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Sensitivity of Japanese Quail (*Coturnix japonica*), Common Pheasant (*Phasianus colchicus*), and White Leghorn Chicken (*Gallus gallus domesticus*) Embryos to *In Ovo* Exposure to TCDD, PeCDF, and TCDF

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Egg injection studies were performed to confirm a proposed model of relative sensitivity of birds to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In this model, species are classified as belonging to one of three categories of sensitivity based on amino acid substitutions in the ligand-binding domain of the aryl hydrocarbon receptor. Embryo lethality and relative potencies of 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) were compared with TCDD for Japanese quail (*Coturnix japonica*; least sensitive), Common pheasant (*Phasianus colchicus*; moderately sensitive), and White Leghorn chicken (*Gallus gallus domesticus*; most sensitive). Doses ranging from 0.044 to 37 pmol/g egg (0.015–12 ng/g egg) were injected into the air cell of eggs prior to incubation. LD_{50} (95% confidence intervals) values, based on rate of hatching for TCDD, PeCDF, and TCDF, were 30 (25–36), 4.9 (2.3–9.2), and 15 (11–24) pmol/g egg for the quail, 3.5 (2.3–6.3), 0.61 (0.28–1.2), and 1.2 (0.62–2.2) pmol/g egg for pheasant, and 0.66 (0.47–0.90), 0.75 (0.64–0.87), and 0.33 (0.23–0.45) pmol/g egg for chicken, respectively. LD_{50}-based relative potencies of PeCDF and TCDF were 6.1 and 2.0 for quail, 5.7 and 2.9 for pheasant, and 0.88 and 2.0 for chicken, respectively. TCDD was not the most potent compound among the species tested, with PeCDF and TCDF being more potent than TCDD in the quail and pheasant. TCDF was the most potent in chicken. Species sensitivity was as expected for TCDD and TCDF, whereas for PeCDF, the chicken and pheasant were similar in sensitivity and both were more sensitive than the quail. Results from companion *in vitro* studies are generally similar to those reported here with a few exceptions.

Key Words: **TCDD; PeCDF; TCDF; egg; bird; toxic equivalency factor; relative potency.**

The current methodology to assess the risk of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally similar chemicals assumes that toxic effects are mediated through the interaction of the chemical with the aryl hydrocarbon receptor (Okey, 2007). This risk assessment approach utilizes toxic equivalency factors or relative potency factors to estimate the toxicity of TCDD-like compounds. TCDD toxic equivalents are calculated as the sum of the product of the concentration of a specific TCDD-like compound and its respective toxic equivalency factor (or relative potency factor depending on the use of the toxic equivalent) for each TCDD-like compound in a mixture (Huwe, 2002; Safe, 1998; Van den Berg *et al.*, 1994, 1998). The toxic equivalency factor for an individual TCDD-like compound is a consensus value based on multiple endpoints from different species belonging to a class of animals (mammals, birds, etc.). Although the toxic equivalency factor gives the relative toxicity of a TCDD-like compound, it is meant to be protective in a risk assessment rather than being predictive. Unlike a toxic equivalency factor, a relative potency factor is based on a species-specific endpoint and is simply the ratio of potency for a TCDD-like compound relative to a reference compound, normally TCDD, which is often assumed to be the most potent of TCDD-like compounds. Although toxic equivalency factors are developed to be protective, the rank order of relative potency factors and toxic equivalency factors are generally similar (Blankenship *et al.*, 2008). In addition, some toxic equivalency factors are based on *in vitro* studies that do not account for the potential species-specific differences between absorption, distribution, metabolism,

and elimination of TCDD-like compounds (Giesy and Kannan, 1998).

Results from acute and chronic *in vivo* studies, as well as recent *in vitro* and *in ovo* studies, indicated substantial differences in the sensitivity of avian species to TCDD-like compounds among species of birds (Head *et al*., 2008; Hervé *et al*., 2010a,b,c; Karchner *et al*., 2006; Kennedy *et al*., 1996; Yang *et al*., 2010). As a result, the current World Health Organization consensus toxic equivalency factor values for individual TCDD-like compounds may over- or underestimate potencies in individual avian species. In fact, Head *et al*. (2008) noted that avian species sensitivities could vary up to 10,000-fold. This variation results in species-specific uncertainties that pose a challenge to risk assessors.

One hypothesis to account for differences in avian sensitivity to TCDD and TCDD-like compounds has proposed that toxicity can be attributed to variations in the affinity of TCDD-like compounds to the ligand-binding domain of the aryl hydrocarbon receptor (Head *et al*., 2008; Karchner *et al*., 2006). The aryl hydrocarbon receptor is a ligand-activated nuclear transcription factor that regulates the expression of a suite of genes, including biotransformation enzymes such as the mixed function monooxygenase enzymes (Hahn, 1998). Karchner *et al*. (2006) demonstrated through the use of chimeric aryl hydrocarbon receptor protein and site-directed mutagenesis that the relative insensitivity of the Common tern (*Sterna hirundo*) to TCDD-like compounds compared with the chicken (250-fold difference) could be explained, in part, by a difference of two amino acids (chicken Ile324/Ser380) in the ligand-binding domain of the aryl hydrocarbon receptor. Head *et al*. (2008) extended these findings by showing that the sensitivity of several avian species to TCDD-like compounds could be predicted based on differences in the substitution of these two amino acids. Those species with an amino acid sequence similar to that of the White Leghorn chicken are considered most sensitive, those with a sequence similar to the Common pheasant (Ile/Ala) are moderately sensitive, and those species with a ligand-binding domain amino acid sequence similar to the Japanese quail (Val/Ala) are least sensitive. Sensitivity classifications and aryl hydrocarbon receptor genotypes do not always correspond to phylogenetic relationships among species surveyed (Head *et al*., 2008).

Presently, World Health Organization toxic equivalency factors for 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) in avian species are 1.0 (Van den Berg *et al*., 1998) based on *in vitro* and *in ovo* studies of PeCDF (Bosveld *et al*., 1992; Sanderson *et al*., 1998) and TCDF (Bosveld *et al*., 1992; Kennedy *et al*., 1996; Poland and Glover, 1977). However, the results of a recent *in vitro* study (Hervé *et al*., 2010a,b,c) indicate the potencies of PeCDF and TCDF relative to TCDD to be greater than 1.0, depending upon the species examined.

The present study was undertaken to (1) assess the relative *in ovo* potencies of TCDF and PeCDF compared with TCDD in Japanese quail (*Coturnix japonica*), Common pheasant (*Phasianus colchicus*), and White Leghorn chicken (*Gallus gallus domesticus*) and (2) confirm, *in ovo*, the proposed avian species sensitivity classification model based primarily on *in vitro* work in all three species (Head and Kennedy, 2010; Head *et al*., 2008; Hervé *et al*., 2010a,b,c; Karchner *et al*., 2006; Kennedy *et al*., 1996).

MATERIALS AND METHODS

Experimental design. This study was divided into three separate experiments, one for each species. The quail experiment consisted of three trials, the chicken study consisted of two trials, and the pheasant study consisted of a single trial because this species is a seasonal breeder and eggs are only available for a short period of time each year.

Doses were chosen to bracket estimated LD_{50} values derived from egg injection studies with TCDD (pheasant [Nosek *et al*., 1993]; chicken [Henshel *et al*., 1997; Powell *et al*., 1996]) or an estimate of relative species sensitivity to TCDD (Japanese quail [Head *et al*., 2008]) and environmentally relevant concentrations for each test compound based on estimated concentrations of TCDD, PeCDF, and TCDF in eggs of house wrens (*Troglodytes aedon*), tree swallows (*Tachycineta bicolor*), and eastern bluebirds (*Sialia sialis*) collected along the Tittabawassee River downstream of Midland, MI (Fredricks *et al*., 2010).

Prior to incubation, nine doses of TCDD and PeCDF and 10 doses of TCDF were injected into Japanese quail eggs, whereas seven doses of each test compound were injected into pheasant or chicken eggs. Doses expressed as picomoles per gram (wt/wt) egg and nanograms per gram (wt/wt) egg are presented in Table 1 for each species. Controls included noninjected and triolein-injected (vehicle control) eggs. There were no differences in embryo mortality between the two types of controls. Therefore, only those eggs injected with the vehicle were included in the statistical analysis. The number of fertile eggs used per dose group for each species is presented in Table 2.

Egg preparation. Pheasant eggs were purchased from McFarlane Pheasants (Janesville, WI), whereas Japanese quail and White Leghorn chicken eggs were obtained from the Michigan State University (MSU) Poultry Research and Teaching Center (East Lansing, MI). All the pheasant eggs were laid on the same day, whereas the quail and chicken eggs were collected over a 1-week period. Eggs were stored in a cooler for no longer than 1 week at 13.5–15°C until 24 h prior to injection. Eggs were weighed to the nearest 0.1 g and then held to a bright light (candling) to detect subtle damage to the shell. Undamaged eggs with mean weights (± 1 SD) of 9.8 ± 0.74 for quail, 29.4 ± 2.1 for pheasants, and 56.3 ± 3.2 for chickens had the center of their air cells marked with pencil to outline the injection site. Each egg was assigned a unique identification number written on the exterior of the shell in pencil.

Preparation of injection solutions and egg injection procedures. In general, preparation of injection solutions and egg injection procedures follow methodology described in Powell *et al*. (1996) with minor modifications. Stock solutions of TCDD, TCDF, and PeCDF (all purchased from Sigma-Aldrich; St Louis, MO) were prepared by dissolving each chemical in triolein (Sigma-Aldrich) that was then cold filtered with a 0.22-μm syringe filter prior to serial dilution. Previous studies in our laboratory indicated that triolein is an effective vehicle for TCDD-like compounds that results in minimal vehicle control mortality (Powell *et al*., 1996). Dosing solutions were formulated based on injection volumes of 2.0, 3.0, and 5.8 μl/egg for quail, pheasant, and chicken, respectively. Previous experience indicates that an injection volume of 0.1–0.2 μl/g egg does not induce excessive embryo mortality (Powell *et al*., 1996). The decision was made to use a fixed injection volume rather than vary volume based on individual egg weight to expedite the injection process. It was decided that variation in egg weight was sufficiently low, allowing for a relatively consistent dose delivery. Following preparation of the dosing

TABLE 1
Doses of TCDD, PeCDF, or TCDF Injected into the Air Cell of Japanese Quail, Common Pheasant, and White Leghorn Chicken Eggs Prior to Incubation

	Japanese quail dose groups		Common pheasant dose groups		White Leghorn chicken dose groups	
Compound	(ng/g egg)	(pmol/g egg)	(ng/g egg)	(pmol/g egg)	(ng/g egg)	(pmol/g egg)
TCDD	0.072	0.22	0.024	0.075	0.016	0.049
	0.16	0.50	0.032	0.10	0.031	0.096
	0.24	0.75	0.072	0.22	0.063	0.19
	0.40	1.2	0.10	0.31	0.13	0.42
	0.92	2.9	0.26	0.82	0.25	0.77
	1.8	5.7	1.0	3.2	0.51	1.6
	3.6	11	2.2	6.7	0.99	3.1
	8.9	28				
	12	37				
PeCDF	0.14	0.42	0.048	0.14	0.015	0.044
	0.31	0.92	0.080	0.24	0.030	0.087
	0.62	1.8	0.13	0.39	0.048	0.14
	0.89	2.6	0.20	0.60	0.11	0.33
	1.8	5.3	0.36	1.1	0.24	0.69
	3.80	11.2	1.4	4.1	0.47	1.4
	3.84	11.3	2.3	6.8	0.85	2.5
	7.3	21				
	7.6	22				
TCDF	0.13	0.42	0.040	0.13	0.023	0.074
	0.19	0.63	0.052	0.17	0.045	0.15
	0.49	1.6	0.088	0.29	0.075	0.25
	0.89	2.9	0.20	0.65	0.16	0.52
	1.5	4.8	0.34	1.1	0.32	1.1
	2.4	7.9	1.5	4.8	0.56	1.8
	2.6	8.6	4.3	14	1.2	4.0
	4.6	15				
	7.2	24				
	9.4	31				

solutions, injection vials were flooded with argon to preserve the triolein, capped and autoclaved. Eggs were injected in a laminar flow hood under sterile conditions (NuAire, Plymouth, MN). The injection site was cleaned with 70% ethanol, a single hole was drilled through the shell into the air cell using a Dremel tool (Model 1100; Robert Bosch Tool Corporation, Racine, WI), and injections were made with a positive displacement pipettor (Gilson, Middleton, WI) with sterile pipette tips that were changed after each injection. The air cell was chosen as the site of injection because of ease and speed of delivery of the chemical into the egg (Heinz *et al*., 2006). The site of injection was then sealed using liquid paraffin wax (Royal Oak Sales, Roswell, GA) applied with a sterile wooden applicator.

Incubation and hatching procedures. Eggs were incubated in a Petersime rotary incubator (Petersime Incubator Co., Gettysburg, OH) and hatched in a Surepip hatcher (Agro Environmental Systems, Dallas, GA) as generally described by Powell *et al*. (1996).

Posthatch procedures. Dry hatchlings were transferred to a Petersime brood unit maintained at 30°C. Chicks were provided water and feed (Purina Mills Game Bird Startena [St Louis, MO] for quail and pheasants and Purina Mills Start & Grow Sunfresh [St Louis, MO] for chickens) *ad libitum*. Prior to transfer to the brood unit, hatchlings were identified with a Swiftack identification tag (Heartland Animal Health, Fair Play, MO) bearing their unique egg number. Chicks were weighed to the nearest 0.1 g, housed by treatment group, and raised for 2 weeks posthatch. Unhatched eggs with no gross indication of embryo development were assumed to be infertile and were not included in mortality calculations.

Necropsy. A subsample of 20 chicks from each dose group from each species was randomly taken from all treatment groups and euthanized by cervical dislocation: 10 at 1 day and 10 at 14 days of age. Livers from all chicks were removed, weighed, and a portion was placed in an I-Chem jar (VWR International, Chicago, IL) and frozen at −20°C for subsequent contaminant analysis. Additional samples of liver from 14-day chicks were placed into a microtube containing RNAlater (Ambion, Austin, TX) for analysis of cytochrome P450 1A4 (*CYP1A4*) and cytochrome P450 1A5 (*CYP1A5*) messenger RNA expression (Yang *et al*., 2010) and a microtube frozen in liquid nitrogen for analysis of ethoxyresorufin *O*-deethylase (EROD) activity (Yang *et al*., 2010).

Contaminant analysis. Concentrations of TCDD, PeCDF, and TCDF in dosing solutions of all three species and in quail liver samples were determined by isotope dilution following the U.S. Environmental Protection Agency's (EPA) method 1613b (Telliard, 1994). Triolein injection solutions were serially diluted with hexane prior to the addition of a mixture of ^{13}C-labeled polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Wellington Laboratories, Guelph, ON, CA). Because of the high dilution factors required to obtain PCDD/F concentrations within the range of the instrument calibration, no additional clean up of the diluted solutions was required. Liver samples (approximately 1 g, wt/wt) were mixed with anhydrous sodium sulfate and fortified with a mixture of ^{13}C-labeled PCDDs and PCDFs (Wellington Laboratories). The samples were then Soxhlet extracted with 400 ml of 1:1 hexane/dichloromethane for 16 h. Extracts were evaporated to near dryness, and the lipid content of each extract was determined gravimetrically by evaporating the entire extract to constant weight. Extracts were then dissolved

TABLE 2
Effects of TCDD, PeCDF, or TCDF Injected into the Air Cell of Japanese Quail, Common Pheasant, and White Leghorn Chicken Eggs Prior to Incubation on Hatchability

	Japanese quail			Common pheasant			White Leghorn chicken		
Compound	Dose (pmol/g egg)	n^a	% Mortality^b	Dose (pmol/g egg)	n^a	% Mortality^b	Dose (pmol/g egg)	n^a	% Mortality^b
Vehicle control	0.0	180	14.4 ^{A}	0.0	74	20.3 ^{A}	0.0	99	16.2 ^{A}
TCDD	0.22	90	17.8 ^{AB}	0.075	69	21.7 ^{A}	0.049	95	11.6 ^{A}
	0.50	95	15.8 ^{A}	0.10	70	22.9 ^{AB}	0.096	97	16.5 ^{A}
	0.75	93	22.6 ^{AB}	0.22	70	22.9 ^{AB}	0.19	97	16.5 ^{A}
	1.2	89	14.6 ^{A}	0.31	73	30.1 ^{AB}	0.42	97	46.4 ^{B}
	2.9	88	13.6 ^{A}	0.82	75	37.3 ^{B}	0.77	96	69.8 ^{C}
	5.7	92	14.1 ^{A}	3.2	69	60.9 ^{C}	1.6	99	72.7 ^{C}
	11	91	26.4 ^{B}	6.7	74	66.2 ^{C}	3.1	91	94.5 ^{D}
	28	88	54.5 ^{C}						
	37	77	66.2 ^{CD}						
PeCDF	0.42	95	21.1 ^{AB}	0.14	69	20.3 ^{A}	0.044	99	12.1 ^{A}
	0.92	90	20.0 ^{B}	0.24	67	34.3 ^{B}	0.087	96	13.5 ^{A}
	1.8	95	11.6 ^{A}	0.39	65	27.7 ^{AB}	0.14	100	14.0 ^{A}
	2.6	94	66.0 ^{C}	0.60	75	69.3 ^{C}	0.33	99	29.3 ^{B}
	5.3	90	70.0 ^{CD}	1.1	77	90.9 ^{D}	0.69	99	51.5 ^{C}
	11.2	88	73.9 ^{CDE}	4.1	76	92.1 ^{D}	1.4	94	79.8 ^{D}
	11.3	44	84.1 ^{DE}	6.8	70	94.3 ^{D}	2.5	96	91.7 ^{E}
	21	85	77.6 ^{DE}						
	22	88	85.2 ^{E}						
TCDF	0.42	93	20.4 ^{AB}	0.13	68	22.1 ^{A}	0.074	95	8.4 ^{A}
	0.63	93	12.9 ^{A}	0.17	72	27.8 ^{AB}	0.15	93	22.6 ^{A}
	1.6	94	17.0 ^{A}	0.29	72	29.2 ^{AB}	0.25	96	46.9 ^{B}
	2.9	90	17.8 ^{AB}	0.65	72	37.5 ^{B}	0.52	98	84.7 ^{C}
	4.8	90	30.0 ^{BC}	1.1	74	70.3 ^{C}	1.1	99	89.9 ^{CD}
	7.9	86	41.9 ^{CD}	4.8	75	92.0 ^{D}	1.8	98	95.9 ^{DE}
	8.6	89	61.8 ^{F}	14	70	88.6 ^{D}	4.0	99	99.0 ^{E}
	15	89	52.8 ^{DE}						
	24	89	71.9 ^{EF}						
	31	91	64.8 ^{EF}						

[a]Sample size (number of fertile eggs).
[b]Values not sharing the same superscript are significantly different ($p < 0.05$).

in 100 ml hexane and treated with 20 ml of concentrated sulfuric acid three times in a separatory funnel. The retained upper hexane layer was then rinsed with two 20-ml aliquots of nanopure water before being dried by passage through anhydrous sodium sulfate and concentrated to approximately 2 ml and sequentially subjected to multilayer silica gel and activated carbon-impregnated silica gel column. The silica gel column was eluted with 200 ml hexane, which was then concentrated and passed through the activated carbon-impregnated silica gel column and eluted with 100 ml of hexane, 100 ml 20% dichloromethane in hexane, and 100 ml toluene. The final eluent was concentrated and fortified with ^{13}C-1,3,6,8-TeCDF for analysis of TCDD, TCDF, and PeCDF. The methodology for the identification and quantification for these compounds as well as the quality assurance and quality control procedures were performed following those of Wan *et al.* (2010).

Analysis of TCDD, PeCDF, and TCDF concentrations in pheasant and chicken liver samples was performed by gas chromatography/high resolution mass spectrometry (GC/HRMS) using a Trace 2000 series gas chromatograph (Thermo Fisher Scientific, Waltham, MA) and a Finnigan MAT-95 double focusing magnetic sector mass spectrometer (Thermo Electron Co., Bremen, Germany). The high resolution gas chromatograph was equipped with a CTC A200S autosampler (Carrboro, NC) and 60 m × 0.25 mm × 0.25 μm DB5-MS GC column. The GC oven was programmed from 160°C (1.5 min hold) to 220°C (hold for 25 min) at 30°C/min, to 240°C (hold for 7 min) at 5°C/min, and to 310°C (hold for 4 min) at 5°C/min. The injection port and interface temperatures were both 280°C, with the helium carrier gas kept constant at 42 psi. The HRMS was equipped with a standard electron impact ion source operating in positive ionization mode. The ionization conditions were electron energy of 42 eV, ion source temperature of 270°C, and acceleration voltage of 4800 V. The mass spectrometer data were obtained in the selected ion monitoring mode at a resolution of 10,000 (10% valley). All calculations were performed via the isotope dilution mass spectrometric procedure. When appropriate, laboratory performance was monitored using the guidelines specified in EPA method 1613b (Telliard, 1994).

Data analysis. All statistical analyses were performed using SAS (Version 9.2; SAS, Cary, NC) with statement of significance based on $p < 0.05$. Categorical data (mortality) were analyzed using Proc Glimmix designed around a fixed-effect model testing for differences among doses. When significant treatment differences were observed, a Tukey's test was used to determine differences between doses. Lethal dose values were calculated using Proc Probit that both estimates and incorporates a natural response threshold parameter (background mortality), identified as *C* (OPTC function), into the curve fitting calculations. A final *C* value was set based on the average of those predicted from each congener to obtain a more accurate natural response rate. Total concentrations of each compound in the livers of 1- and 14-day chicks

TABLE 3
Lethal Dose (LD) Estimates (95% CI) for Japanese Quail, Common Pheasant, and White Leghorn Chicken Embryos Exposed to TCDD, PeCDF, or TCDF *In Ovo* Prior to Incubation

Species	Compound	LD_{20} (pmol/g egg)	LD_{50} (pmol/g egg)	LD_{80} (pmol/g egg)
Japanese quail	TCDD	15 (10–18)	30 (25–36)	60 (46–97)
	PeCDF	1.4 (0.21–2.8)	4.9 (2.3–9.2)	18 (9.4–77)
	TCDF	4.6 (2.2–6.7)	15 (11–24)	52 (31–160)
Common pheasant	TCDD	0.6 (0.29–0.90)	3.5 (2–6.3)	22 (11–77)
	PeCDF	0.2 (0.042–0.42)	0.6 (0.28–1.2)	1.7 (0.93–6.3)
	TCDF	0.3 (0.091–0.59)	1.2 (0.62–2.2)	4.5 (2.1–15)
White Leghorn chicken	TCDD	0.3 (0.14–0.39)	0.7 (0.47–0.90)	1.7 (1.2–2.8)
	PeCDF	0.4 (0.27–0.44)	0.8 (0.64–0.87)	1.6 (1.3–2.0)
	TCDF	0.2 (0.090–0.23)	0.3 (0.23–0.45)	0.7 (0.51–1.1)

Note. LD values calculated using a Probit model incorporating background mortality (Japanese quail = 14.6%, Common pheasant = 17.9%, and White Leghorn chicken = 12.5%) into the curve fitting calculations.

TABLE 4
Lethal Dose (LD) Estimates (95% CI) Expressed as Nanograms Compound per Gram Egg for Japanese Quail, Common Pheasant, and White Leghorn Chicken Embryos Exposed to TCDD, PeCDF, or TCDF *In Ovo* Prior to Incubation

Species	Compound	LD_{20} (ng/g egg)	LD_{50} (ng/g egg)	LD_{80} (ng/g egg)
Japanese quail	TCDD	4.8 (3.2–5.8)	9.7 (8.0–12)	19 (15–31)
	PeCDF	0.48 (0.071–095)	1.7 (0.78–3.1)	6.1 (3.2–26)
	TCDF	1.4 (0.67–2.1)	4.6 (3.4–7.3)	16 (9.5–49)
Common pheasant	TCDD	0.18 (0.93–0.29)	1.2 (0.74–2.0)	7.1 (3.5–25)
	PeCDF	0.075 (0.014–0.14)	0.21 (0.10–0.41)	0.58 (0.32–2.1)
	TCDF	0.095 (0.028–0.18)	0.37 (0.19–0.67)	1.4 (0.73–4.6)
White Leghorn chicken	TCDD	0.087 (0.045–0.13)	0.21 (0.15–0.29)	0.55 (0.39–0.90)
	PeCDF	0.12 (0.092–0.15)	0.26 (0.22–0.30)	0.54 (0.44–0.68)
	TCDF	0.049 (0.028–0.070)	0.10 (0.070–0.14)	0.21 (0.16–0.34)

Note. LD values calculated using a Probit model incorporating background mortality (Japanese quail = 14.6%, Common pheasant = 17.9%, and White Leghorn chicken = 12.5%) into the curve fitting calculations.

were analyzed using a linear regression model (Proc Reg). A single liver concentration with an *R* student value greater than 7 was considered an outlier and removed from the data set.

Calculation of relative potency and sensitivity values. The use of relative potency values to compare potencies of TCDD-like compounds within a particular species has been described in Van den Berg *et al.* (1998). In the present study, relative potency values were derived as the ratio of the LD_{50} value for TCDD and the LD_{50} of the compound of interest; in this case, PeCDF or TCDF. To evaluate compound-specific differences between species, relative sensitivity values were calculated as the ratio of the LD_{50} value of the presumed most sensitive species (chicken) and the LD_{50} for the species of interest (quail or pheasant).

RESULTS

Effects of TCDD, PeCDF, and TCDF on Mortality

In ovo administration of TCDD, PeCDF, or TCDF caused a dose-related increase in embryo mortality for the Japanese quail, Common pheasant, and White Leghorn chicken (Table 2, Fig. 1). Embryo mortality in the vehicle control group was 14% for the quail, 20% for the pheasant, and 16% for the chicken (Table 2). Dose-response curves based on lethality, calculated as the ratio of the number of dead embryos to the number of fertile eggs for each dose group, and LD_{50} values (95% confidence intervals [CI]) were adjusted for background mortality (Tables 3 and 4, Fig. 1).

Relative Potencies and Species Sensitivity

Based on mortality, TCDD was not the most potent of the three compounds assessed in this study in Japanese quail, Common pheasant, or White Leghorn chicken (Fig. 1). In the quail, the order of chemical potency was PeCDF > TCDF > TCDD based on relative potency values of 6.1 for PeCDF and 2.0 for TCDF (Table 5, Fig. 1a). In the pheasant, the order of chemical potency was PeCDF > TCDF > TCDD based on relative potency values of 5.7 for PeCDF and 2.9 for TCDF (Table 5, Fig. 1b). In the chicken, the order of chemical potency was TCDF > TCDD ≈ PeCDF based on relative potency values of 2.0 and 0.88 for TCDF and PeCDF, respectively (Table 5, Fig. 1c). The order of species sensitivity was relatively consistent for all three compounds based on relative sensitivity values (Table 6). For TCDD, the order of species sensitivity was chicken > pheasant > quail based on relative sensitivity values of 0.19 for the pheasant and 0.022 for the quail; for PeCDF, the order of sensitivity was pheasant ≈ chicken > quail based on relative sensitivity values of 1.2 for the pheasant and 0.15 for the quail; and for TCDF, the order of species sensitivity was chicken > pheasant > quail based on relative sensitivity values of 0.28 for the pheasant and 0.022 for the quail.

Concentrations of TCDD, PeCDF, and TCDF in livers of chicks

Figure 2 illustrates the relationship between the injected dose and hepatic concentration of each compound in 1- and 14-day chicks. In 1-day chicks, the correlation between dose and liver concentration was significant in all cases with the exception of chickens exposed to TCDF. The significant correlations between injected dose and hepatic concentration were weak in Japanese quail for all three compounds (R^2 < 0.25 was designated as weak). At 14 days of age, most of the treatment groups had significant correlations between dose and hepatic concentration, with the exception of pheasants exposed to TCDD and quail exposed to TCDF. All the significant correlations at 14 days had R^2 values greater than 0.25.

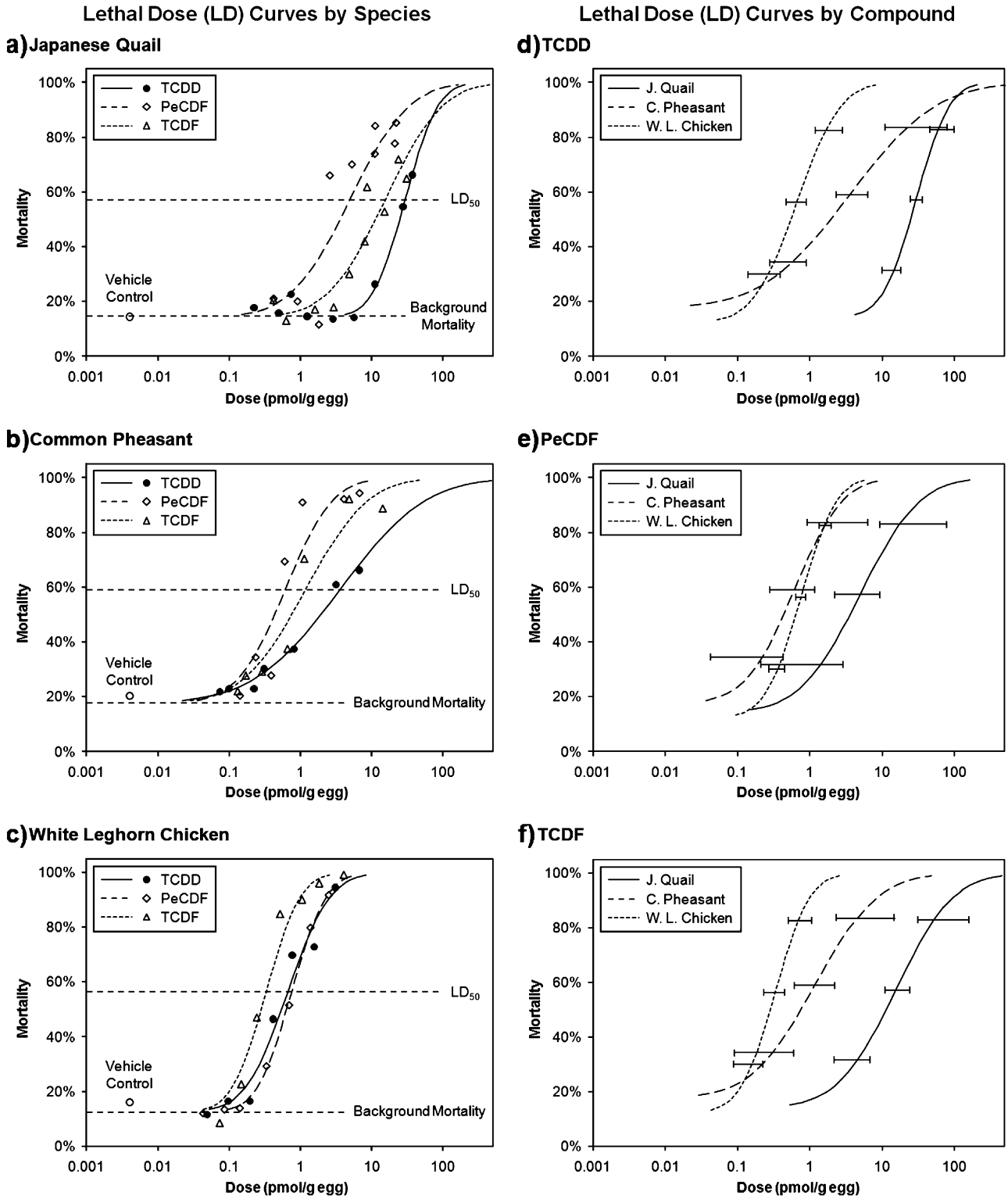

FIG. 1. Mortality of Japanese quail, Common pheasant, and White Leghorn chicken eggs injected with TCDD, TCDF, or PeCDF prior to incubation by species (a,b,c) and by compound (d,e,f). Mortality curves take into account the rate of background mortality for each species.

TABLE 5
Relative Potency (RePs) Values for PeCDF and TCDF Compared with TCDD Based on Lethal Dose and Effective Concentration (EC) Estimates in Japanese Quail, Common Pheasant, and White Leghorn Chicken Embryos after *In Ovo* Exposure Prior to Incubation

Species	Compound	LD_{20} ReP	LD_{50} ReP	LD_{80} ReP	EC_{50} ReP
Japanese quail	TCDD	1.0	1.0	1.0	1.0
	PeCDF	11	6.1	3.3	13[a]
	TCDF	3.3	2.0	1.2	0.1[a]
Common pheasant	TCDD	1.0	1.0	1.0	1.0
	PeCDF	2.6	5.7	13	3.4[a], 15[b]
	TCDF	1.8	2.9	4.9	0.8[a], 0.7[b]
White Leghorn chicken	TCDD	1.0	1.0	1.0	1.0
	PeCDF	0.75	0.88	1.1	0.9[a], 0.5[c]
	TCDF	1.7	2.0	2.5	0.9[a], 0.6[c]

[a]Based on *in vitro* EC_{50} values for maximal EROD induction from Hervé *et al.* (2010b).
[b]Based on *in ovo* EC_{50} values for *CYP1A4* messenger (mRNA) expression from Yang *et al.* (2010).
[c]Based on *in ovo* EC_{50} values for *CYP1A5* mRNA expression from Yang *et al.* (2010).

TABLE 6
Relative Sensitivity (ReS) Values of TCDD, PeCDF, and TCDF for Common Pheasant and Japanese Quail Compared with White Leghorn Chicken

Compound	Species	LD_{20} ReS	LD_{50} ReS	LD_{80} ReS	EC_{50} ReS
TCDD	White Leghorn chicken	1.0	1.0	1.0	1.0
	Common pheasant	0.47	0.19	0.077	0.2[a]
	Japanese quail	0.018	0.022	0.028	0.09[a]
PeCDF	White Leghorn chicken	1.0	1.0	1.0	1.0
	Common pheasant	1.6	1.2	0.94	0.8[a]
	Japanese quail	0.26	0.15	0.089	1.3[a]
TCDF	White Leghorn chicken	1.0	1.0	1.0	1.0
	Common pheasant	0.52	0.28	0.15	0.2[a]
	Japanese quail	0.035	0.022	0.013	0.01[a]

[a]Based on *in vitro* effective concentration (EC_{50}) values for maximal EROD induction from Hervé *et al.* (2010b).

DISCUSSION

The study described herein was part of a group of collaborative studies designed to further validate, at the molecular (Yang *et al.*, 2010), *in vitro* (Hervé *et al.*, 2010a,b) and *in ovo* levels, the proposed avian sensitivity classification model based on differences in the amino acid sequence of the aryl hydrocarbon receptor ligand-binding domain. Each study used the same species from each of the proposed sensitivity classes and the same three TCDD-like compounds. The ultimate goal of this line of research is to firmly establish a predictive tool that reduces the uncertainty associated with avian species sensitivity to TCDD-like compounds for ecological risk assessment.

We show here that PeCDF is the most potent compound (approximately sixfold compared with TCDD) followed by TCDF (two- to threefold compared with TCDD) in terms of embryotoxicity in both the Japanese quail and the Common pheasant, whereas TCDF is more potent (approximately twofold) than TCDD and PeCDF in the chicken. Furthermore, we demonstrate that the chicken is the most sensitive species to *in ovo* TCDD and TCDF exposure, followed by the pheasant and then the quail, supporting the species sensitivity classification model. The chicken and pheasant are equally sensitive to PeCDF, whereas the quail is approximately six- to eightfold less sensitive.

Control Mortality Data

Mortality of vehicle control embryos (Table 2) was similar to historical or control mortality values from other studies using the Japanese quail, Common pheasant, or White Leghorn chicken. In the present study, vehicle control mortality in Japanese quail was 14%, comparable to the historical hatchability of untreated Japanese quail eggs at the MSU Poultry Research and Teaching Center. In the Common pheasant, vehicle control mortality in the present study was 20%, which was half of the value reported by Nosek *et al.* (1993). One explanation for the differences in control mortality between these two studies could be the difference in vehicles. In the study by Nosek *et al.* (1993), TCDD was partitioned into 1,4-dioxane before it was injected into the egg, whereas triolein, a naturally occurring triglyceride of oleic acid, was used in the present study. The 1,4-dioxane vehicle control mortality was 38% (30/80) when the site of injection was albumin and 50% when the site of injection was the yolk (40/80). The site of injection can also explain the difference in mortality in that yolk injection typically results in greater mortality than air cell injection (Henshel *et al.*, 1997). The natural rate of embryo mortality for the Common pheasant has been reported to be approximately 30% (Fant, 1957). In the present study, mortality of vehicle control White Leghorn chicken embryos was 16%. This is within the range reported in other chicken egg injection studies where triolein was used as the vehicle. Mortalities of embryos exposed to this vehicle by yolk sac injection were 23% (13/56) and 13%, respectively (Blankenship *et al.*, 2003; Powell *et al.*, 1996).

Effects of TCDD, PeCDF, and TCDF on Mortality

At present, little information is available about *in ovo* toxicity of TCDD, PeCDF, and TCDF in Galliform species other than the chicken. The LD_{50} values (95% CI) for the Japanese quail of 30 (25, 36) pmol TCDD/g egg, 4.9 (2.3, 9.2) pmol PeCDF/g egg, and 15 (11, 24) pmol TCDF/g egg reported here (Table 3) are the first published for these

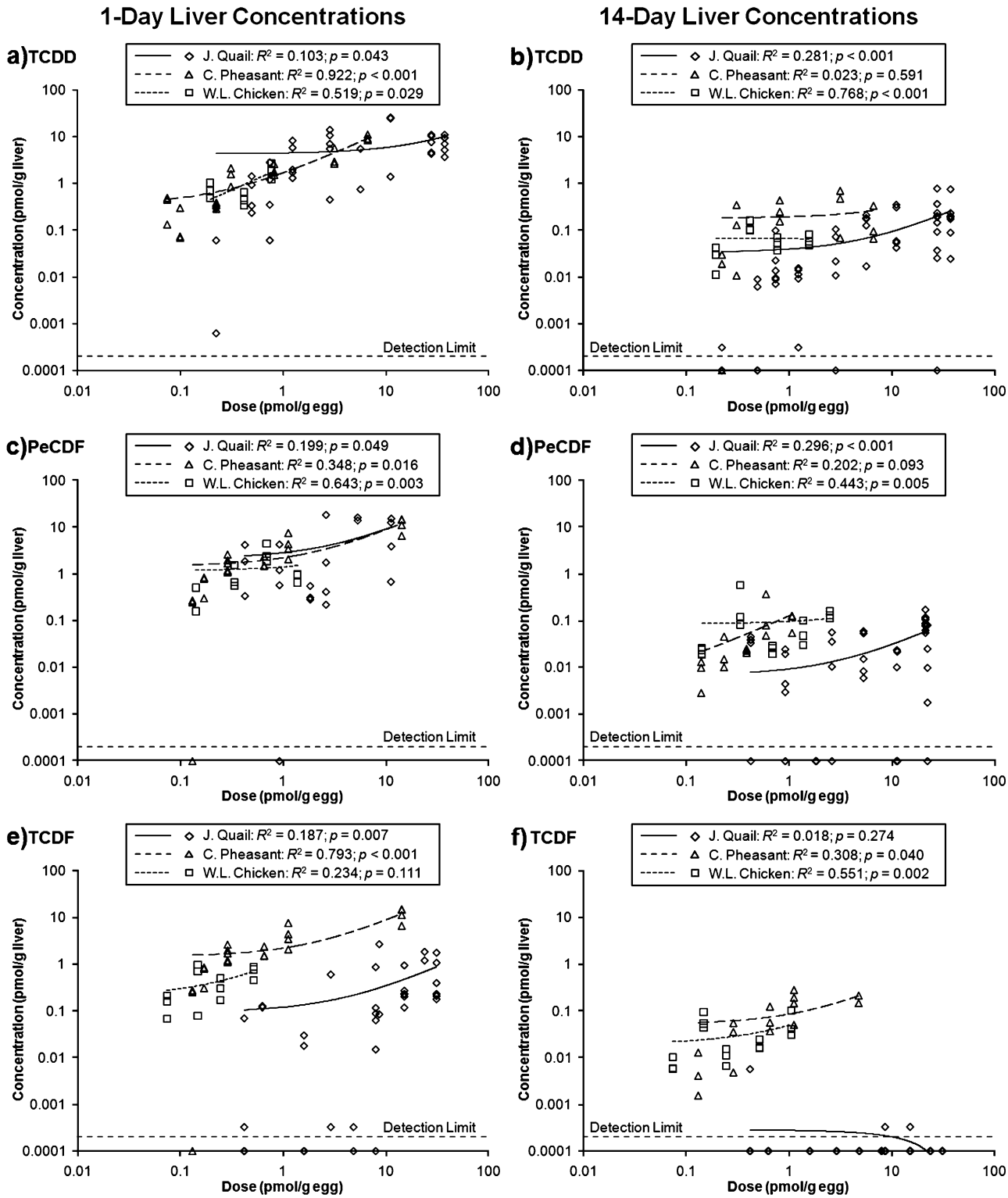

FIG. 2. Concentrations of TCDD (a,b), PeCDF (c,d), or TCDF (e,f) in the livers of 1- and 14-day-old Japanese quail, Common pheasant, and White Leghorn chicken. Linear regression lines, R^2, and associated p values are presented for each species for both age groups.

compounds in this species. The LD_{50} values of 3.5 (2.3, 6.3) and 0.66 (0.47, 0.90) pmol TCDD/g egg for the Common pheasant and White Leghorn chicken (Table 3), respectively, are similar to those reported in other *in ovo* toxicity studies. For pheasants, Nosek *et al.* (1993) reported an LD_{50} of 4.2 pmol TCDD/g egg when injected into the albumin (within the 95%

CI of the LD_{50} reported here) and 6.8 pmol TCDD/g egg when injected into the yolk. In chickens, Verrett (1976) and Powell *et al.* (1996) both reported an LD_{50} of 0.47 pmol/g egg, which approximates the lower 95% CI in the present study, whereas Allred and Strange (1977) reported an LD_{50} of 0.75 pmol TCDD/g egg. Injection into the air cell resulted in an LD_{50} value of 0.92 pmol TCDD/g egg, whereas injection into the yolk resulted in an LD_{50} of 0.38 pmol TCDD/g egg (Henshel *et al.*, 1997). At present, there are no other published reports on the *in ovo* toxicity of PeCDF or TCDF in either the Common pheasant or the White Leghorn chicken.

Relative Potencies of PeCDF and TCDF

The first objective of the present study was to assess the relative *in ovo* potencies of TCDF and PeCDF compared with TCDD in the quail, pheasant, and chicken. PeCDF was the most potent compound followed by TCDF in both the Japanese quail and the Common pheasant, whereas TCDF was more potent than TCDD and PeCDF in the chicken (Table 5). Relative potencies based on effective $concentration_{50}$ values from companion *in vitro* studies are generally consistent with the results of this study in that they indicated TCDD was not the most potent TCDD-like compound in quail, pheasant, or chicken.

In Japanese quail, Hervé *et al.* (2010b) reported PeCDF to be the most potent chemical (relative potency = 13) and TCDF to be the least potent (relative potency = 0.1) based on EROD induction in primary hepatocyte cultures, whereas the *in ovo* data reported here indicate TCDD to be less potent than TCDF (Table 5).

In the pheasant, both Hervé *et al.* (2010b) and Yang *et al.* (2010) reported PeCDF to be the most potent based on EROD induction (relative potency = 3.4) or *CYP1A4* expression (relative potency = 15) in primary hepatocyte cultures, which agrees with the *in ovo* results of the present study. The potency of TCDF in the pheasant, based on EROD induction (relative potency = 0.8) and *CYP1A4* expression (relative potency = 0.7), was comparable to TCDD (Hervé *et al.*, 2010b; Yang *et al.*, 2010) (Table 5). Similarly, Kennedy *et al.* (1996) reported a relative potency value of 0.8 for TCDF based on maximal EROD induction in primary cultures of pheasant hepatocytes. In contrast, the *in ovo* data presented here indicate TCDF was almost threefold more potent than TCDD.

In the chicken, the relative potencies among the three compounds were similar based on EROD induction (Hervé *et al.*, 2010b) or *CYP1A5* expression (Yang *et al.*, 2010) in hepatocyte cultures (Table 5). These results are consistent with those reported by Bosveld *et al.* (1992) and Kennedy *et al.* (1996) who assessed EROD induction in hepatocytes as well. In contrast, *in ovo* results indicated that TCDF was twofold more potent than TCDD and PeCDF. The greater potency of TCDF *in ovo* compared with *in vitro* potency among these species indicates that the *in vitro* approach may not always accurately reflect *in vivo* toxicity.

Relative Sensitivity of Japanese Quail and Common Pheasant Compared with White Leghorn Chicken

The second objective of this study was to confirm, *in ovo*, the proposed avian species sensitivity classification model based on *in vitro* work. The order of species sensitivity was chicken > pheasant > quail based on relative sensitivity values for TCDD and TCDF (Table 6, Figs. 1d and 1f). The order of species sensitivity to PeCDF was pheasant ≈ chicken > quail (Table 6, Fig. 1e).

The order of species sensitivity for TCDD and TCDF reported in this study is the same as that based on *in vitro* studies. The Japanese quail was reported to be 11-fold less sensitive than the chicken based on induction of EROD activity in primary hepatocyte cultures, and the pheasant was fivefold less sensitive (Table 6) (Hervé *et al.*, 2010b). For PeCDF, the Japanese quail and pheasant are similar to the White Leghorn chicken in sensitivity based on relative sensitivity values of 1.3 and 0.8, respectively, derived from hepatocyte EROD induction data (Table 6) (Hervé *et al.*, 2010b).

Concentrations of TCDD, PeCDF, and TCDF in Liver

With the exception of TCDF-exposed 14-day Japanese quail, there was a positive association between the concentrations of all three compounds in the liver to the dose injected (Fig. 2). In 14-day quail, only 3 of the 69 samples (4.3%) had detectable concentrations of TCDF, in contrast to 1-day quail where 30 of the 38 samples (79%) had detectable concentrations. These results suggest that this species has the ability to metabolize and/or eliminate TCDF to a greater extent than TCDD or PeCDF. In general, differences in concentrations of all three compounds between 1- and 14-day chicks can be attributed to growth dilution when concentrations for both age groups are normalized for growth using the following equations:

$$\text{1day growth normalized concentration} = \text{1day concentration} \times (\text{1day chick mass}/\text{14day chick mass}),$$

$$\text{14day growth normalized concentration} = \text{1day concentration} \times (\text{14day chick mass}/\text{1day chick mass}).$$

For example, using means from the 0.29 pmol TCDF/g egg dose group of pheasants, the original hepatic TCDF concentration in 1-day chicks of 1.62 pmol/g liver is converted to 0.355 pmol/g liver and the original TCDF concentration in 14-day chicks of 0.0717 pmol/g liver is converted to 0.327 pmol/g liver. Thus, when adjusted for growth, the two concentrations are very similar. It should be noted that concentrations reported here are representative of only those embryos surviving until

hatch; thus, these values may include bias associated with embryo mortality.

Differences in the metabolism of TCDF and other TCDD-like compounds have been reported in other avian species. In cormorant populations residing in environments contaminated with both PeCDF and TCDF, preferential metabolism of TCDF is implied in that liver and muscle tissue had elevated concentrations of PeCDF and minimal concentrations of TCDF (Kubota *et al.*, 2005, 2006). Bald eagle tissues containing the greatest concentrations of TCDD also contained the least concentrations of TCDF (Kumar *et al.*, 2002). These observations are consistent with upregulation of hepatic *CYP450* genes in eagles exposed to elevated concentrations of TCDD that suggested enhanced metabolism of TCDF.

Results of this and companion studies indicate that (1) the potency of TCDD-like chemicals in birds varies among species and TCDD is not necessarily the most potent in this class of compounds and (2) the avian sensitivity classification scheme based on amino acid substitutions in the ligand-binding domain of the aryl hydrocarbon receptor deserves serious consideration as a tool for ecological risk assessment. The variation in potency of TCDD-like compounds within species highlights the potential uncertainty associated with the use of toxic equivalency factors in risk assessment. Categorization of a greater number of avian species in terms of their sensitivity to TCDD-like compounds accompanied by adequate *in vitro* and, when possible, *in ovo* confirmation should reduce the error inherent in assigning risk associated with environmental exposure.

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