japanese quail reached a plateau, but maximal responses in chicken and pheasant were less than 50% of the positive control. This result made it more difficult to make comparisons among species.

*EC50 of AhR1 constructs to PCDD/Fs exposure*

In the present study, EC50 was calculated by the systematic framework proposed by Villeneuve et al. [33] with some modifications for comparing the potencies of DLCs and sensitivities among avian species (Figure 2, Table 3). If DLCs did not exhibit the same efficacies (maxima), an EC50 value could not be calculated. The potencies of TCDD, 1, 2, 3, 7, 8-PeCDD, 2,3,4,7,8-PeCDF were the greatest while that of OCDF was the least. The EC50 values of OCDF were 10 to 30-fold less than that of 1,2,3,4,6,7,8-HpCDD, which was nearest to OCDF in the same species. The rank order of EC50 values among species was approximately Japanese quail ≥ ring-necked pheasant > chicken. For TCDD, EC50 values differed by almost an order of magnitude among the avian species.

It was postulated that, for responses of the avian species exposed to mixtures from the environment for which the constituents are unknown, types of DLCs causing observed potencies could be inferred based on ordination of the sample. This type of analysis would be useful when conducting a potency balance study to determine if all of the DLCs in the mixture had been identified.

While this type of bioassay is not meant to replace instrumental analyses, it could be useful in
identifying unknowns or at least suggesting the type of DLCs for which to look, particularly in areas of the world where access to HRGC-HRMS is limited or unavailable [35, 36]. To test this hypothesis, EC50 values of more compounds and more species would be necessary.

*ReS of AhR constructs to PCDD/Fs*

The ReS values based on both EC10 or EC50 of some PCDD/Fs demonstrated distinct rank orders of sensitivity among cells containing AhR1 constructs of birds that were different from that of TCDD (Table 1). Generally, the rank order of sensitivities of species to dioxins was chicken > ring-necked pheasant > Japanese quail, which was consistent with results of previous studies (exposed to TCDD, 2,3,7,8-TCDF, PCB 126 or PCB 77) [13, 20, 37]. Especially for TCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF, the rank order of sensitivities was in agreement with results of a previous study [6]. But, 2,3,4,7,8-PeCDF had a different rank order of relative sensitivities, depending on endpoints used [10]. For instance, based on induction of EROD activity, the rank order of sensitivity to 2,3,4,7,8-PeCDF was chicken > ring-necked pheasant > Japanese quail. However, based on expression of CYP1A4 mRNA, when exposed to PeCDF, the pheasant was predicted to be equally sensitive as the chicken [10]. Rank orders of ReS50 values for 1,2,3,7,8-PeCDD, 1,2,3,4,6,7,8-HpCDD, and 1,2,3,7,8-PeCDF between the ring-necked pheasant and Japanese quail were generally similar. Among the 3 avian species studied, the construct containing chicken AhR1 was the most sensitive, 1- to 10-fold more sensitive than the pheasant. The ring-necked pheasant construct was more sensitive (0.9 to 7.5 times) than that of the Japanese quail construct. However, for 1,2,3,7,8-PeCDF, sensitivities based on pheasant and Japanese quail constructs were almost equal to
the chicken construct. Rank orders of sensitivities of birds among PCDD/Fs and other DLCs might be due to the ligand-specific differences in AhR conformation [9, 13].

ReP of PCDD/Fs in different avian AhR1 constructs

The EC values, maximal responses, and the ReP values for each PCDD/Fs and AhR1 construct are provided (Table 3). PCDD/Fs demonstrated diverse relative potencies in AhR-mediated activity among AhR1 constructs bioassays. Of the 9 DLCs tested, only 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF had been studied previously in the LRG [20]. The ReP values of 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF were consistent with those reported previously (Table 3). Based on ReP values (Table 3), the rank order of PCDD/Fs potency was TCDD > 2,3,4,7,8-PeCDF > 2,3,7,8-TCDF ≈ 1,2,3,7,8-PeCDD > 1,2,3,7,8,9-HxCDD > 1,2,3,4,6,7,8-HpCDD > OCDF for the chicken AhR1 construct, 2,3,4,7,8-PeCDF > 1,2,3,7,8-PeCDD > 2,3,7,8-TCDF ≈ TCDD > 1,2,3,7,8-PeCDD > 1,2,3,7,8,9-HxCDD > 1,2,3,4,6,7,8-HpCDD for the pheasant AhR1 construct, 2,3,4,7,8-PeCDF > 1,2,3,7,8-PeCDD > 1,2,3,7,8-PeCDF > 2,3,7,8-TCDF > 1,2,3,7,8,9-HxCDD > TCDD ≈ 1,2,3,4,6,7,8-HpCDD > OCDF for the Japanese quail AhR1 construct. Rank orders of PCDD/Fs were generally similar among AhR1 constructs of chicken and ring-necked pheasant with an exception of TCDD (Table 3). Rank orders of PCDD/Fs were generally similar among the ring-necked pheasant and Japanese quail AhR1 constructs with a few exceptions of TCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF (Table 3). For the chicken AhR1 construct, ReP values of all PCDD/Fs were less than that for TCDD. Except for OCDD and OCDF, ReP values of other PCDD/Fs in cells containing the Japanese quail AhR1 construct were greater than of TCDD, with values 1- to 22-fold greater than that of TCDD. There were differences between the 3 kinds of AhR1 constructs for PCDD/Fs with an
exception of 2,3,7,8-TCDF. ReP values were different by an order of magnitude among the avian species. Inter-species variation of ReP values for PCDD/Fs among birds might be due to differences in the AhR amino acid sequence, leading to different sizes of the AhR1 binding cavity or differences in ligand-receptor conformation among avian species [13].

Since TCDD is generally considered to be the most potent inducer of AhR-mediated responses in all taxa and has therefore been used as the reference DLCs to which all other DLCs are compared to derive ReP and TEFs [5]. However, the present study found that 2,3,4,7,8-PeCDF, 1,2,3,7,8-PeCDD and 2,3,7,8-TCDF have greater potency than TCDD in pheasant and especially, the potency of all dioxins selected in the present study but OCDD and OCDF were greater than that of TCDD for Japanese quail. In Japanese quail, ReP values for 2,3,4,7,8-PeCDF and 1,2,3,7,8-PeCDD were almost 20-fold greater than TCDD. The greater potency of 2,3,4,7,8-PeCDF compared to TCDD observed in the present study is consistent with results of previous studies that demonstrated that 2,3,4,7,8-PeCDF was more potent than TCDD in Japanese quail primary hepatocytes [6]. Besides Japanese quail, there were also similar phenomena observed for other species, such as double-crested cormorant and Forster’s tern (Sterna forsteri) [38], green frog (Rana esculenta) [39] and herring gull [7].

In order to carry out assessments of hazard and risk to wild species exposed to mixtures of DLCs, the findings included in the present study and previous studies, demonstrate the importance of assessing relative potencies of DLCs and inter-species sensitivities among species other than the chicken. Meanwhile, the AhR1-LRG assay based on constructs in COS-7 cells makes it possible to develop species and chemical-specific ReP for use in assessments of hazards or risks of DLCs to birds.
Because the chicken is thought to be among the most sensitive birds to the effects of DLCs, it has been used as a surrogate for birds when conducting hazard and risk assessments. Thus, an overestimate of the potential risks and inappropriate decisions might result [40].

**Combined effects of PCDD/Fs on avian species**

The TU values for binary mixtures of TCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF overlapped with the additivity belt, which showed that mixture effects of PCDD/Fs were additive (Figure 3). Results of previous studies have also found the same combined effect of 2,3,4,7,8-PeCDF mixed with 1,2,3,4,7,8-HxCDF or 2,3,4,5,3′,4′-hexachlorobiphenyl respectively [16]. Although concentration of each congener of PCBs and PCDD/Fs might vary among environmental mixtures, the overall combined potency can be estimated by a simple additive model [18].

Here, effects of mixtures based on EC50, demonstrated that they were additive, which might be due to the fact that all of the DLCs tested had effects mediated solely via the AhR. In the present study, binary mixtures (B1-B7) among TCDD, 2,3,7,8-TCDF or 2,3,4,7,8-PeCDF were made up based on contribution rates (1:4, 1:1, 4:1) to TEQs respectively (Table 2). The binary mixtures samples (B1-B7) among TCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF all resulted in similar ReP values (Figure 4). These mixtures, when ordinated in the 2 dimensional spaces demonstrated that the mixtures ordinated near to the dominant DLCs in the various mixtures. For instance, B1 was near TCDD, B1 and B5 were near to 2,3,4,7,8-PeCDF, while B4 and B7 were close to 2,3,7,8-TCDF. Thus, if a substance is dominant in the mixtures of DLCs substances, ReP values of mixtures will be similar to that of the dominant substance. Therefore, our results suggested that ReP values of environmental samples among different species could be used to predict the dominant DLCs.

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In summary, results of the present study have contributed to a growing number of RePs for DLCs among avian species that can be used in the mass balance analysis of environmental samples. Effects of binary mixtures of TCDD, 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF were strictly additive. TCDD was found to not be the most potent inducer of toxic effects, especially for Japanese quail. Potencies of most DLCs, including 1,2,3,7,8-PeCDD, 1,2,3,7,8,9-HxCDD, 2,3,7,8-TCDF, 1,2,3,7,8-PeCDF and 2,3,4,7,8-PeCDF were more potent than TCDD. The rank order of sensitivities of birds to DLCs was chicken > ring-necked pheasant > Japanese quail. The ReP values together with the inter-species variations based on the AhR-LRG assays could provide valuable information for the hazards and risk assessments of complex environmental pollutants exposed to wild birds.

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Data availability—The data can be obtained from the corresponding author (howard50003250@yahoo.com).
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2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. \textit{Toxicol Sci} 113:380-391.


Figure 1. Concentration-dependent effects of 2,3,7,8-tetra-chlorodibenzo-p-dioxin (TCDD), polychlorinated dibenzo-p-dioxins (PCDDs) or polychlorinated dibenzofurans (PCDFs) on luciferase activity based on hydrocarbon receptor 1 (AhR1) in COS-7 cells transfected with chicken, ring-necked pheasant or Japanese quail AhR1 constructs. Data are presented as percent response values relative to that of a 300 nM TCDD positive control for each of the avian constructs.

Concentration-response curves for the PCDD/Fs are only presented for constructs that showed a significant ($p < 0.05$), concentration-dependent increase in luciferase activity relative to the DMSO response. Points represent mean, positive control-normalized luciferase ratios obtained from 3 independent experiments, each with 4 technical replicates per concentration of PCDD/Fs or TCDD.

1,2,3,7,8-PeCDD = 1,2,3,7,8-pentachlorodibenzo-p-dioxin; 1,2,3,7,8,9-HxCDD = 1,2,3,7,8,9-hexachlorodibenzo-p-dioxin; 1,2,3,4,6,7,8-HpCDD = 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin; OCDD = 1,2,3,4,6,7,8,9-octachlorodibenzo-p-dioxin;

2,3,7,8-TCDF = 2,3,7,8-tetrachlorodibenzofuran; 1,2,3,7,8-PeCDF = 1,2,3,7,8-pentachlorodibenzofuran; 2,3,4,7,8-PeCDF = 2,3,4,7,8-pentachlorodibenzofuran; OCDF = 1,2,3,4,6,7,8,9-octachlorodibenzofuran; DMSO = dimethyl sulfoxide; Bars represent standard error.

Figure 2. Effective concentration (EC50) value of polychlorinated dibenzo-p-dioxins (PCDDs) or polychlorinated dibenzofurans (PCDFs) in avian species, based on a luciferase reporter gene (LRG) assay with aryl hydrocarbon receptor 1 (AhR1). EC50 values represented the average of three replicates obtained from three 96-well plates for each compound. TCDD = 2,3,7,8-tetra-chlorodibenzo-p-dioxin; 1,2,3,7,8-PeCDD = 1,2,3,7,8-pentachlorodibenzo-p-dioxin;

1,2,3,7,8,9-HxCDD = 1,2,3,7,8,9-hexachlorodibenzo-p-dioxin; 1,2,3,4,6,7,8-HpCDD = 1,2,3,4,6,7,8-pentachlorodibenzofuran;
1,2,3,4,6,7,8-heptachlorodibenzo-\(p\)-dioxin; 2,3,7,8-TCDF = 2,3,7,8-tetrachlorodibenzoofuran;

1,2,3,7,8-PeCDF = 1,2,3,7,8-pentachlorodibenzoofuran; 2,3,4,7,8-PeCDF = 

2,3,4,7,8-pentachlorodibenzoofuran; OCDF = 1,2,3,4,6,7,8,9-octachlorodibenzoofuran.

Figure 3. Evaluation of binary mixture toxicity of 2,3,7,8-tetra-chlorodibenzo-\(p\)-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzoofuran (2,3,7,8-TCDF) and 2,3,4,7,8-pentachlorodibenzoofuran (2,3,4,7,8-PeCDF). Axes are TU of TCDD, 2,3,7,8-TCDF, 2,3,4,7,8–PeCDF to which 3 avian species were separately exposed. The solid line is the additive equivalent curve, while the area delineated by the 2 dashed lines is the belt of additive effects. Connecting TU of 2 compounds and applying 95% confidence intervals generates an additivity belt. TU = toxic unit.

Figure 4. Scatter diagram of relative potency (ReP) values for polychlorinated dibenzo-\(p\)-dioxins (PCDDs) or polychlorinated dibenzofurans (PCDFs) and binary mixtures of 2,3,7,8-tetra-chlorodibenzo-\(p\)-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzoofuran (2,3,7,8-TCDF) and 2,3,4,7,8-pentachlorodibenzoofuran (2,3,4,7,8-PeCDF). The figure on the right is a partial enlargement of the figure on the left. Binary mixtures (B1-B7) among TCDD, 2,3,7,8-TCDF or 2,3,4,7,8-PeCDF were made up based on contribution rates (1:4, 1:1, 4:1) to toxic equivalents (TEQs) respectively.

1,2,3,7,8-PeCDD = 1,2,3,7,8-pentachlorodibenzo-\(p\)-dioxin; 1,2,3,7,8,9-HxCDD = 

1,2,3,7,8,9-hexachlorodibenzo-\(p\)-dioxin; 1,2,3,4,6,7,8-HpCDD = 

1,2,3,4,6,7,8-heptachlorodibenzo-\(p\)-dioxin; 1,2,3,7,8-PeCDF = 1,2,3,7,8-pentachlorodibenzoofuran; 

OCDF = 1,2,3,4,6,7,8,9-octachlorodibenzoofuran.
**ABLE LEGENDS**

Table 1. Relative sensitivities (ReS) of hydrocarbon receptor 1 (AhR1) constructs among chicken, ring-necked pheasant, and Japanese quail exposed to polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in the AhR1 luciferase reporter gene (AhR1-LRG) assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS No.</th>
<th>ReS</th>
<th>ReS10</th>
<th>ReS50</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-tetra-chlorodibenzo-p-dioxin</td>
<td>TCDD 1746-01-6</td>
<td>1.0</td>
<td>0.20</td>
<td>0.10</td>
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<tr>
<td>1,2,3,7,8-pentachlorodibenzo-p-dioxin</td>
<td>1,2,3,7,8-PeCDD 40321-76-4</td>
<td>1.0</td>
<td>0.41</td>
<td>0.50</td>
</tr>
<tr>
<td>1,2,3,7,8,9-hexachlorodibenzo-p-dioxin</td>
<td>1,2,3,7,8,9-HxCDD 19408-74-3</td>
<td>1.0</td>
<td>0.22</td>
<td>0.34</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin</td>
<td>1,2,3,4,6,7,8-HpCDD 35822-46-9</td>
<td>1.0</td>
<td>0.62</td>
<td>0.52</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8,9-octachlorodibenzo-p-dioxin</td>
<td>OCDD 3268-87-9</td>
<td>1.0</td>
<td>0.060</td>
<td>NC</td>
</tr>
<tr>
<td>2,3,7,8-tetrachlorodibenzofuran</td>
<td>2,3,7,8-TCDF 51207-31-9</td>
<td>1.0</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>1,2,3,7,8-pentachlorodibenzofuran</td>
<td>1,2,3,7,8-PeCDF 57117-41-6</td>
<td>1.0</td>
<td>0.47</td>
<td>1.0</td>
</tr>
<tr>
<td>2,3,4,7,8-pentachlorodibenzofuran</td>
<td>2,3,4,7,8-PeCDF 57117-31-4</td>
<td>1.0</td>
<td>0.20</td>
<td>0.57</td>
</tr>
<tr>
<td>1,2,3,4,6,7,8,9-octachlorodibenzofuran</td>
<td>OCDF 39001-02-0</td>
<td>1.0</td>
<td>0.51</td>
<td>0.71</td>
</tr>
</tbody>
</table>

*ReS based on mean EC10 and EC50 values (ReS10 and ReS50) calculated from the AhR1-LRG assays are presented.*

*NC = not calculated since EC10 or EC50 value is not available.*

EC = effective concentration.
Table 2. Binary mixtures (B1-B7) of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,7,8-tetrachlorodibenzo furan (2,3,7,8-TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (2,3,4,7,8-PeCDF)$^a$

<table>
<thead>
<tr>
<th>mixture</th>
<th>%TEQs</th>
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<tr>
<td></td>
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<tr>
<td>B1</td>
<td>4</td>
</tr>
<tr>
<td>B2</td>
<td>1</td>
</tr>
<tr>
<td>B3</td>
<td>1</td>
</tr>
<tr>
<td>B4</td>
<td>4</td>
</tr>
<tr>
<td>B5</td>
<td>/</td>
</tr>
<tr>
<td>B6</td>
<td>/</td>
</tr>
<tr>
<td>B7</td>
<td>/</td>
</tr>
</tbody>
</table>

$^a$B1 to B7 were made up by TCDD, 2,3,7,8-TCDF or 2,3,4,7,8-PeCDF based on contribution rates (1:4, 1:1, 4:1) to toxic equivalents (TEQs) respectively.

TEQs = toxic equivalents.
Table 3. Relative potency (ReP), effective concentration (EC) and maximal response values of polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in hydrocarbon receptor 1 (AhR1) constructs of chicken, ring-necked pheasant and Japanese quail

<table>
<thead>
<tr>
<th>AhR</th>
<th>Compound</th>
<th>EC10±SE (nM)</th>
<th>EC50±SE (nM)</th>
<th>ReP10</th>
<th>ReP50</th>
<th>ReP80</th>
<th>RePavg</th>
<th>RePrange</th>
<th>Maximal response±SE(%)</th>
<th>ReP50a</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCDD</td>
<td></td>
</tr>
<tr>
<td><strong>Chicken</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCDD</td>
<td>0.068±0.005</td>
<td>0.22±0.04</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0–1.0</td>
<td>100±2.2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1,2,3,7,8-PeCDD</td>
<td>0.062±0.003</td>
<td>0.46±0.08</td>
<td>1.1</td>
<td>0.43</td>
<td>0.40</td>
<td>0.64</td>
<td>0.40–1.1</td>
<td>93±3.2</td>
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</tr>
<tr>
<td></td>
<td>1,2,3,7,8,9-HxCDD</td>
<td>0.49±0.03</td>
<td>3.4±0.1</td>
<td>0.14</td>
<td>0.053</td>
<td>0.08</td>
<td>0.091</td>
<td>0.080–0.14</td>
<td>49±2.5</td>
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<tr>
<td></td>
<td>1,2,3,4,6,7,8-HpCDD</td>
<td>1.7±0.09</td>
<td>9.4±0.09</td>
<td>0.039</td>
<td>0.018</td>
<td>0.036</td>
<td>0.031</td>
<td>0.036–0.039</td>
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<tr>
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<td>59±0.1</td>
<td>NCd</td>
<td>1.2E-03</td>
<td>NAf</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>16±4.5</td>
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<tr>
<td></td>
<td>2,3,7,8-TCDF</td>
<td>0.075±0.002</td>
<td>0.43±0.07</td>
<td>0.90</td>
<td>0.54</td>
<td>0.24</td>
<td>0.56</td>
<td>0.24–0.90</td>
<td>83±2.5</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>1,2,3,7,8-PeCDF</td>
<td>0.33±0.08</td>
<td>3.7±0.07</td>
<td>0.20</td>
<td>0.057</td>
<td>0.036</td>
<td>0.099</td>
<td>0.036–0.20</td>
<td>106±4.1</td>
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<td>2,3,4,7,8-PeCDF</td>
<td>0.043±0.002</td>
<td>0.25±0.08</td>
<td>1.6</td>
<td>0.83</td>
<td>1.2</td>
<td>1.2</td>
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<tr>
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<td>9.3E-04</td>
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<td>1.5E-03–2.3E-03</td>
<td>41±5.5</td>
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<td><strong>Pheasant</strong></td>
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<tr>
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<td>TCDD</td>
<td>0.34±0.003</td>
<td>2.8±0.05</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
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<tr>
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<td>1,2,3,7,8-PeCDD</td>
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<td>0.93±0.04</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2–2.7</td>
<td>113±2.2</td>
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<tr>
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<td>1,2,3,7,8,9-HxCDD</td>
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<td>10±0.03</td>
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<td>0.23</td>
<td>0.30</td>
<td>0.23</td>
<td>0.16–0.30</td>
<td>76±1.6</td>
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<tr>
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<td>1,2,3,4,6,7,8-HpCDD</td>
<td>2.8±0.4</td>
<td>18±0.04</td>
<td>0.12</td>
<td>0.12</td>
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<td>0.12–0.13</td>
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<td>976±3</td>
<td>NC</td>
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<td>0.32±0.005</td>
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<td>1.1</td>
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<td>0.70±0.02</td>
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<td>0.48</td>
<td>0.74</td>
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<td>0.48–0.90</td>
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<td>4.3</td>
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<td>1.6–4.3</td>
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<tr>
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<td>OCDF</td>
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<td>0.013</td>
<td>0.010</td>
<td>5.7E-03–0.013</td>
<td>41±1.7</td>
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<tr>
<td><strong>Quail</strong></td>
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<td>21±0.05</td>
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<td>1.0–1.0</td>
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<td>1,2,3,7,8-PeCDD</td>
<td>0.23±0.06</td>
<td>0.98±0.02</td>
<td>19</td>
<td>19</td>
<td>16</td>
<td>18</td>
<td>16–19</td>
<td>121±1.2</td>
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<tr>
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<td>1,2,3,7,8,9-HxCDD</td>
<td>4.6±0.06</td>
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<td>0.97</td>
<td>1.2</td>
<td>1.5</td>
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<td>0.97–1.5</td>
<td>71±1.8</td>
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<td>1,2,3,4,6,7,8-HpCDD</td>
<td>4.1±0.08</td>
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<td>0.95</td>
<td>1.5</td>
<td>1.2</td>
<td>0.95–1.5</td>
<td>97±1.3</td>
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<tr>
<td></td>
<td>OCDD</td>
<td>NC</td>
<td>NC</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>5.1±0.45</td>
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</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Compound</th>
<th>EC10 (μM)</th>
<th>EC50 (μM)</th>
<th>EC80 (μM)</th>
<th>Maximal Response (%)</th>
<th>ReP50</th>
<th>RePrange</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,7,8-TCDF</td>
<td>0.99 ± 0.01</td>
<td>9.4 ± 0.02</td>
<td>4.5</td>
<td>2.3–4.7</td>
<td>77 ± 1.1</td>
<td>3</td>
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<tr>
<td>1,2,3,7,8-PeCDF</td>
<td>0.92 ± 0.03</td>
<td>3.3 ± 0.03</td>
<td>4.8</td>
<td>4.8–5.4</td>
<td>92 ± 2.0</td>
<td>_</td>
</tr>
<tr>
<td>2,3,4,7,8-PeCDF</td>
<td>0.33 ± 0.004</td>
<td>0.92 ± 0.02</td>
<td>13</td>
<td>13–20</td>
<td>100 ± 1.5</td>
<td>20</td>
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<tr>
<td>OCDF</td>
<td>141 ± 2.5</td>
<td>484 ± 0.02</td>
<td>0.031</td>
<td>0.03–0.049</td>
<td>56 ± 1.2</td>
<td>_</td>
</tr>
</tbody>
</table>

ECx and maximal response values represent the average of three replicates ± standard error (SE) obtained from three 96-well plates for each compound.

Maximal response observed for the PCDD/Fs expressed as a percentage of the mean of the maximal response observed for TCDD.

| a | Mean relative potency (RePavg) values and ranges of ReP (RePrange) values were calculated from EC10-, EC50- and EC80-based ReP values. No RePrange values were presented when only the ReP10 could be calculated. |
| b | ReP50 values were from previous study[20]. |
| c | NC = not calculated because the maximal response was not reached. |
| d | NA = ReP estimates not available to calculate the value. |

TCDD = 2,3,7,8-tetra-chlorodibenzo-\(p\)-dioxin; 1,2,3,7,8-PeCDD = 1,2,3,7,8-pentachlorodibenzo-\(p\)-dioxin; 1,2,3,7,8,9-HxCDD = 1,2,3,7,8,9-hexachlorodibenzo-\(p\)-dioxin; 1,2,3,4,6,7,8-HpCDD = 1,2,3,4,6,7,8-heptachlorodibenzo-\(p\)-dioxin; OCDD = 1,2,3,4,6,7,8,9-octachlorodibenzo-\(p\)-dioxin; 2,3,7,8-TCDF = 2,3,7,8-tetrachlorodibenzofuran; 1,2,3,7,8-PeCDF = 1,2,3,7,8-pentachlorodibenzofuran; 2,3,4,7,8-PeCDF = 2,3,4,7,8-pentachlorodibenzofuran; OCDF = 1,2,3,4,6,7,8,9-octachlorodibenzofuran.
Figure 1.
Figure 2.
Figure 3.
Figure 4.