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DEVELOPMENTAL AND POSTHATCH EFFECTS OF IN OVO EXPOSURE TO 2,3,7,8-TCDD, 2,3,4,7,8-PECDF, AND 2,3,7,8-TCDF IN JAPANESE QUAIL (*COTURNIX JAPONICA*), COMMON PHEASANT (*PHASIANUS COLCHICUS*), AND WHITE LEGHORN CHICKEN (*GALLUS GALLUS DOMESTICUS*) EMBRYOS

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Abstract—An egg injection study was conducted to confirm a proposed model of relative sensitivity of three avian species to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-like chemicals. It was previously reported that the order of species sensitivity to in ovo exposure to TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF) at doses ranging from 0.044 to 37 picomoles (pmol)/g egg was the chicken (*Gallus gallus domesticus*), common pheasant (*Phasianus colchicus*), and Japanese quail (*Coturnix japonica*) based on embryo mortality and hepatic enzyme induction. In the present study, the incidence of developmental deformities, changes in body and relative organ masses, and organ pathology of hatchlings as additional indicators of species sensitivity were assessed; in addition, embryo mortality in the three species was categorized by stage of development. Embryo mortality varied temporally with significant increases generally occurring after organogenesis and just prior to hatching. A significant increase in the percentage of developmental deformities was observed only in Japanese quail exposed to TCDF. Body and relative organ masses of quail, pheasants, and chickens dosed in ovo with TCDD, PeCDF, or TCDF were not consistently affected. Chemical-related pathology occurred only in livers of quail at the greatest doses of each compound. These results indicated that the incidence of developmental deformities, changes in body and relative organ masses and organ pathology could not be used as indicators of species sensitivity or chemical potency. Environ. Toxicol. Chem. 2011;30:1659–1668. © 2011 SETAC

Keywords—2,3,7,8-Tetrachlorodibenzo-*p*-dioxin 2,3,4,7,8-Pentachlorodibenzofuran 2,3,7,8-Tetrachlorodibenzofuran Egg injection Deformity

INTRODUCTION

Currently, elevated concentrations of polychlorinated dibenzofurans (PCDFs) and measurable concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been detected in several freshwater ecosystems of the Great Lakes region [1–3]. Historically, exposure to these and other TCDD-like compounds has been linked to the impairment of reproductive performance in avian species, including the double-crested cormorant (*Phalacrocorax auritus*) [4], herring gull (*Larus argenatatus*) ([5,6], common tern (*Sterna hirundo*) [7], Caspian tern (*Hydroprogne caspia*) [8], and Forster's tern (*Sterna forsteri*) [9]. Unfortunately, characterization of the effects of TCDD-like compounds in birds in contaminated areas remains a significant challenge. This is due, in part, to environmental concentrations that differ spatially and temporally [10,11] as well as to differences in species-specific sensitivity [12–14].

The toxicity of TCDD and TCDD-like compounds is thought to be due to their interactions with the aryl hydrocarbon receptor (AhR), a ligand-activated nuclear transcription factor [15]. Among birds, variations in the amino acid sequence of the ligand-binding domain of the AhR have been associated with differences in sensitivity to TCDD-like compounds [16,17]. Those species with an amino acid sequence similar to that of the white leghorn chicken (*Gallus gallus domesticus*) are considered the most sensitive, those similar to the common pheasant (*Phasianus colchicus*) are moderately sensitive, and those similar to the Japanese quail (*Coturnix japonica*) are least sensitive. However, phylogenetic relationships among species do not always correspond to sensitivity classifications or AhR genotypes [17].

Clinical signs of exposure to TCDD and TCDD-like compounds are similar among avian species. These include increased embryonic and chick mortality, growth retardation, and developmental abnormalities such as bill deformities, club feet, missing eyes, and defective feathering [10,18,19]. Other clinical signs include subcutaneous, pericardial and peritoneal edema, hepatomegaly, hepatic necrosis, porphyria, and the induction of several mixed-function monooxygenase enzymes [6,20–22]. The manifestation of these signs in water birds of the Great Lakes area is referred to as Great Lakes Embryo Mortality, Edema, and Deformities Syndrome (GLEMEDS), which is consistent with chick edema disease previously described in commercial poultry [18,20]. The majority of these effects have been noted in wild populations of birds exposed to complex environmental mixtures of TCDD-like compounds or various avian species in laboratory settings exposed to commercial polychlorinated biphenyl (PCB) mixtures or individual TCDD-like congeners. Few studies have addressed avian exposure to specific TCDD-like congeners of the PCDF family.

All Supplemental Data may be found in the online version of this article.

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Currently, risk assessments of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) are based on ethoxyresorufin-*O*-deethylase (EROD)-induction potential from egg injection and hepatocyte studies [12,22–24]. More recently, a series of collaborative studies was conducted to assess differences in relative potency among these compounds, including TCDD, and species sensitivity among the Japanese quail, common pheasant, and white leghorn chicken; Galliform species from each of the proposed avian sensitivity classification categories. Estimates of lethal doses (LD) derived from embryo mortality after in ovo exposure [14] and effective concentration (EC) estimates based on EROD, cytochrome P450 1A4 (CYP1A4), or CYP1A5 induction (in ovo [14,25]; in vitro [13]) were used to compare sensitivities of species and potencies of compounds. Median lethal dose values for TCDD, PeCDF, and TCDF were 30, 4.9, and 15 pmol/g egg for quail, 3.5, 0.61, and 1.2 pmol/g egg for pheasant and 0.66, 0.75, and 0.33 pmol/g egg for chicken, respectively. These results confirmed the presumed order of species sensitivity (most sensitive to least sensitive) of chicken > pheasant > quail. Relative potencies of PeCDF and TCDF compared to TCDD based on LD50 values were 6.1 and 2.0 for quail, 5.7 and 2.9 for pheasant, and 0.88 and 2.0 for chicken, respectively [14].

In the present study we examined developmental and post-hatching endpoints resulting from in ovo exposure to TCDD, PeCDF, or TCDF, and compared these endpoints between the Japanese quail, common pheasant, and white leghorn chicken to determine if any of these endpoints could be used to assess species sensitivity and/or chemical potency in a way similar to embryo lethality and enzyme induction [13,14,25]. Endpoints included the incidence and types of developmental deformities, body and relative organ masses of 1- and 14-d-old chicks, and pathology of the liver, heart, brain, bursa, and spleen from 14-d-old chicks. Additionally, the stage at which embryo mortality occurred during incubation was examined.

MATERIALS AND METHODS

Experimental design

The present study, which was approved by the Michigan State University Institutional Animal Care and Use Committee, was divided into three separate experiments, one for each species, as described in greater detail in Cohen-Barnhouse et al. [14]. 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 each year. Nine doses of TCDD and PeCDF and 10 doses of TCDF were injected into Japanese quail eggs (100 eggs per dose group; 200 eggs in the vehicle control group), while seven doses of each test compound were injected into pheasant (80 eggs per dose group including vehicle control) and chicken eggs (100 eggs per dose group including vehicle control). The number of fertile eggs in each dose group by species is presented in Table 1. Doses were based on previous egg injection studies [26–28], estimates regarding species sensitivity [17], and environmentally relevant concentrations of each test compound in wild avian species [3].

Egg preparation

Pheasant eggs were purchased from McFarlane Pheasants while Japanese quail and white leghorn chicken eggs were

Table 1. Doses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (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	(pmol/g egg)	*n*	(pmol/g egg)	*n*	(pmol/g egg)	*n*
Vehicle control[a]	0.0	180	0.0	74	0.0	99
TCDD	0.22	90	0.075	69	0.049	95
	0.5	95	0.1	70	0.096	97
	0.75	93	0.22	70	0.19	97
	1.2	89	0.31	73	0.42	97
	2.9	88	0.82	75	0.77	96
	5.7	92	3.2	69	1.6	99
	11	91	6.7	74	3.1	91
	28	88				
	37	77				
PeCDF	0.42	95	0.14	69	0.044	99
	0.92	90	0.24	67	0.087	96
	1.8	95	0.39	65	0.14	100
	2.6	94	0.6	75	0.33	99
	5.3	90	1.1	77	0.69	99
	11.2	88	4.1	76	1.4	94
	11.3	44	6.8	70	2.5	96
	21	85				
	22	88				
TCDF	0.42	93	0.13	68	0.074	95
	0.63	93	0.17	72	0.15	93
	1.6	94	0.29	72	0.25	96
	2.9	90	0.65	72	0.52	98
	4.8	90	1.1	74	1.1	99
	7.9	86	4.8	75	1.8	98
	8.6	89	14	70	4	99
	15	89				
	24	89				
	31	91				

[a] Vehicle control = triolein.

obtained from the Michigan State University (MSU) Poultry Research and Teaching Center. All pheasant eggs were laid on the same day, while quail and chicken eggs were collected over a one-week period. Eggs were stored in a cooler for no longer than one week at 13.5 to 15.0°C until 24 h prior to injection. Eggs were weighed to the nearest 0.1 g and were candled to detect any subtle damage to the shell. Undamaged eggs with mean weights (±1 standard deviation [SD]) of 9.8 ± 0.74 g for quail, 29.4 ± 2.1 g for pheasants, and 56.3 ± 3.2 g for chickens had the center of their air cells marked in pencil to outline the injection site. Eggs were assigned a unique identification number written in pencil on the exterior of the shell.

Injection solutions and procedures

The preparation of injection solutions and egg injection procedures are described in Cohen-Barnhouse et al. [14]. Stock solutions of TCDD, TCDF, and PeCDF (Sigma-Aldrich) were prepared by dissolving each chemical in triolein. Solutions were then cold-filtered with a 0.22 μm syringe filter prior to serial dilution. 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 an injection volume of 0.1 to 0.2 μl/g egg does not induce excessive embryo mortality [27]. Following preparation of dosing solutions, injection vials were flooded with argon to preserve the triolein, capped, and sterilized in an autoclave. The injection site was cleaned with 70% ethanol immediately before eggs were injected in a laminar flow hood (NuAire). A single hole was drilled through the shell into the air cell using a Dremel® tool (Robert Bosch Tool). Injections were made with a positive displacement pipettor (Gilson) and the sterile pipette tip was changed after each injection. The site of injection was then sealed using heated paraffin (Royal Oak Sales) applied with a sterile wooden applicator. Incubation was initiated after all eggs were injected.

Incubation and hatching procedures

Eggs were placed injection site up in a Petersime® rotary incubator (Petersime Incubator). Incubation parameters were standard for commercial operations (37.6°C with 50–60% relative humidity). Eggs were automatically rotated every 2 h for 13 d (quail), 17 d (pheasant), or 16 d (chicken). Three days prior to the expected hatching date, eggs were transferred to the hatching trays of a Surepip hatcher (Agro Environmental Systems). The internal environment of the hatcher was maintained at 37.6°C with 60 to 70% relative humidity. There was one treatment group per hatching tray. Dividers were inserted in each tray to allow placement of eggs into individual compartments. Eggs were examined for evidence of hatching from 1 d prior to the expected hatching date to 2 d beyond anticipated hatching.

Embryo necropsy

Embryos that failed to hatch were opened to assess the time of mortality. Prior to opening, all eggs were candled to check for fertility and possible damage that may have occurred during transport or incubation. Embryos were categorized into one of five stages of development (quail: embryonic day [ED] 0 to ED 3, ED 4 to ED 6, ED 7 to ED 10, ED 11 to ED 13, ED 14 to pipping; pheasants: ED 0 to ED 5, ED 6 to ED 10, ED 11 to ED 15, ED 16 to ED 20, or ED 21 to pipping; chickens: ED 0 to ED 4, ED 5 to ED 8, ED 9 to ED 12, ED 13 to ED 16, and ED 17 to pipping) based on key developmental characteristics.

Hatchling necropsy

A subsample of 10 chicks from each dose group from each species was randomly taken from all treatment groups and euthanized by cervical dislocation at 1 d and 14 d of age. Livers from all chicks were removed, weighed, and a portion was placed in an I-Chem® jar (VWR International) on ice for subsequent contaminant analysis [14]. Additional samples of liver from 14-d-old chicks were placed into a microtube containing RNAlater® (Ambion) for analysis of *CYP1A4* and *CYP1A5* mRNA expression [25], a microtube frozen in liquid nitrogen for analysis of EROD activity [25], and a vial with 10% buffered formalin for histological evaluation. Livers from all dose groups, as well as hearts, spleens, and bursas from the control and greatest dose groups for each compound, were assessed for pathological changes.

Contaminant analysis

Concentrations of TCDD, PeCDF, and TCDF in dosing solutions for all three species were determined as described previously [14]. In general, congener concentrations were determined by isotope dilution following the U.S. Environmental Protection Agency's (U.S. EPA) method 1613b [29]. Triolein injection solutions were serially diluted with hexane prior to the addition of a mixture of [^{13}C]PCDDs and [^{13}C]PCDFs (Wellington Laboratories). The methodology for the identification and quantification of these compounds, as well as the quality assurance and quality control (QA/QC) procedures, are described in Wan et al. [30].

Data analysis

All statistical analyses were performed using SAS® (v. 9.2) with statements of significance based on $p < 0.05$. Categorical data (stage of embryo death) 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. Due to the nature of binomial analysis, when the total incidence of a particular stage in the control group was equal to zero, a dummy variable with an incidence of one was added to allow for comparisons between doses. Relative organ masses (percent of body mass) were compared across dose groups, and as percentage data (P), subjected to arcsine square root transformation [$x = \sin^{-1}(\sqrt{P})$] prior to statistical analysis. The reported means and 95% confidence intervals of chick relative organ masses were back transformed [$P = (\sin(x))^2$] to the scale of observation. Differences between body and relative organ masses were compared using a mixed linear model (Proc Glimmix) and compared against control values using Dunnett's test. Differences between or among trials within species, when appropriate, were taken into account within each analysis.

RESULTS

Stage of embryo mortality

In the Japanese quail, common pheasant, and white leghorn chicken vehicle control groups, most embryonic mortality occurred near the beginning or end of incubation (first and last stages). This pattern remained consistent, with 15.4% (4/26) of embryo mortality occurring between ED 0 and ED 3 and 73.1% (19/26) occurring between ED 14 and pipping in quail; 20.0% (3/15) of embryo mortality occurring between ED 0 and ED 5 and 60.0% (9/15) between ED 21 and pipping in pheasants; and 18.8% (3/16) of embryo mortality occurring between ED 0 and

ED 4 and 62.5% (10/16) between ED 19 and pipping in chickens.

The mortality of embryos exposed to the three compounds of interest varied temporally throughout incubation in all three species (Figs. 1–3). In general, embryo mortality had two peaks. The first occurred around the second developmental stage and the second occurred during the last developmental stage, just prior to hatching. In Japanese quail, a significant increase in the incidence of embryo mortality was observed between ED 4 and ED 10 for all three compounds at doses greater than 11 pmol TCDD/g egg ($p < 0.0264$), 1.8 pmol PeCDF/g egg ($p < 0.0255$), and 7.9 pmol TCDF/g egg ($p < 0.0064$) when compared to the vehicle control (Figs. 1–3). A significant increase in embryo mortality during the ED 14 to pipping stage also occurred in those embryos exposed to TCDF between 7.9 and 15 pmol/g egg ($p < 0.0022$) and in the 2.6 pmol PeCDF/g egg dose group ($p = 0.0125$) (Figs. 2, 3). In the common pheasant, significant increases in embryo mortality occurred between ED 6 and ED 10 at doses greater than 0.82 pmol TCDD/g egg ($p < 0.0001$), 0.39 pmol PeCDF/g egg ($p < 0.0080$), and 0.65 pmol TCDF/g egg ($p < 0.0001$) (Figs. 1–3). In the white leghorn chicken, there was significantly greater embryo mortality between ED 0 and ED 4 in the 3.1 pmol TCDD/g egg dose group ($p = 0.0011$) and at doses greater than 1.1 pmol TCDF/g egg ($p < 0.0027$). All three compounds caused significantly greater embryo mortality between ED 5 and ED 8 at doses greater than 0.19 pmol TCDD/g egg ($p < 0.0246$), 0.34 pmol PeCDF/g egg ($p < 0.0008$), and 0.15 pmol TCDF/g egg ($p < 0.0085$). In addition, significantly greater embryo mortality between ED 9 and ED 12 occurred at doses greater than 0.77 pmol TCDD/g egg ($p < 0.0435$), and between 0.25 and 4.0 pmol TCDF/g egg ($p < 0.0095$) (Figs. 1–3). For surviving hatchlings of all three species, posthatch mortality was not significantly different from that of the vehicle control.

Deformities

The percentage and types of deformities that occurred in quail, pheasants, and chickens exposed in ovo to TCDD, PeCDF, or TCDF are illustrated in Figures 4 to 6 and presented in Supplemental Data, Tables S1–4. The percent of deformed quail, pheasant, and chicken vehicle control embryos was 1.7, 1.4, and 0.0%, respectively. The percent of deformed embryos resulting from exposure to TCDD, PeCDF, and TCDF was 2.2, 3.5, and 4.6%, respectively, for quail, 1.6, 1.4, and 1.4%, respectively, for pheasants and 0.60, 0.88, and 0.44%, respectively, for chickens. The only significant increases in the percentage of deformities compared to vehicle controls occurred in Japanese quail exposed to TCDF at doses of 2.9 ($p = 0.0266$), 4.8 ($p = 0.0055$), 8.6 ($p = 0086$), 15 ($p = 0.0259$), and 24 pmol TCDF/g egg ($p = 0.0032$). Examination of Figures 4 to 6 suggests for Japanese quail that doses of TCDD and TCDF resulting in the greatest number of deformities were also those doses that resulted in the greatest mortality, whereas the incidence of deformed embryos in the PeCDF group tended to occur at intermediate doses. In pheasants exposed to TCDD, the incidence of deformed embryos occurred over the range of doses, whereas for PeCDF and TCDF, deformities tended to be associated with intermediate doses. For the chicken, the relatively small percentage of deformed embryos occurred across the range of doses.

Morphological deformities were grouped into four categories: cranial, bill, trunk, and limb (Supplemental Data, Tables S1–4). Cranial deformities included microphthalmos and anophthalmos (deformed or absences of eyes), anencephaly or exencephaly (absence or partial exposure of the brain), or acephalia (absence of head). Deformities of the bill were characterized by incomplete development or crossing of the upper and lower bill. Trunk deformities included edema, gastroschisis (exposed abdominal cavity), and achondroplasia (dwarfism), while limb deformities included curled toes, club feet, and supernumerary appendages. Of the 2,476 quail embryos dosed with TCDD, PeCDF, or TCDF, 3.2% were deformed. The majority of total deformities ($n = 105$) were of the bill (36%) and limbs (43%), with fewer instances of cranial (15%) and

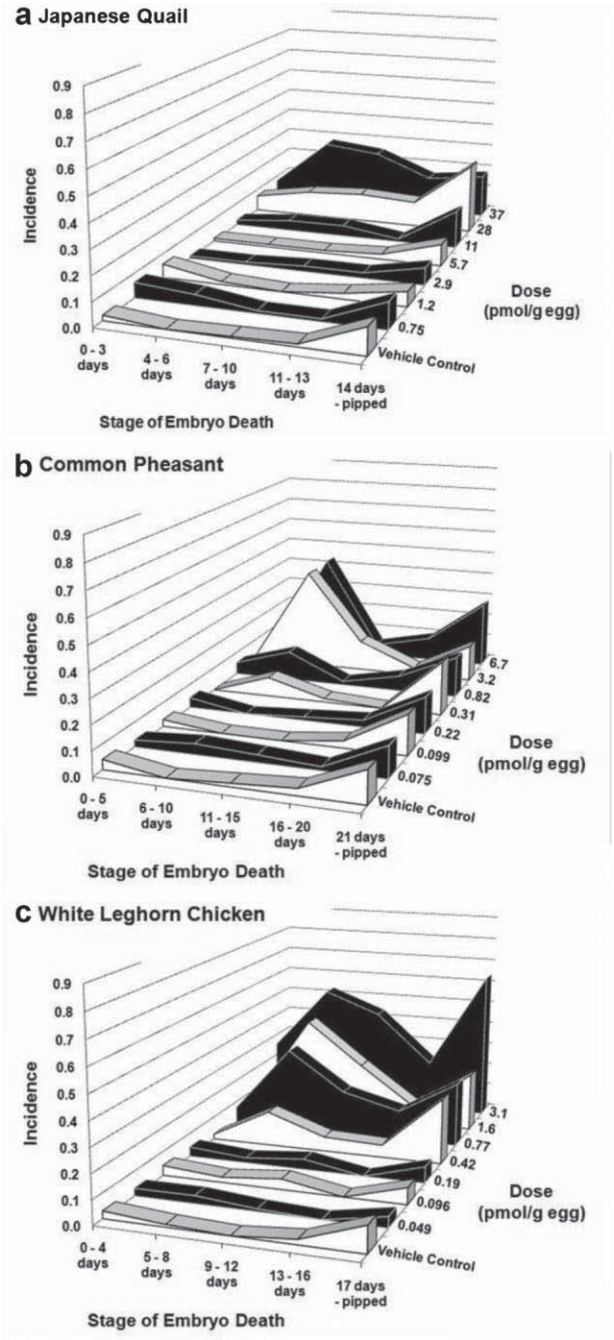

Fig. 1. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on stage of mortality in Japanese quail (**a**), common pheasant (**b**), and white leghorn chicken (**c**) embryos. Five stages were identified based on the appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence equals the number of observed mortalities in a stage/(total number of fertile eggs – embryo mortalities from previous stages).

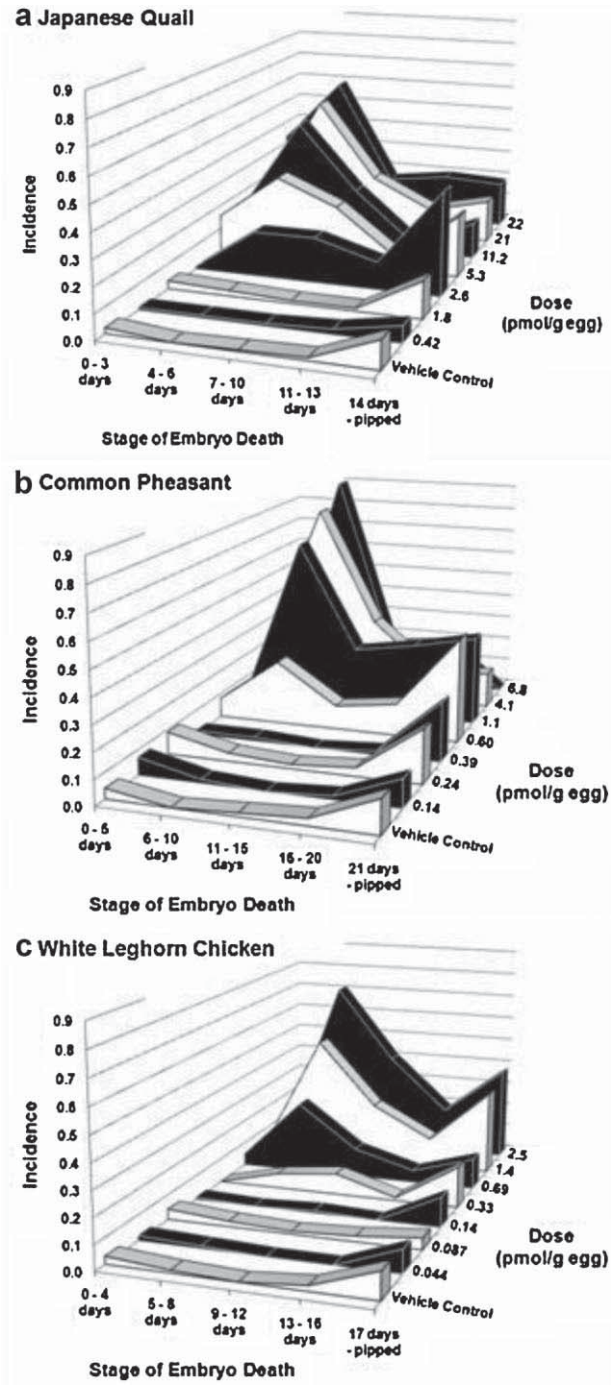

Fig. 2. Effect of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) on stage of mortality in Japanese quail (**a**), common pheasant (**b**), and white leghorn chicken (**c**) embryos. Five stages were identified based on the appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence equals the number of observed mortalities in a stage/(total number of fertile eggs – embryo mortalities from previous stages).

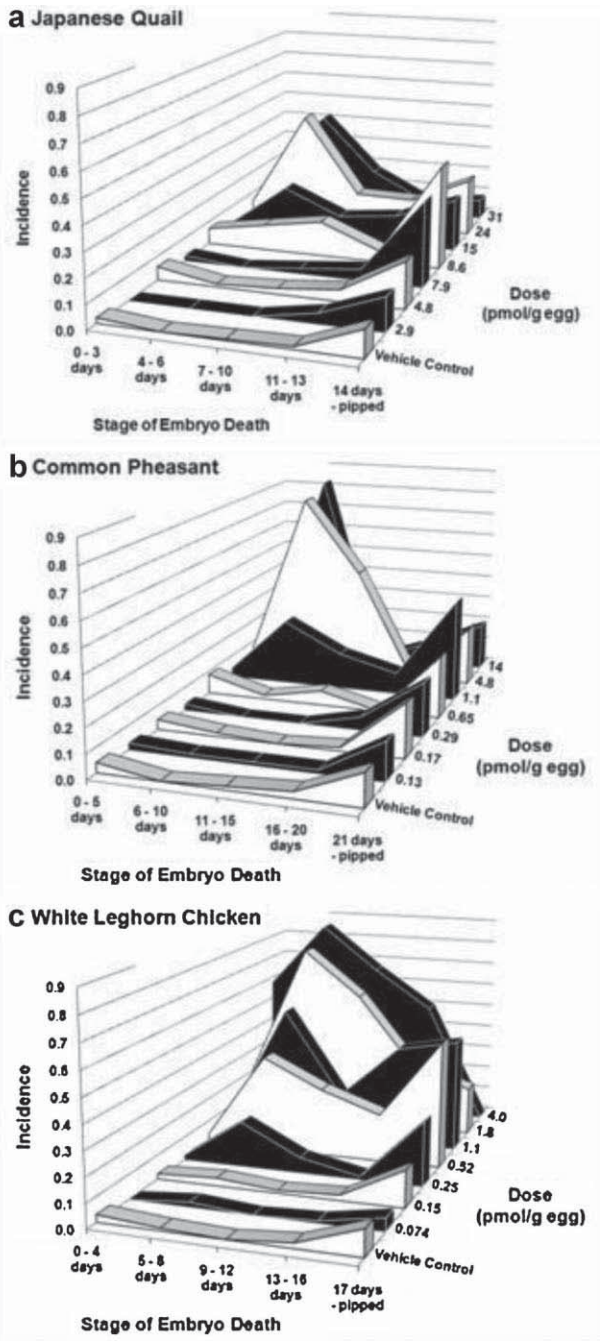

Fig. 3. Effect of 2,3,7,8-tetrachlorodibenzofuran (TCDF) on stage of mortality in Japanese quail (**a**), common pheasant (**b**), and white leghorn chicken (**c**) embryos. Five stages were identified based on the appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence equals the number of observed mortalities in a stage/(total number of fertile eggs – embryo mortalities from previous stages).

trunk (7%) deformities. Within the vehicle control group, there was one instance of curled toes and one embryo with gastroschisis. In pheasants, of the 1,502 exposed embryos, 1.5% were deformed, and similar to the quail, the majority of total deformities ($n = 33$) were of the bill (27%) and limb (55%). Cranial and trunk deformities each made up 9% of the total deformities. One instance of curled toes occurred within the pheasant vehicle control group. Deformities occurred in 0.64% of 2,033 exposed chicken embryos. In contrast to quail and pheasants, the majority of total deformities ($n = 16$) were trunk (50%) compared to cranial (19%), bill (25%), and limb (6%) deformities. However, in contrast to the quail and pheasant, in which the three chemicals resulted in similar deformities, bill deformities were most predominant in chicken embryos exposed to TCDD and trunk deformities were most predominant in those exposed to PeCDF and TCDF. No deformities were observed in embryos from chicken eggs injected with the vehicle.

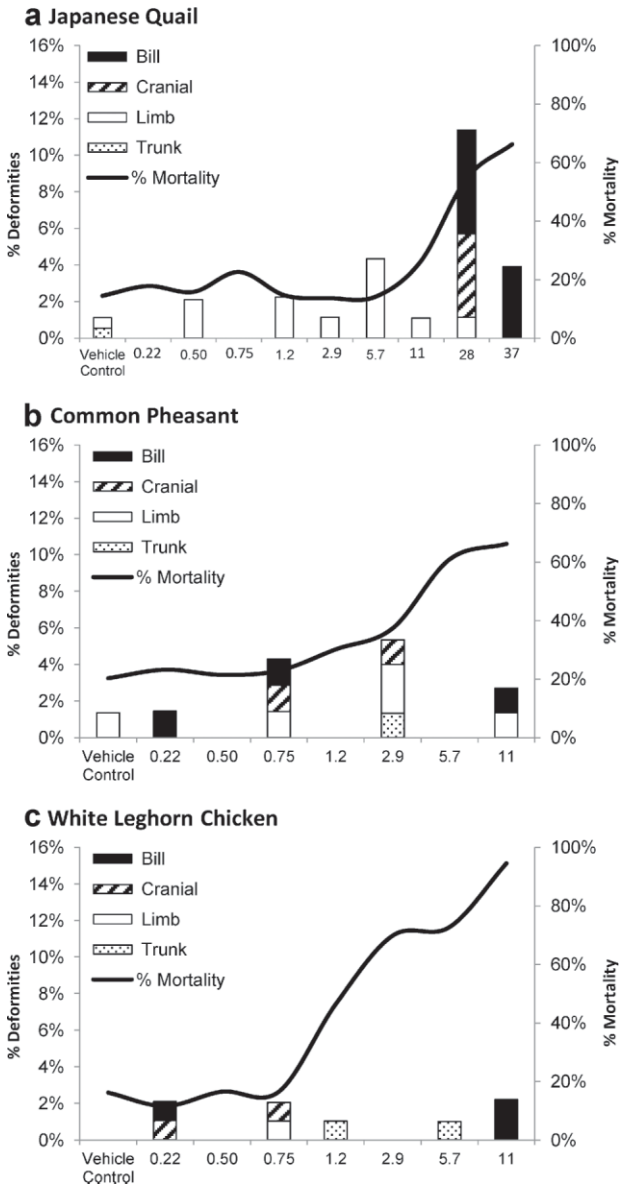

Fig. 4. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the percent incidence of deformities in Japanese quail (**a**), common pheasant (**b**), and white leghorn chicken (**c**) embryos. Cranial deformities include exencephaly, anophthalmos, or microphthalmos. Bill deformities include incomplete or lack of upper/lower beak or crossbill. Trunk deformities include edema, gastroschisis, or achondroplasia. Limb deformities include club foot, curled toes, or extra limb development.

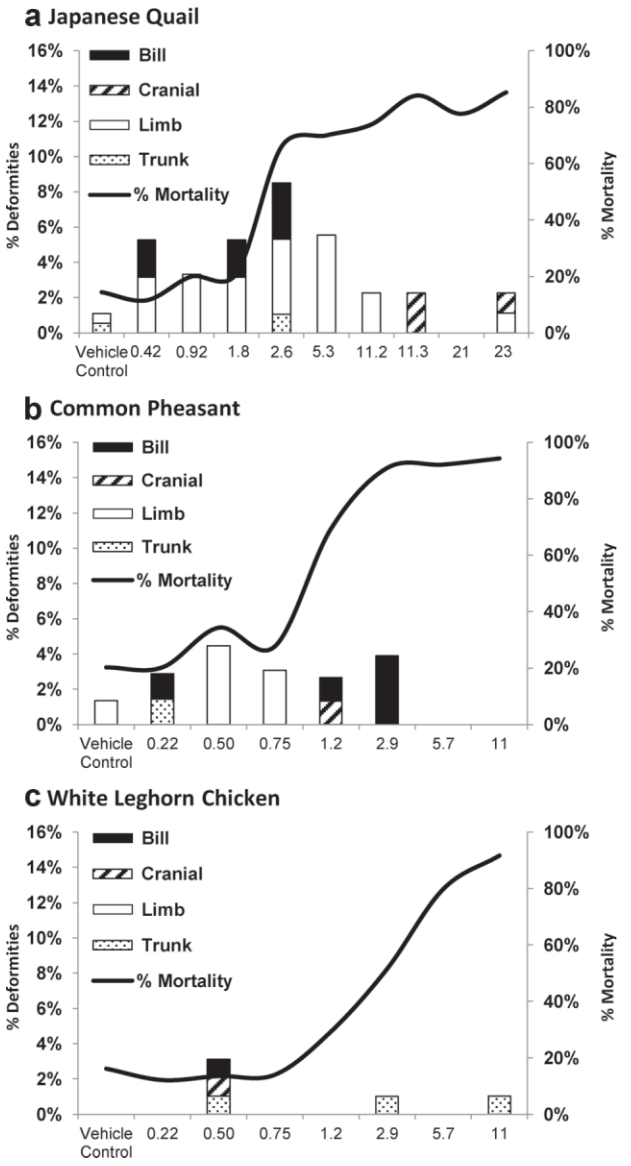

Fig. 5. Effect of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) on the percent incidence of deformities in Japanese quail (**a**), common pheasant (**b**), and white leghorn chicken (**c**) embryos. Cranial deformities include exencephaly, anophthalmos, or microphthalmos. Bill deformities include incomplete or lack of upper/lower beak or crossbill. Trunk deformities include edema, gastroschisis, or achondroplasia. Limb deformities include club foot, curled toes, or extra limb development.

Body mass

Relatively few differences in the body mass (expressed as mean ± 1 SD) of 1- and 14-d-old chicks were observed in Japanese quail, common pheasants, or white leghorn chickens. In 1-d-old quail and pheasant hatchlings exposed to any of the three compounds, body masses were not significantly different compared to vehicle controls (quail, 6.9 ± 0.66 g; pheasant, 20.0 ± 1.8 g) (Supplemental Data, Tables S5, 6). In 1-d-old chicken hatchlings, only those injected with 0.77 pmol TCDD/g egg had an average body mass significantly less ($p = 0.0446$) than that of the vehicle control (39.5 ± 2.4 g) (Supplemental Data, Table S7).

In 14-d-old quail, the 28 ($p < 0.0001$) and 37 pmol TCDD/g egg ($p < 0.0001$), 11.3 ($p < 0.0001$) and 21 pmol PeCDF/g egg ($p < 0.0001$), and 0.63 ($p = 0.0146$), 2.9 ($p = 0.0485$), 15 ($p = 0.0141$), and 31 pmol TCDF/g egg ($p < 0.0001$) dose groups had body masses significantly greater than that of the vehicle control group (56.2 ± 7.4 g) (Supplemental Data, Table S5). Body masses of 14-d-old pheasants and chickens were significantly less than those of their respective vehicle control groups (pheasant, 88.5 ± 12.2 g; chicken, 114.9 ± 12.5 g) at doses of 0.31 pmol TCDD/g egg ($p < 0.0001$) and 0.60 pmol PeCDF/g egg ($p = 0.0037$) for the pheasant and 1.6 ($p = 0.0035$) and 3.1 pmol TCDD/g egg ($p = 0.0057$) and 1.1 pmol TCDF/g egg ($p = 0.0166$) for the chicken (Supplemental Data, Tables S6, 7).

Liver mass and pathology

Differences in relative liver mass (expressed as percent body mass, mean [95% confidence interval]) in all three species were not associated with dose. In 1-d-old quail, mean relative liver

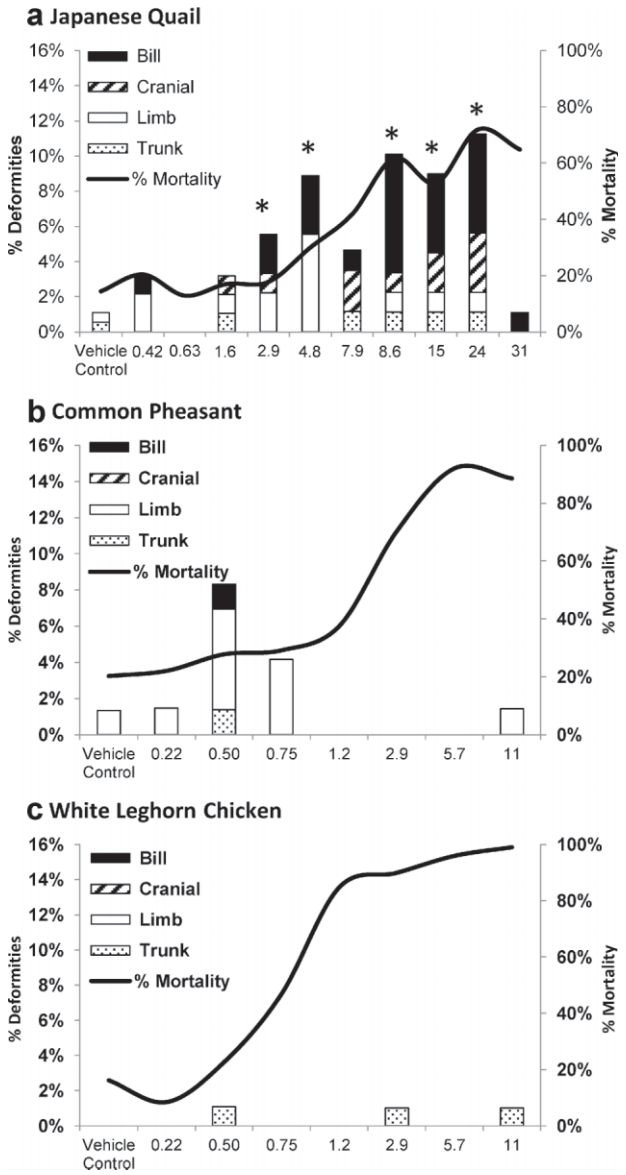

Fig. 6. Effect of 2,3,7,8-tetrachlorodibenzofuran (TCDF) on the percent incidence of deformities in Japanese quail (**a**), common pheasant (**b**), and white leghorn chicken (**c**) embryos. Cranial deformities include exencephaly, anophthalmos, or microphthalmos. Bill deformities include incomplete or lack of upper/lower beak or crossbill. Trunk deformities include edema, gastroschisis, or achondroplasia. Limb deformities include club foot, curled toes, or extra limb development. Bars marked with an asterisk are significantly different from vehicle control.

mass significantly greater than that of the vehicle control (4.20 [3.52, 4.87]) occurred at 28 pmol TCDD/g egg ($p = 0.0040$) and 7.9 ($p = 0.0397$) and 15 pmol TCDF/g egg ($p = 0.0154$), while liver mass significantly less than the vehicle control value occurred at doses of 1.8 ($p = 0.0356$), 2.6 ($p = 0.0125$), and 5.3 pmol PeCDF/g egg ($p = 0.0229$). Fourteen days later, only the 7.9 ($p < 0.0001$) and 31 pmol TCDF/g egg ($p = 0.0003$) dose groups had relative liver masses significantly greater than the vehicle control (2.78 [2.52, 3.04]) (Supplemental Data, Table S8). Relative liver masses in 1-d-old pheasants were significantly greater than the vehicle control (3.02 [2.73, 3.32]) at doses of 0.60 ($p = 0.0143$), 1.1 ($p = 0.0542$), and 4.1 pmol PeCDF/g egg ($p = 0.0007$). Those doses resulting in significantly greater relative masses compared to the vehicle control (2.59 [2.47, 2.71]) in 14-d-old chicks included the 0.075 ($p = 0.0177$) and 0.31 pmol TCDD/g egg ($p < 0.0001$), and 0.60 pmol PeCDF/g egg ($p = 0.0079$) dose groups (Supplemental Data, Table S9). In 1- and 14-d-old chickens, relative liver masses were not significantly different than those of the vehicle control (1-d-old: 2.93 [2.37, 3.50], 14-d-old: 2.86 [2.57, 3.15]) (Supplemental Data, Table S10).

No hepatic lesions of the liver were associated with TCDD, PeCDF, or TCDF exposure in either the common pheasant or white leghorn chicken. Hepatic vacuolation due to lipid accumulation across all dose groups, including vehicle controls, was noted for both species. However, this was associated with age rather than compound exposure. Histological examination of hepatic tissue from Japanese quail also indicated hepatic vacuolation across all groups. In addition, incidences were noted of focal bile duct hyperplasia, binucleation, and karyomegalic (enlarged hepatocyte nuclei) and necrotic hepatocytes that seemed most prevalent in birds exposed to TCDD and TCDF (Supplemental Data, Table S11).

Heart, brain, bursa, and spleen mass and pathology

Differences in relative mass of the heart, brain, bursa, and spleen (expressed as percent body mass, mean [95% confidence interval]) in 14-d-old chicks of all three species were not associated with dose. In quail, relative heart mass was significantly greater ($p = 0.0476$) in the 0.50 pmol TCDD/g egg dose group when compared to the vehicle control (0.791 [0.712, 0.871]). Relative brain mass was also significantly greater ($p = 0.0489$) than the vehicle control (0.891 [0.806, 0.977]) in the 8.6 pmol TCDF/g egg dose group (Supplemental Data, Table S12). Relative bursa mass was significantly less in the 28 ($p = 0.0489$) and 37 pmol TCDD/g egg ($p = 0.0388$) dose groups and the 0.42 pmol PeCDF/g egg ($p = 0.0298$) dose group when compared to the vehicle control (0.090 [0.076, 0.105], while relative spleen mass was not significantly different when compared to the vehicle control (0.038 [0.030, 0.045]) (Supplemental Data, Table S13). In pheasants, relative heart and bursa masses were significantly greater when compared to the vehicle control (heart: 1.62 [1.39, 1.85], bursa: 0.163 [0.132, 0.193]) in the 0.31 pmol TCDD/g egg (heart: $p = 0.0535$, bursa: $p = 0.0472$) dose group. No significant differences were observed between vehicle control relative brain (0.784 [0.755, 0.812] and spleen (0.084 [0.057, 0.111]) masses compared to treatment dose groups (Supplemental Data, Tables S14, 15). Differences in relative organ masses of chickens included significantly greater relative heart masses in the 0.25 ($p = 0.0195$), 0.52 ($p = 0.0150$), 1.1 ($p = 0.0098$), and 4.0 pmol TCDF/g egg ($p = 0.0187$) dose groups when compared to the vehicle control (1.15 [1.08, 1.22]) and significantly greater relative brain mass (vehicle control: 0.653 [0.570, 0.736]) in the 3.1 pmol TCDD/g egg ($p = 0.0141$) dose group (Supplemental Data, Table S16). No significant differences occurred in relative bursa and spleen masses between treatment groups and the vehicle control (bursa: 0.499 [0.381, 0.517], spleen: 0.107 [0.092, 0.122] (Supplemental Data, Table S17). No histological lesions were associated with TCDD, PeCDF, or TCDF exposure in the heart, brain, bursa, or spleen of all three species.

DISCUSSION

In general, results of companion studies indicated that the chicken was most sensitive to in ovo exposure to TCDD, PeCDF, and TCDF, the pheasant was of intermediate sensitivity, and the Japanese quail was least sensitive, based on embryo

mortality and hepatic enzyme induction [13,14,25]. Furthermore, these studies indicated that PeCDF and TCDF were more potent than TCDD in the quail and pheasant and TCDF was the most potent in the chicken based on the same endpoints. Results reported here indicated that the incidence of developmental deformities and changes in body and relative organ masses and organ pathology in 1-d and/or 14-d-old white leghorn, common pheasant, and Japanese quail chicks exposed to TCDD, PeCDF, or TCDF in ovo could not be used to assess species sensitivity and compound potency because changes in these parameters, unlike like embryo mortality and enzyme induction, were not consistently related to dose within species and/or compound.

Stage of embryo mortality

Embryo mortality in avian species is perhaps the most consistent feature of exposure to TCDD or TCDD-like compounds [18]. However, few studies have examined the particular stage(s) of development during which mortality occurs. In the present study, dose-dependent increases in embryo mortality during the second stage of development in each species tested indicated that exposure to TCDD, PeCDF, or TCDF resulted in a consistent embryo mortality pattern (Figs. 1–3) and that the lethality of these compounds was more apparent after the avian embryo had completed organogenesis [31,32]. Additionally, a relatively great percentage of late-stage embryos of all three species exposed to TCDD, PeCDF, or TCDF failed to hatch after pipping. Similar findings were reported for chicken and pheasant embryos experimentally exposed to a variety of commercial PCB mixtures as reported in Gilbertson et al. [18] as well as for herring gull and Forster's tern populations contaminated with TCDD-like compounds [9,33].

Deformities

The average incidence of deformities in the present study resulting from in ovo exposure to TCDD, PeCDF, or TCDF (3.4% for Japanese quail, 1.5% for pheasants, and 0.6% for chickens) was less compared to other egg injection studies assessing the effects of TCDD-like compounds. For example, Powell et al. [27,34] reported that injection of TCDD and TCDD-like PCB congeners (3,3′,4,4′-tetrachlorobiphenyl [PCB 77] and 3,3′4,4′,5-pentachlorobiphenyl [PCB 126]) into the yolk of chicken eggs at doses comparable to those used in the present study resulted in approximately 7 to 12% deformed embryos. Hoffman et al. [7] reported that injection of PCB 77 and PCB 126 into the air cell of chicken, American kestrel (*Falco sparverius*), and common tern eggs on day 4 of incubation resulted in 38, 55, and 29% deformed embryos, respectively. These differences in percentage of deformed embryos among studies could reflect methodological differences such as vehicle used (corn oil, triolein), site of injection (yolk, air cell), and/or time of injection (prior to incubation, ED 4). In the present study, only in Japanese quail exposed to TCDF was there a significant increase in the percentage of deformed embryos, which tended to be associated with the greater doses. Interestingly, the Japanese quail is considered the least sensitive species of the three tested based on LD50 values [14]. Because the chicken had the least percentage of deformed embryos, these results suggest that the incidence of teratogenicity was inversely related to species sensitivity based on embryo lethality. Hoffman et al. [7] reported similar results in that the percentage of deformed embryos tended to be greater for the less sensitive kestrel compared to the more sensitive chicken. Both Powell et al. [27] and Hoffman et al. [7] commented that the percentage of deformed embryos in the chicken was less at greater doses because of increased mortality. Thus, the increased incidence of deformities in less-sensitive species could be due to the greater opportunity for embryo development prior to hatching.

In terms of compound potency, PeCDF was considered the most potent in Japanese quail based on embryo lethality [14], yet the percentage of deformed embryos resulting from exposure to PeCDF only approached statistical significance compared to the vehicle control. Similarly, Hoffman et al. [7] reported that PCB 77, which was less potent than PCB 126 in both chickens and kestrels based on LD50 values, resulted in the same average number of deformities in the chicken as PCB 126 and a slightly greater percentage in the kestrel.

In general, the types of deformities observed in the embryos of all three species exposed to TCDD, PeCDF, and TCDF in the present study were similar to those reported in other studies assessing the effects of in ovo exposure to TCDD and/or TCDD-like compounds in chickens [7,27,34–36], pheasants [37], and other wild avian species [7,10,18]. In the present study, limb and bill deformities were the most predominant deformities in the quail and pheasant. In the chicken, bill deformities were predominant in TCDD-dosed embryos and trunk deformities were more prevalent in PeCDF- and TCDF-dosed embryos (Figs. 4–6, Supplemental Data, Tables S1–4). Both Powell et al. [27,34] and Hoffman et al. [7] stated that the most frequent malformations in the chicken as a result of in ovo exposure to TCDD and/or TCDD-like PCB congeners were of the bill. The most frequent type of deformity reported for the kestrel and tern exposed in ovo to TCDD-like PCB congeners also related to the bill [7].

Body mass

No consistent dose-related changes occurred in 1- and 14-d body mass of any of the three species exposed to any of the three compounds with the exception of 14-d-old chickens in the two greatest TCDD dose groups (1.6 and 3.1 pmol/g egg), which had significant decreases in body mass (Supplemental Data, Tables S5–7). Body mass results from other egg injection studies are variable. Blankenship et al. [36] reported no effect on body mass in chickens exposed to 0.5 pmol TCDD/g egg in ovo. Similarly, Powell et al. [27] reported no effect on body mass of chickens exposed in ovo to TCDD or PCB 126 at doses as great as 2.0 pmol TCDD/g egg. In contrast, Henshel et al. [28] reported that chickens exposed in ovo to TCDD at doses greater than 0.31 pmol/g egg had decreases in body mass.

Organ mass and pathology

In general, the changes in relative organ mass reported here (increases in relative liver and heart mass and decreased relative bursa mass) were consistent with the types of changes reported by others after in ovo exposure to TCDD-like chemicals [27,34,38,39], but the changes were not consistently related to dose (Supplemental Data, Tables S8–10, S12–17). The hepatic lesions that occurred in the quail were similar to those described in herring gulls and chickens exposed to TCDD-like chemicals [18,34]. While lesions tended to be more prevalent in birds in the greater dose groups for each compound, no consistent dose–response relationships were observed.

In summary, the results of the present study indicate that the magnitude of in ovo effects of TCDD, PeCDF, and TCDF on embryo development, body and organ masses, and tissue morphology in the quail, pheasant, and chicken were not species- or compound-specific. Thus, these parameters cannot be used to assess species sensitivity or compound potency as has been

demonstrated for embryo mortality and hepatic enzyme induction in companion studies.

SUPPLEMENTAL DATA

The Supplemental Data includes 17 tables. (460 KB).

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