IN OVO EXPOSURE OF JAPANESE QUAIL, COMMON PHEASANT AND WHITE LEGHORN CHICKEN EMBRYOS TO 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN, 2,3,4,7,8- PENTACHLORODIBENZOFURAN AND 2,3,7,8-TETRACHLORODIBENZOFURAN

By

Andrew Michael Cohen-Barnhouse

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Animal Science

2010

ABSTRACT

IN OVO EXPOSURE OF JAPANESE QUAIL, COMMON PHEASANT AND WHITE LEGHORN CHICKEN EMBRYOS TO 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN, 2,3,4,7,8- PENTACHLORODIBENZOFURAN AND 2,3,7,8-TETRACHLORODIBENZOFURAN

By

Andrew Michael Cohen-Barnhouse

A series of egg injection studies was conducted to confirm a proposed model of relative avian-species sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and two furan congeners in three Galliform species. This classification model predicts sensitivity to TCDD and TCDD-like compounds based on key amino acids of the ligand-binding domain of the aryl hydrocarbon receptor; where, those species with amino acid sequences similar to that of the White Leghorn chicken (*Gallus gallus domesticus*) will be most sensitive, those similar to the Common pheasant (*Phasianus colchicus*) will be moderately sensitive, and those with amino acid sequences similar to the Japanese quail (*Corturnix japonica*) will be least sensitive to TCDD-like toxicity. Doses ranging from 0.044 to 37 pmol/g egg were injected into the air cell of eggs prior to incubation. Relative potency and species sensitivity was determined between compounds and species from lethal dose estimates derived from embryo mortality. Developmental stages of embryo mortality, incidences of deformities, body weight, and relative organ weight and histopathology of liver, heart, brain, bursa and spleen tissues were also evaluated.

To my grandparents,
Dr. Harry and Miztie P. Cohen

ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Steve Bursian, for his assistance, guidance, and occasional push in the right direction throughout my Masters program. I would also like to thank the rest of my committee members: Dr. Mathew Zwiernik for his assistance and guidance throughout this project, Dr. Scott Fitzgerald for both his critical review of this project's histopathological assessment and my education in this field, and Dr. Michael Orth, for always being there to answer random questions, as well as his good humor.

I would like to give a special thanks to Angelo Napolitano, Farm Manager of the MSU Poultry Research and Teaching Center and mentor, as well as Mrs. Jane Link, without whom this project may have never been completed. I am extremely thankful to the following for various assistance during this project: Anthony Satkowiak, Amanda Jenison, Brianna Groubert, Caroline Davis, Katie Link, Michelle Dawes, Hayley Frey, Maria Diez Leon, Dusty Tazalaar, Rita Seston, Tim Fredricks, Jeremy Moore, Patrick Bradley, Tracy Hescott, Rebecca Meagher, Douglas Crump, Jessica Hervé and Caroline Egloff.

Finally, I would like to thank my family and friends for their love and support; my mother, Judith Cohen, without whom I never would have made it, my father, Mark Barnhouse, for his encouragement along the way, and both the Napolitano and Campbell families, for always providing a home away from home.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

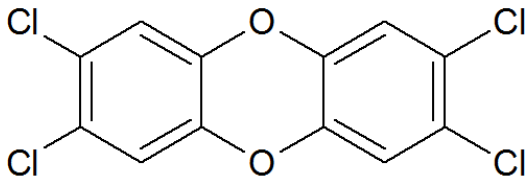
LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
BM	Body mass
EC50	Effective concentration: concentration resulting in 50% response
EROD	Ethoxyresorufin *O*-deethylase
LD50	Lethal Dose: dose at which 50% mortality occurs
LBD	Ligand binding domain
MSU	Michigan State University
PCB	Polychlorinated biphenyl
PeCDF	2,3,4,7,8-pentachlordibenzofuran
ReP	Relative potency
ReS	Relative sensitivity
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TEF	Toxic equivalency factor
TEQ	TCDD toxic equivalency
WHO	World Health Organization

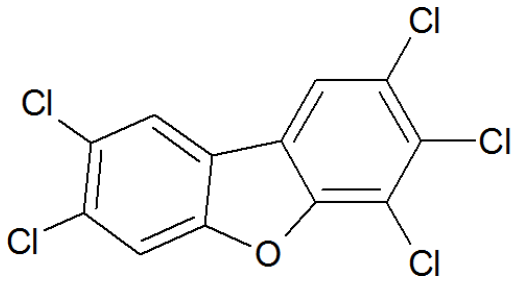
INTRODUCTION

Currently, elevated concentrations of polychlorinated dibenzofurans and measurable concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Figure 1) have been detected in several freshwater ecosystems throughout the Great Lakes region as a result of industrial activities (Kumar *et al*. 2002; Zwiernik *et al*., 2008; Fredricks *et al*., 2010). Historically, avian exposure to TCDD and other TCDD-like compounds was linked to impairment of reproductive performance in several species of avian wildlife. As a result, species including the Double-crested cormorant (*Phalacrocorax auritus*) (Fox *et al.* 1991), Herring gull (*Larus argenatatus*) (Fox *et al*., 1978, 1988), Common tern (*Sterna hirundo*) (Hoffman *et al.*, 1998), Caspian tern (*Hydroprogne caspia*) (Ludwig *et al.*, 1996) and Forster's tern (*Sterna forsteri*) (Hoffman *et al*., 1987) experienced localized population decline. As avian sensitivities have been shown to range from 100- to 10,000-fold between species (Head *et al*., 2008) and population-level studies cannot be conducted for every species in a given area, methods minimizing the uncertainty associated with the exposure and effects of TCDD and TCDD-like compounds are greatly needed.

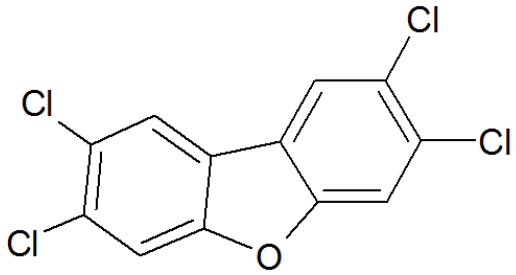
Current risk assessment protocols for TCDD and TCDD-like compounds utilizes toxic equivalency factors (TEFs) (based on multiple endpoints from different species belonging to a class of animal) or relative potency factors (RePs) (the ratio of potency for a TCDD-like compound relative to TCDD) to estimate the toxicity of these compounds. These factors go into the calculation of TCDD toxic equivalents (TEQ); where the toxic potency of a mixture of TCDD-



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)
Molecular Weight: 321.97096 g/mol
$TEF_{WHO\text{-}Avian}$: 1.0



2,3,4,7,8-Pentachlorodibenzofuran (PeCDF)
Molecular Weight: 340.41662 g/mol
$TEF_{WHO\text{-}Avian}$: 1.0



2,3,7,8-Tetrachlorodibenzofuran (TCDF)
Molecular Weight: 305.97156 g/mol
$TEF_{WHO\text{-}Avian}$: 1.0

Figure 1. The structure, molecular weight and avian-specific 1998 World Health Organization (WHO) toxicity equivalency factors ($TEF_{WHO\text{-}Avian}$) for TCDD, PeCDF and TCDF.

like compounds is estimated by multiplying the concentration of individual congeners by their respective TEF. The sum of these TEQs estimates the total TCDD-like toxicity for any given mixture (Gupta, 2007). At present, World Health Organization toxic equivalency factors for 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) for avian species are 1.0 (Van den Berg *et al*., 1998) based on several *in vitro* and related *in ovo* studies (for PeCDF: Bosveld *et al*., 1992; Sanderson *et al*., 1998; for TCDF; Poland and Glover, 1977; Bosveld *et al*., 1992; Kennedy *et al*., 1996) (Figure 1). As these TEFs are based, in part, on *in vitro* studies, they do not account for complete organism or species-specific differences in absorption, distribution, metabolism, and elimination of TCDD-like compounds (Giesy and Kannan, 1998). In addition, results from acute and chronic *in vivo* studies, as well as recent *in vitro* and *in ovo* studies, have shown great differences in sensitivity to these compounds among species of birds (Head *et al*., 2008; Cohen-Barnhouse *et al., 2010;* Hervé *et al*., 2010; Yang *et al*., 2010). As a result, the current TEF values may over or under estimate the potencies of PeCDF and TCDF in individual avian species.

The toxicity of TCDD and TCDD-like compounds has been linked to their interactions with the aryl hydrocarbon receptor (AhR). The AhR is a ligand-activated nuclear transcription factor that regulates the expression of a suite of genes including biotransformation enzymes such as mixed–function monooxygenases (Hahn, 1998). After TCDD or a TCDD-like compound diffuses across the plasma membrane, the binding of the ligand to the AhR, in association with chaperone proteins including two hsp90 (heat shock protein of 90kDa), the X-associated protein 2 (XAP2), and p23 (a co-chaperone protein of 23 kDa), induces a conformational change allowing the complex to

translocate into the nucleus (Denison *et al.*, 2002; Denison and Nagy, 2003) (Figure 2). The toxicity of TCDD-like compounds has been linked to their affinity to the AhR with the most toxic being those with the greatest binding strength (Okey *et al*., 1994). Once in the nucleus, the chaperone proteins dissociate and the AhR–ligand bind to the AhR nuclear translocator (Arnt) and other factors that induce the conversion of the complex into a form that binds to DNA with high affinity at specific sites called dioxin responsive elements (DREs). Upon binding, the transcription of genes encoding cytochrome P450 enzymes in the CYP1A family and other AhR–responsive genes, located upstream to DREs, is initiated (Denison *et al.*, 2002; Denison and Nagy, 2003) (Figure 2).

Research assessing AhR–mediated responses, such as the induction of ethoxyresorufin-*O*-deethylase (EROD) activity in hepatocyte cultures of different avian species by TCDD-like compounds, has shown variations in species sensitivity based on these endpoints (Bronström and Reutergardh, 1986; Bronström, 1988; Bronström and Lund, 1988). Kennedy *et al.* (1996) suggested this methodology might be useful for estimating the sensitivity of avian species to the embryotoxic effects elicited by TCDD and TCDD-like compounds.

Recent molecular studies provided a mechanistic basis for the hypothesis that EROD induction potential might be useful in predicting TCDD sensitivity for individual species of birds. Karchner *et al*. (2006) demonstrated through the use of chimeric AhR proteins and site-directed mutagenesis that the relative

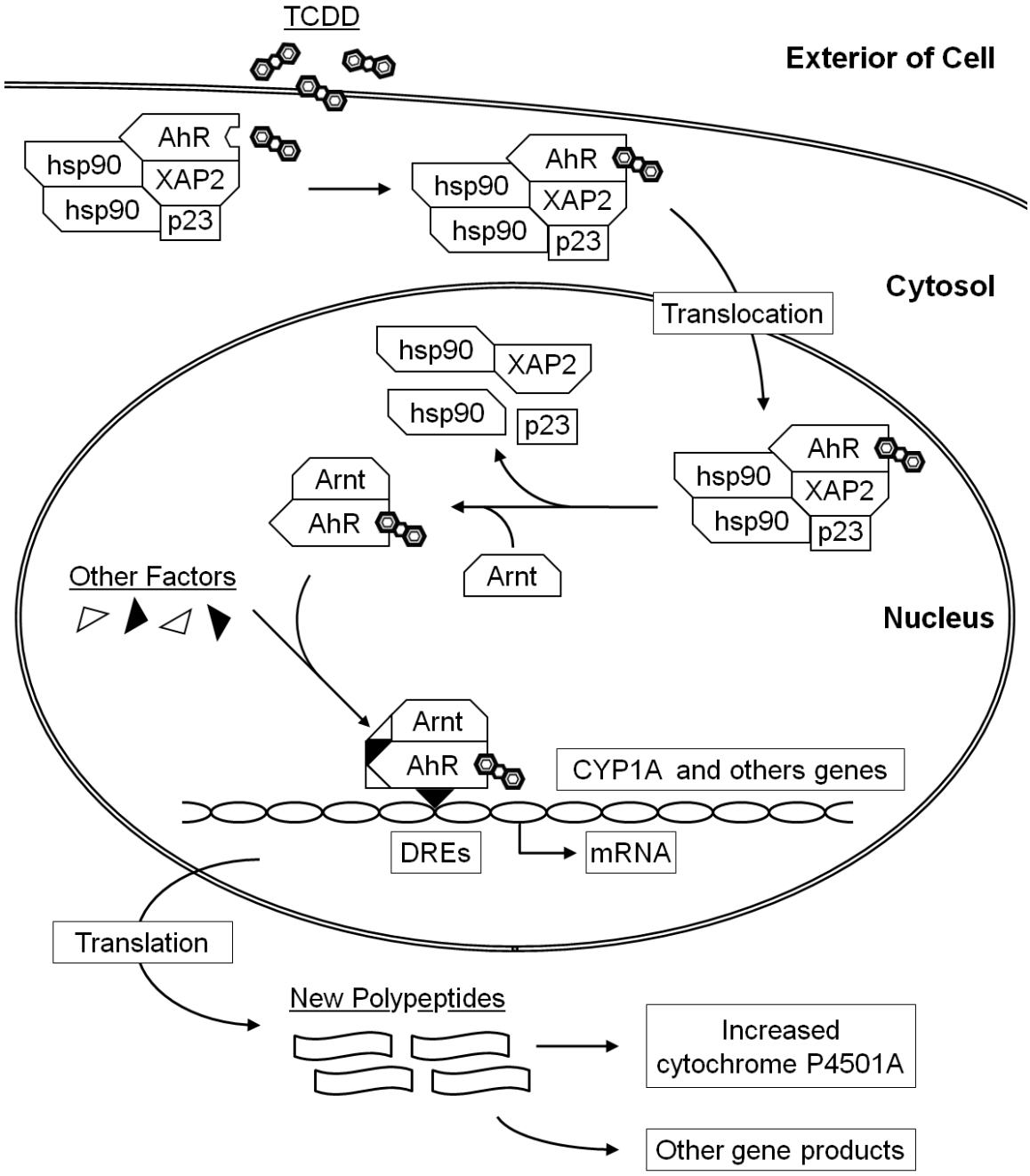


Figure 2. The proposed mechanism of action for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and TCDD-like compounds. Adapted from Gupta (2007).

insensitivity of the common tern (*Sterna hirundo*) to TCDD-like compounds compared to the chicken (250-fold difference) could be explained, in part, by a difference in two amino acids in the ligand binding domain (LBD) of the AhR: Ile324 and Ser380 in the chicken and Val325 and Ala381 in the tern. Expanding upon these findings, Head *et al*. (2008) determined that variations of these two amino acid residues (Ile324 and Ser380) could predict embryonic sensitivity to TCDD-like compounds and categorized species based on similarities in their amino acid sequence of the AhR LBD.

In avian species surveyed, three categories of TCDD-like sensitivity were determined based on the amino acid sequence of the AhR LBD (Figure 3) (Head *et al*., 2008). Those species with an amino acid sequence similar to that of the White Leghorn chicken (*Gallus gallus domesticus*), having the Ile/Ser genotype, were considered most sensitive. Species sharing the Ile/Ala genotype of the Common pheasant (*Phasianus colchicus*), including the wild turkey (*Meleagris gallopavo*) and Eastern bluebird (*Sialia sialis*), were considered to have intermediate sensitivity. Species with LBD amino acid sequences similar to the Japanese quail (*Coturnix japonica*) (the Val/Ala genotype), including the American kestrel (*Falco sparverius*), Common tern, Double-crested cormorant (*Phalacrocorax auritis*), Herring gull (*Larus argentatus*), Wood duck (*Aix sponsa*) and mallard (*Anas platyrhynchos*), were considered least sensitive. However, phylogenetic relationships among species did not always correspond to sensitivity classifications or AhR genotypes (Head *et al.*, 2008).

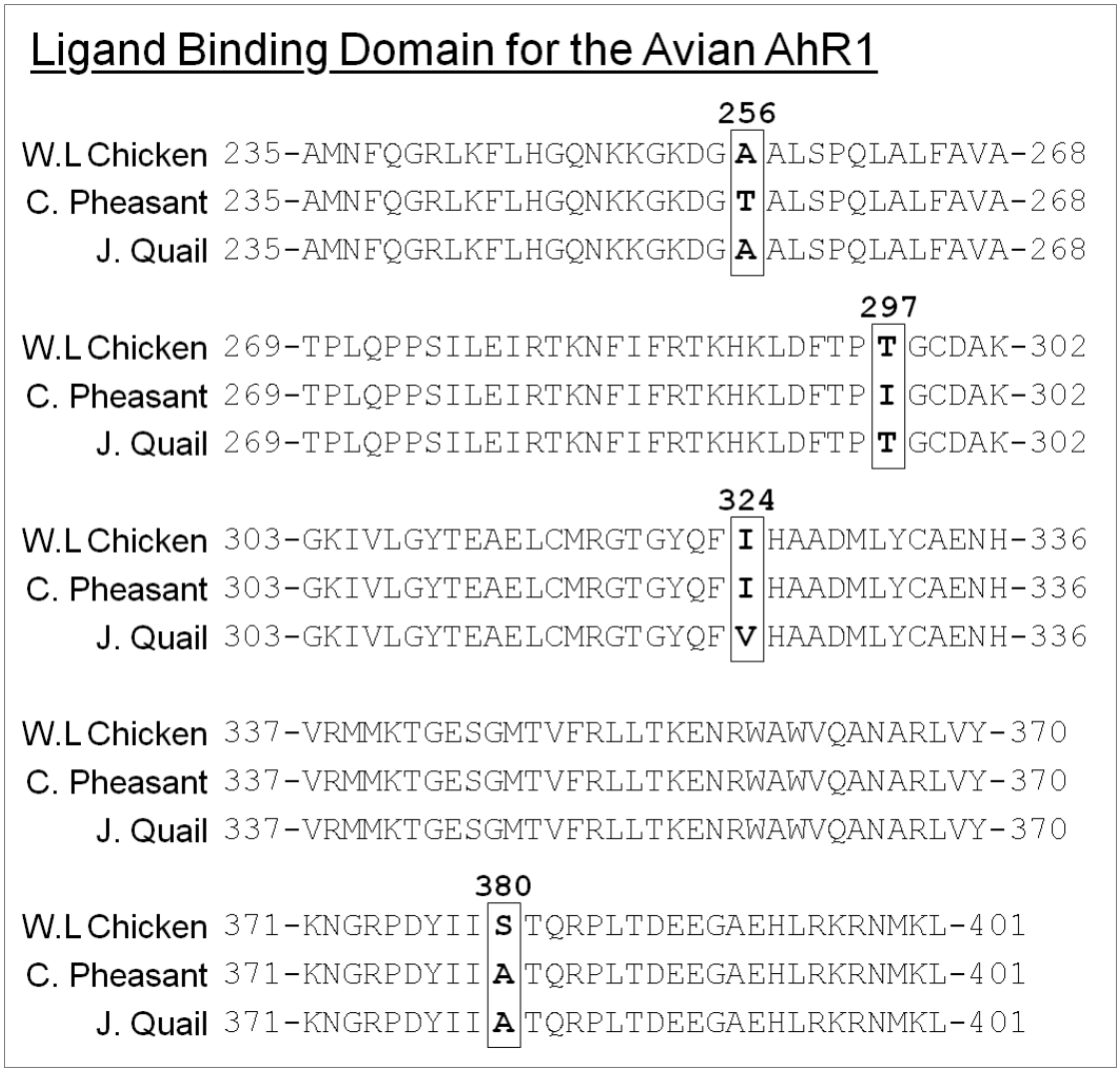


Figure 3. Amino acid sequence of the ligand binding domain (LBD) of the aryl hydrocarbon receptor (AhR) in Japanese quail, Common pheasants and White Leghorn chickens. Differences are noted for amino acid residues at positions 256, 297, 324 and 380. Adapted from Head *et al*. (2008).

The study herein was part of a group of collaborative studies using the Japanese quail, Common pheasant and White Leghorn chicken to further validate this model at the molecular (Yang *et al.*, 2010), *in vitro* (Hervé *et al.*, 2010a) and *in ovo* (Cohen-Barnhouse *et al.*, 2010) levels for TCDD and two TCDD-like compounds; PeCDF and TCDF. These particular compounds were chosen because of their significant contribution to the congener profile of the contaminated area of interest, the Tittabawassee River, MI, USA (Giesy *et al.*, 1997; Zwiernik *et al.*, 2008; Fredricks *et al.*, 2010). However, the ultimate goal of this line of research is to firmly establish a predictive tool reducing the uncertainty associated with avian species sensitivity to TCDD-like compounds for ecological risk assessment.

The first objective of this study was to assess the relative *in ovo* potencies of TCDF and PeCDF compared to TCDD, based on lethal dose (LD) 50 estimates derived from embryo mortality in the quail, pheasant and chicken. The second objective was to validate the proposed avian species sensitivity classification model that is based primarily on *in vitro* work (Kennedy *et al.*, 1996; Head *et al.*, 2008; Hervé *et al.*, 2010) in all three species. This was to be accomplished by determining relative species sensitivity (ReS) values evaluating the potencies of each compound in the quail and pheasant relative to the chicken (presumed to be the most sensitive species). The third objective of this study was to assess differences in embryotoxicity and post-hatching endpoints resulting from the *in ovo* exposures to all three compounds and to compare these endpoints between each of the species tested. These endpoints included the stage at which embryo mortality occurred as defined by key developmental characteristics, the occurrence and type of

embryo and chick deformities, 1- and 14-day old chick body mass, and histology and mass of liver, heart, brain, bursa and spleen tissues.

LITERATURE CITED

Literature Cited

Bosveld, A.T.C., Van den Berg, M., Theelen, R.M.C. (1992). Assessment of the EROD inducing potency of eleven 2,3,7,8- substituted PCDD/Fs and three coplanar PCBs in the chick embryo. *Chemosphere* **25**: 911-916.

Bronström, B. (1988). Sensitivity of embryos from duck, goose, herring gull, and various chicken breeds to 3,3′,4,4′-tetrachlorobiphenyl. *Poult. Sci.* **63**:52-57.

Bronström, B., and Anderson, L. (1988). Toxicity and 7-ethoxyresorufin-*O*-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos. *Arch. Toxicol.* **62**:263-266.

Bronström, B., and Reutergardh, L. (1986). Differences in sensitivity of some avian species to the embryotoxicity of a PCB, 3,3′,4,4′-tetrachlorobiphenyl, injected into the eggs. *Environ. Pollut.* **42**:37-45.

Cohen-Barnhouse, A.M. Bursian, S.J., Link, J.E., Fitzgerald, S.D., Kennedy, S.W., Hervé, J., Giesy, J.P., Wiseman, S.,Yang, Y., Jones, P.D., Wan, Y., Collins, B., Newsted, J.L., Kay, D,. Zwiernik, M.J. (2010). Sensitivity of Japanese Quail (*Coturnix japonica*), Common Pheasant (*Phasianus colchicus*) and White Leghorn Chicken (*Gallus gallus domesticus*) Embryos to *In Ovo* Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) and 2,3,7,8-Tetrachlorodibenzofuran (TCDF). *Toxicol. Sci.* (*submitted*)

Denison, M.S. and Nagy S.R. (2003). Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Ann. Rev. Pharmacol. Toxicol.* **43**:309-334.

Denison, M.S., Pandini, A., Nagy S.R., Baldwin, E.P., Bonati, L. (2002). Ligand binding and activation of the Ah receptor. *Chem. Biol. Interact*. **141**:3-24.

Fox, G.A., Collins, B., Hayakawa, E., Weseloh, D.V., Ludwig, J.P., Kubiak, T.J., Erdman, T.C. (1991). Reproductive outcomes in colonial fish-eating birds: a biomarker for developmental toxins in Great Lakes food chains. II. Spatial variation in the occurrences and prevalence of bill defects in young double-crested cormorants in the Great Lakes, 1979-1987. *J. Great Lakes Res.* **17**:158-167.

Fox, G.A., Gilman, A.P., Peakall, D.B., Anderka, F.W. (1978). Behavioral abnormalities of nestling Lake Ontario herring gulls. *J. Wildlife Manage.* **42**:477-483.

Fox, G.A., Kennedy, S.W., Norstrom, R.J., Wigfield, D.C. (1988). Porphyria in herring gulls: a biochemical response to chemical contamination of Great Lakes food chains. *Toxicol. Chem*. **7**:831-839.

Gupta, R.C. (2007). Veterinary Toxicology: Basic and Clinical Principles. New York; Elsevier Inc. 640-660 p.

Hahn, M.E. (1998). The aryl hydrocarbon receptor: a comparative perspective. *Comp. Biochem. Physiol.* **121**(Part C):23-53.

Head, J.A. and Kennedy, S.W. (2010) Correlation between an in vitro and in vivo measure of dioxin sensitivity in birds. *Ecotoxicology.* DOI 10.1007/S10646-009-0421-3:6

Head, J.A., Hahn, M.E., Kennedy, S.W. (2008). Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ. Sci. Technol.* **42**:7535-7541.

Hervé, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., and Kennedy, S.W. (2010). Cytochrome P450A induction by 2,3,4,8-tetrachlorodibenzo-*p*-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. *Toxicol. Sci*. **113**:380-391.

Hoffman, D.J., Melancon, M.J., Klein, P.N., Eisemann, J.D., Spann, J.W. (1998). Comparative developmental toxicity of planar polychlorinated biphenyl congeners in chickens, American kestrels, and common terns. *Environ. Toxicol. Chem.* **17**:747-757.

Hoffman, D.J., Rattner, G.A., Sileo, L., Docherty, D., Kubiak, T.J. (1987). Embryo toxicity, teratogenicity and aryl hydrocarbon hydroxylase activity in Forster's terns on Green Bay, Lake Michigan. *Environ. Res*. **42**:176-184.

Giesy, J.P., Jude, D.J., Tillitt, D.E., Gale, R.W., Meadows, J.C., Zajieck, J.L., Peterman, P.H., Verbrugge, D.A., Sanderson, J.T., Schwartz, T.R., Tuchman, M.L. (1997). Polychlorinated dibenzo-*p*-dixoins, dibenzofurans, biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin equivalents in fishes from Saginaw Bay, Michigan. *Environ. Toxicol. Chem*. **16**:713-724.

Giesy, J.P. and Kannan, K. (1998). Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): Implications for risk assessment. *Crit. Rev. Toxicol*. **28**:511-569.

Gupta, R.C. (2007). Veterinary Toxicology: Basic and Clinical Principles. New York; Elsevier Inc. 640-660 p.

Karchner, S.I., Franks, D.G., Kennedy, S.W., Hahn, M.E. (2006). The molecular basis for differential dioxin sensitivity in birds: Role of the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci*. **103**:6252-6257.

Kennedy, S.W., Lorenzen, A., Jones, S.P., Hahn, M.E., Stegeman, J.J. (1996). Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. AppI. Pharmacol.* **141**:214-230.

Kumar, K.S., Kannan, K., Giesy, J.P., Masunaga, S. (2002). Distribution and elimination of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, and *p,p*'-DEE in tissues of Bald Eagles from the Upper Peninsula of Michigan. *Environ. Sci. Tech.* **36**:2789-2796.

Ludwig, J.P., KuritaMatsuba, H., Ludwig, M.E., Summer, C.L., Giesy, J.P., Tillitt, D.E., Jones, P.D. (1996). Deformities, PCBs, and TCDD-Equivalents in double-crested cormorants (*Phalacrocorax auritus*) and Caspian terns (*Hydroprogne caspia*) of the upper Great Lakes 1986-1991: Testing a cause-effect hypothesis. *J. Great Lakes Res*. **22**:172-197.

Okey, A.B., Riddick, D.S., Harper, P.A. (1994). The Ah receptor: mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds. *Toxicol. Lett*. **70**:1-22.

Poland, A. and Glover, E. (1977). Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: a study of the structure-activity relationship. *Mol. Pharmacol.* **13**:924-938.

Sanderson, J.T., Kennedy, S.W., Giesy, J.P. (1998). In vitro induction of ethoxyresorufin-*O*-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. *Environ. Toxicol. Chem*. **17**:2006–2018.

Van den Berg, M., Birnbaum, L.S., Bosveld, A.T.C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W. *et al*. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Persp*. **106**:775–792.

Yang, Y., Wiseman, S., Cohen-Barnhouse, A.M., Wan, Y., Jones, P., Newsted, J.L., Kay, D.P., Kennedy, S.W., Zwiernick, M.J., Bursian, S.J., Giesy, J.P. (2010). Effects of *in ovo* exposure of White-1 leghorn Chicken, Common pheasant and Japanese quail to TCDD, 2,3,4,7,8-PeCDF and 2,3,7,8-TCDF on CYP1A induction. *Environ. Toxicol. Chem.* **29**:1490-1502.

Zwiernik, M.J., Kay, D.P., Moore, J., Beckett, K.J., Khim, J.S., Newsted, J.L., Roark, S.A., Giesy, J.P. (2008) Exposure and effects assessment of resident mink (*Mustela vison*) exposed to polychlorinated dibenzofurans and other dioxin-like compounds in the Tittabawassee River basin, Midland, Michigan, USA. *Environ. Toxicol. Chem.*. **27**:2076-2087.

CHAPTER 1

Acute Sensitivity of Japanese Quail (*Coturnix japonica*), Common Pheasant (*Phasianus colchicus*) and White Leghorn Chicken (*Gallus gallus domesticus*) Embryos to *In Ovo* Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8- Pentachlorodibenzofuran (2,3,4,7,8-PeCDF) and 2,3,7,8-Tetrachlorodibenzofuran (2,3,7,8-TCDF)

AUTHORS

A.M. Cohen-Barnhouse*, M.J. Zwiernik*, J.E. Link*, S.D. Fitzgerald†, S.W. Kennedy¶, J.P. Giesy‡, S. Wiseman‡, Y. Yang‡, P.D. Jones‡§, Y. Wan‡, J. Hervé¶, B. Collins¶, J.L. Newsted‖, D. Kay‖, S.J. Bursian*

*Department of Animal Science, Michigan State University, East Lansing, MI, USA

† Department of Pathobiology and Diagnostic Investigation, Diagnostic Center for Population and Animal Health, Michigan State University, East Lansing, MI, USA

‡Department of Veterinary Biomedical Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, SK, CA

§ School of Environment and Sustainability, University of Saskatchewan, Saskatoon, SK, CA

¶ National Wildlife Research Centre, Environment Canada, Ottawa, ON, CA

‖ ENTRIX Inc., East Lansing, MI, USA

This chapter has been submitted as a manuscript to:

The Journal of Toxicological Sciences

July, 2010

ABSTRACT

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 to TCDD for Japanese quail (*Corturnix 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 to 12 ng/g egg) were injected into the air cell of eggs prior to incubation. LD50 (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. 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 chemical of the three in the chicken. Species sensitivity was as expected for TCDD and TCDF while for PeCDF, the chicken and pheasant were similar in sensitivity and both were more sensitive than the quail.

INTRODUCTION

The current methodology to assess the risk of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally similar chemicals assumes 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 individual TCDD-like compounds. To predict the potency of environmental mixtures total 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 (Safe, 1998; Van den Berg *et al*., 1994, 1998; Huwe, 2002). The toxic equivalency factor for an individual TCDD-like compound is a consensus value which may be based on multiple endpoints from different species belonging to a class of animals (mammals, birds etc). While 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. While 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 whole animal responses including species-specific differences between in 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, show differences in sensitivity to TCDD-like compounds among species of birds (Head *et al*., 2008; Hervé *et al*., 2010; Yang *et al*., 2010). As a result, current World Health Organization toxic equivalency factor values may over or under estimate the potencies of these compounds in individual avian species. In addition, such differences pose a challenge to risk assessors as avian sensitivities range from 100- to 10,000-fold between species (Head *et al*., 2008). One hypothesis to account for differences in avian sensitivity to TCDD and TCDD-like compounds is that toxicity can be attributed to variations in the affinity of TCDD-like compounds to the ligand-binding domain of the aryl hydrocarbon receptor (Karchner *et al*., 2006; Head *et al*., 2008). 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). Head *et al*. (2008) showed the sensitivity of avian species to TCDD-like compounds could be predicted based on the amino acid sequence of the aryl hydrocarbon receptor LBD. 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 are moderately sensitive, and those species with a LBD amino acid sequence similar to the Japanese quail are least sensitive.

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* studies of PeCDF (Bosveld *et al*., 1992; Sanderson *et al*., 1998), and TCDF (Poland and Glover, 1977; Bosveld *et al*., 1992; Kennedy *et al*., 1996). However, the results of a recent *in vitro* study (Hervé *et al*., 2010) 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 to 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 (Kennedy *et al*., 1996; Head *et al*., 2008; Hervé *et al*., 2010a,b).

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 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, and the chicken study consisted of two trials.

Doses were chosen to bracket estimated LD_{50} values derived from egg injection studies with TCDD (pheasant [Nosek *et al.* 1993]; chicken [Powell *et al.* 1996; Henshel *et al*., 1997]) 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, USA (Fredricks *et al*., 2010).

Prior to incubation, 9 doses of TCDD and PeCDF and 10 doses of TCDF were injected into Japanese quail eggs, while 7 doses of each test compound were injected into pheasant or chicken eggs. Doses expressed as pmol/g (ww) egg and ng/g (ww) egg are presented in Table 1 for each species. Controls included non-injected 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, USA) while Japanese quail and White Leghorn chicken eggs were obtained from the Michigan State University (MSU) Poultry Research and Teaching Center (East Lansing, MI, USA). All the pheasant eggs were laid on the same day while the 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 – 15.0 °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, USA) 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 have 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, 3 and 6 µL/egg for quail, pheasant and chicken, respectively. Previous experience indicated an injection volume of 0.1 to 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. The variation in egg weight was sufficiently low to allow for a relatively consistent dose delivery. Following preparation of the dosing 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, USA). 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, USA) and injections were made with a positive displacement pipettor (Gilson, Middleton, WI, USA) 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, USA) applied with a sterile wooden applicator.

Incubation and Hatching Procedures

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

Post-hatch Procedures

Dry hatchlings were transferred to a Petersime brood unit maintained at 30.0°C where clean feed and water were available *ad libitum*. Chicks were provided water and feed (Purina Mills Game Bird Startena [St. Louis, MO, USA] for quail and pheasants and Purina Mills Start & Grow Sunfresh [St. Louis, MO, USA] 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, USA) bearing their unique egg number. Chicks were weighed to the nearest 0.1 g, housed by treatment group and raised for two weeks post-hatch. Unhatched eggs with no gross indication of embryo development were assumed to be infertile and removed from the study.

Necropsy

A sub-sample of 10 chicks from each dose group from each species was randomly taken from all treatment groups and euthanized by cervical dislocation at both 1- and 14-d of age. Livers from all chicks were removed, weighed and a portion was placed in an I-Chem jar (VWR International, Chicago, IL, USA) on ice for subsequent contaminant analysis. Additional samples of liver from 14-d chicks were placed into; a microtube containing RNAlater (Ambion, Austin, TX, USA) for analysis of CYP1A4 and CYP1A5 mRNA expression (Yang *et al*., 2010), a microtube frozen in liquid nitrogen for analysis of ethoxyresorufin O-deethylase (EROD) activity (Yang *et al*., 2010), and a vial with 10% buffered formalin for histological evaluation.

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 US 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 PCDDs and PCDFs (Wellington Laboratories, Guelph, ON, CA). Due to 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, ww) were mixed with anhydrous sodium sulfate and fortified with a mixture of ^{13}C-labeled PCDDs and PCDFs (Wellington Laboratories, Guelph, ON, CA). 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 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 (QA/QC) procedures were performed following those of Wan *et aI*. (2010).

Analysis of TCDD, PeCDF and TCDF concentrations in pheasant and chicken liver samples was performed by GC/HRMS using a Trace 2000 series gas chromatograph (Thermo Fisher Scientific, Waltham, MA, USA) and a Finnigan MAT-95 double focusing magnetic sector mass spectrometer (Thermo Electron Co., Bremen, Germany). The HRGC was equipped with a CTC A200S autosampler (Carrboro, NC, USA) and 60 m x 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 5.0°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 EI 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 SIM mode at a resolution of 10,000 (10% valley). All calculations were performed via the isotope-dilution mass spectrometric procedure. When appropriate, the system and 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, USA) 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-d chicks 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. Differences between trials within a species, when appropriate, were taken into account within each analysis.

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 LD50 value for TCDD and the LD50 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 LD50 value of the presumed most sensitive species (chicken) and the LD50 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 (Figures 4-6). Embryo mortality in the vehicle control group was 14% for the quail, 18% for the pheasant, and 16% for the chicken (Table 2). Significantly greater mortality of quail embryos occurred at doses greater than 5.7 pmol TCDD/g egg, 1.8 pmol PeCDF/g egg, and 2.9 pmol TCDF/g egg when compared to the vehicle control (Table 2). For pheasant embryos, significantly greater mortality occurred at doses greater than 0.31 pmol TCDD/g egg, 0.39 pmol PeCDF/g egg, and 0.29 pmol TCDF/g egg when compared to the vehicle control (Table 2). Mortality of chicken embryos was significantly greater than that of the vehicle control at doses greater than 0.19 pmol TCDD/g egg, 0.14 pmol PeCDF/g egg, and 0.15 pmol TCDF/g egg (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 LD50 values (95% confidence intervals) were adjusted for background mortality (Tables 3 and 4; Figures 7-12).

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 (Figures 7-9). 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). 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). In the chicken, the order of chemical potency was TCDF > TCDF ≈ PeCDF based on relative potency values of 2.0 and 0.88 for TCDF and PeCDF, respectively (Table 5). The order of species sensitivity from greatest to least 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 0.18 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 0.021 for the quail.

Concentrations of TCDD, PeCDF and TCDF in livers of chicks

Figures 9 through 14 illustrate the relationship between the injected dose and hepatic concentration of each compound in 1- and 14-d chicks. In 1-d chicks (Figures 10, 12 and 14), the correlation between dose and liver concentration was significant in all cases with the exception of chickens exposed to TCDF. The significant correlation between injected dose and hepatic concentration was weak in Japanese quail exposed to all three compounds and chickens exposed to TCDF when a correlation less than 0.5 (R^2 = 0.25) was designated as weak. At 14-d of age, the correlation between dose and hepatic concentration was not significant for pheasants exposed to TCDD and PeCDF as well as quail exposed to TCDF (Figures 11, 13, and 15). All of the significant correlations had R^2 values greater than 0.25.

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.[a]

	Japanese Quail Dose Groups		Common Pheasant Dose Groups		White Leghorn Chicken Dose Groups	
Compound[a]	(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	0.099	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				

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF)

Table 2. Effects of TCDD, PeCDF or TCDF injected into the air cell of Japanese Quail eggs prior to incubation on embryo mortality.[a]

Compound[a]	Dose (pmol/g egg)	# dead / # fertile	% Mortality[b]
Vehicle Control	0.00	26 / 180	14.4 A
TCDD	0.22	16 / 90	17.8 AB
	0.50	15 / 95	15.8 A
	0.75	21 / 93	22.6 AB
	1.2	13 / 89	14.6 A
	2.9	12 / 88	13.6 A
	5.7	13 / 92	14.1 A
	11	24 / 91	26.4 B
	28	48 / 88	54.5 C
	37	51 / 77	66.2 CD
PeCDF	0.42	20 / 95	21.1 AB
	0.92	18 / 90	20.0 B
	1.8	11 / 95	11.6 A
	2.6	62 / 94	66.0 C
	5.3	63 / 90	70.0 CD
	11.2	65 / 88	73.9 CDE
	11.3	37 / 44	84.1 DE
	21	66 / 85	77.6 DE
	22	75 / 88	85.2 E
TCDF	0.42	19 / 93	20.4 AB
	0.63	12 / 93	12.9 A
	1.6	16 / 94	17.0 A
	2.9	16 / 90	17.8 AB
	4.8	27 / 90	30.0 BC
	7.9	36 / 86	41.9 CD
	8.6	55 / 89	61.8 F
	15	47 / 89	52.8 DE
	24	64 / 89	71.9 EF
	31	59 / 91	64.8 EF

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Values that do not share the same letter are significantly different ($p < 0.05$)

Table 3. Effects of TCDD, PeCDF or TCDF injected into the air cell of Common Pheasant eggs prior to incubation on embryo mortality.[a]

Compound[a]	Dose (pmol/g egg)	# dead / # fertile	% Mortality[b]
Vehicle Control	0.0	15 / 74	20.3 A
TCDD	0.024	15 / 69	21.7 A
	0.032	16 / 70	22.9 AB
	0.072	16 / 70	22.9 AB
	0.10	22 / 73	30.1 AB
	0.26	28 / 75	37.3 B
	1.0	42 / 69	60.9 C
	2.2	49 / 74	66.2 C
PeCDF	0.048	14 / 69	20.3 A
	0.080	23 / 67	34.3 B
	0.13	18 / 65	27.7 AB
	0.20	52 / 75	69.3 C
	0.36	70 / 77	90.9 D
	1.4	70 / 76	92.1 D
	2.3	66 / 70	94.3 D
TCDF	0.040	15 / 68	22.1 A
	0.052	20 / 72	27.8 AB
	0.088	21 / 72	29.2 AB
	0.20	27 / 72	37.5 B
	0.34	52 / 74	70.3 C
	1.5	69 / 75	92.0 D
	4.3	62 / 70	88.6 D

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Values that do not share the same letter are significantly different ($p < 0.05$)

Table 4. Effects of TCDD, PeCDF or TCDF injected into the air cell of White Leghorn Chicken eggs prior to incubation on embryo mortality.[a]

Compound[a]	Dose (pmol/g egg)	# dead / # fertile	% Mortality[b]
Vehicle Control	0.0	16 / 99	16.2 A
TCDD	0.016	11 / 95	11.6 A
	0.031	16 / 97	16.5 A
	0.063	16 / 97	16.5 A
	0.13	45 / 97	46.4 B
	0.25	67 / 96	69.8 C
	0.51	72 / 99	72.7 C
	0.99	86 / 91	94.5 D
PeCDF	0.015	12 / 99	12.1 A
	0.030	13 / 96	13.5 A
	0.048	14 / 100	14.0 A
	0.11	29 / 99	29.3 B
	0.24	51 / 99	51.5 C
	0.47	75 / 94	79.8 D
	0.85	88 / 96	91.7 E
TCDF	0.023	8 / 95	8.42 A
	0.045	21 / 93	22.6 A
	0.075	45 / 96	46.9 B
	0.16	83 / 98	84.7 C
	0.32	89 / 99	89.9 CD
	0.56	94 / 98	95.9 DE
	1.2	98 / 99	99.0 E

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Values that do not share the same letter are significantly different ($p < 0.05$)

Table 5. Lethal dose (LD) estimates [95% confidence interval] expressed as pmol compound/g egg for Japanese Quail, Common Pheasant and White Leghorn Chicken embryos exposed to TCDD, PeCDF or TCDF *in ovo* prior to incubation.[a]

Species	Compound[a]	LD20 (pmol/g egg)	LD50 (pmol/g egg)	LD80 (pmol/g egg)
J. 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]
C. Pheasant	TCDD	0.57 [0.29 - 0.90]	3.5 [2.3 - 6.3]	22 [11 - 77]
	PeCDF	0.22 [0.042 - 0.42]	0.61 [0.28 - 1.2]	1.7 [0.93 - 6.3]
	TCDF	0.31 [0.091 - 0.59]	1.2 [0.62 - 2.2]	4.5 [2.1 - 15]
W.L. Chicken	TCDD	0.27 [0.14 - 0.39]	0.66 [0.47 - 0.90]	1.7 [1.2 - 2.8]
	PeCDF	0.36 [0.27 - 0.44]	0.75 [0.64 - 0.87]	1.6 [1.3 - 2.0]
	TCDF	0.16 [0.090 - 0.23]	0.33 [0.23 - 0.45]	0.69 [0.51 - 1.1]

Note. Lethal dose (LD) values calculated using a Probit model incorporating background mortality (J. Quail = 14.6%, C. Pheasant = 17.9% and W.L. Chicken = 12.5%) into the curve fitting calculations.

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF)

Table 6. Lethal dose (LD) estimates [95% confidence interval] expressed as ng compound/g egg for Japanese Quail, Common Pheasant and White Leghorn Chicken embryos exposed to TCDD, PeCDF or TCDF *in ovo* prior to incubation.[a]

Species	Compound[a]	LD20 (ng/g egg)	LD50 (ng/g egg)	LD80 (ng/g egg)
J. 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]
C. 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]
W.L. 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. Lethal dose (LD) values calculated using a Probit model incorporating background mortality (J. Quail = 14.6%, C. Pheasant = 17.9% and W.L. Chicken = 12.5%) into the curve fitting calculations.

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF)

Table 7. Relative potency (RePs) values for PeCDF and TCDF compared to TCDD based on lethal dose (LD) 50 estimates in Japanese Quail, Common Pheasant and White Leghorn Chicken embryos after *in ovo* exposure prior to incubation.[a]

Species	Compound[a]	LD20 ReP	LD50 ReP	LD80 ReP	EC50 ReP
J. Quail	TCDD	1.0	1.0	1.0	1.0
	PeCDF	11	6.1	3.3	13[b]
	TCDF	3.3	2.0	1.2	0.1[b]
C. Pheasant	TCDD	1.0	1.0	1.0	1.0
	PeCDF	2.6	5.7	13	3.4[b], 15[c]
	TCDF	1.8	2.9	4.9	0.8[b], 0.7[c]
W.L. Chicken	TCDD	1.0	1.0	1.0	1.0
	PeCDF	0.75	0.88	1.1	0.9[b], 0.5[d]
	TCDF	1.7	2.0	2.5	0.09[b], 0.6[d]

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF)

[b] Based on *in vitro* EC50 values for maximal EROD-induction from Hervé *et al.* (2010)

[c] Based on *in ovo* EC50 values for *CYP1A4* mRNA expression from Yang *et al.* (2010).

[d] Based on *in ovo* EC50 values for *CYP1A5* mRNA expression from Yang *et al*. (2010).

Table 8. Relative sensitivity (ReS) values of TCDD, PeCDF and TCDF for Common Pheasant and Japanese Quail compared to White Leghorn Chicken.[a]

Compound[a]	Species	LD20 ReS	LD50 ReS	LD80 ReS	EC50 ReS
TCDD	W.L. Chicken	1.0	1.0	1.0	1.0
	C. Pheasant	0.47	0.19	0.077	0.2[b]
	J. Quail	0.018	0.022	0.028	0.09[b]
PeCDF	W.L. Chicken	1.0	1.0	1.0	1.0
	C. Pheasant	1.6	1.2	0.94	0.8[b]
	J. Quail	0.26	0.15	0.089	1.3[b]
TCDF	W.L. Chicken	1.0	1.0	1.0	1.0
	C. Pheasant	0.52	0.28	0.15	0.2[b]
	J. Quail	0.035	0.022	0.013	0.01[b]

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF)

[b] Based on *in vitro* EC50 values for maximal EROD-induction from Hervé *et al.* (2010)

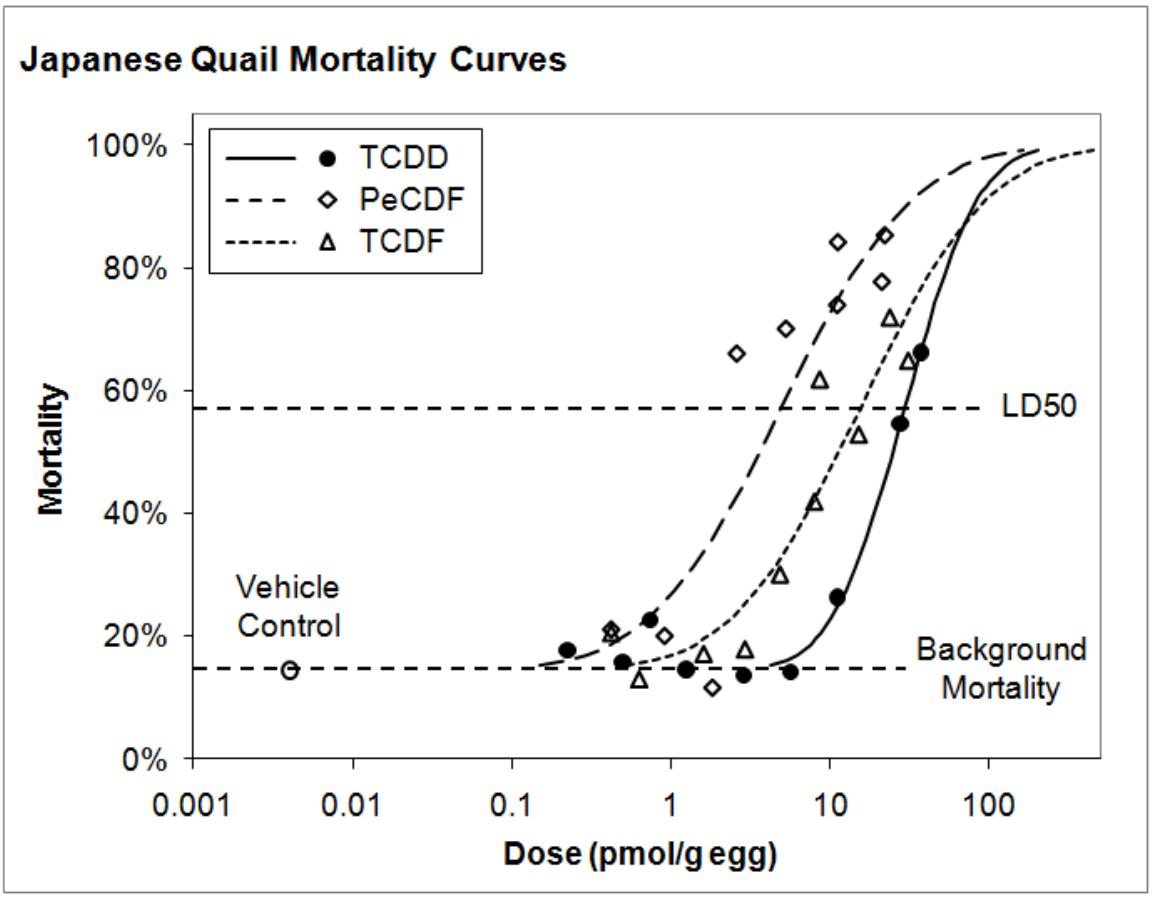


Figure 4. Mortality of Japanese quail eggs injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation. Triolein used as vehicle control. Mortality curves take into account the rate of background mortality (14.6%).

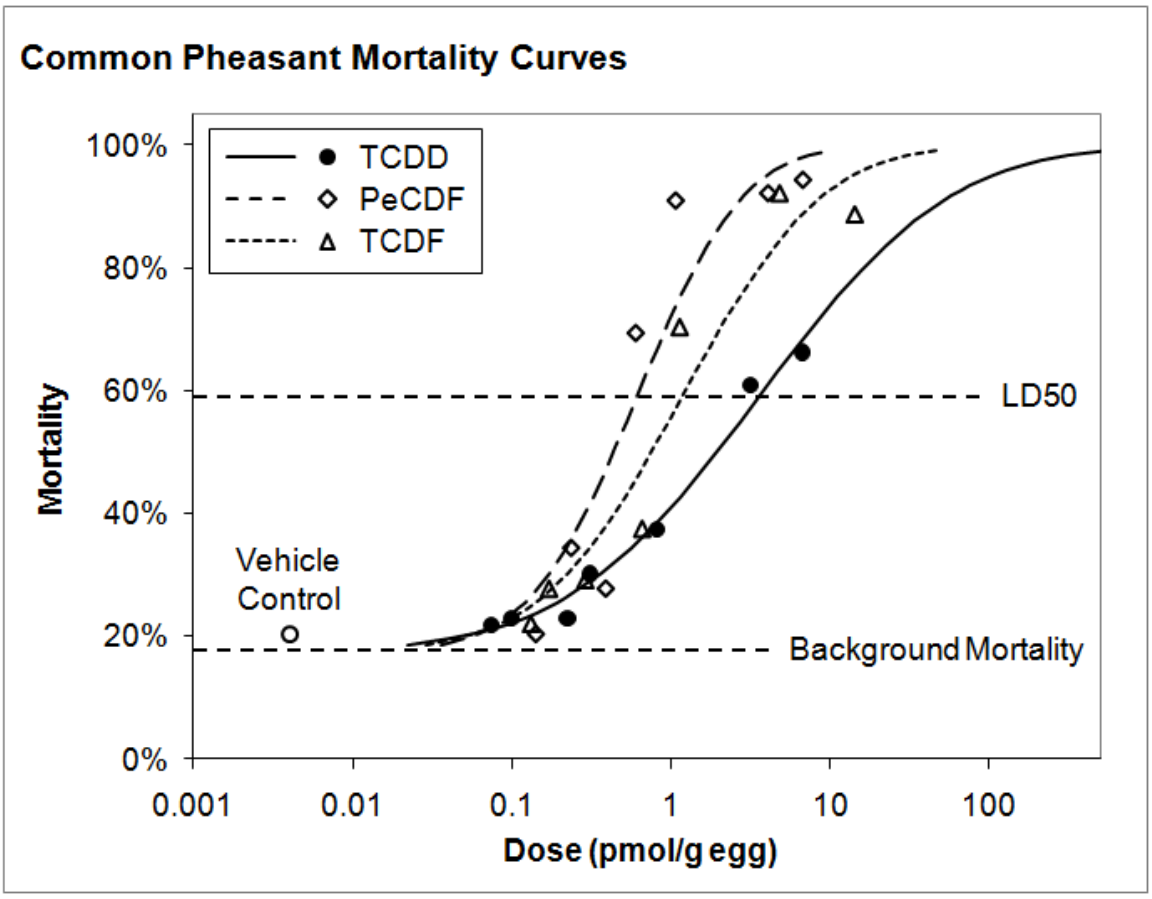


Figure 5. Mortality of Common pheasant eggs injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation. Triolein used as vehicle control. Mortality curves take into account the rate of background mortality (17.9%).

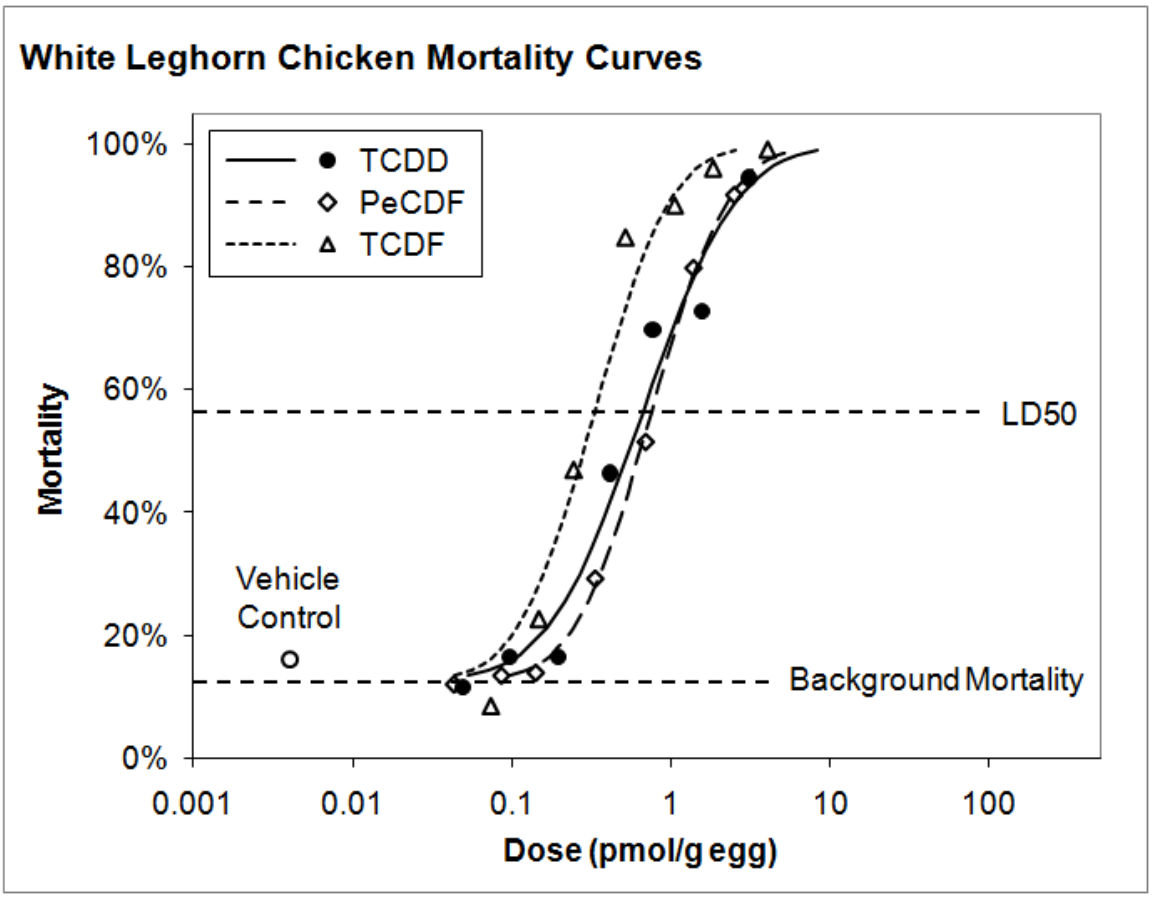


Figure 6. Mortality of White Leghorn chicken eggs injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation. Triolein used as vehicle control. Mortality curves take into account the rate of background mortality (12.5%).

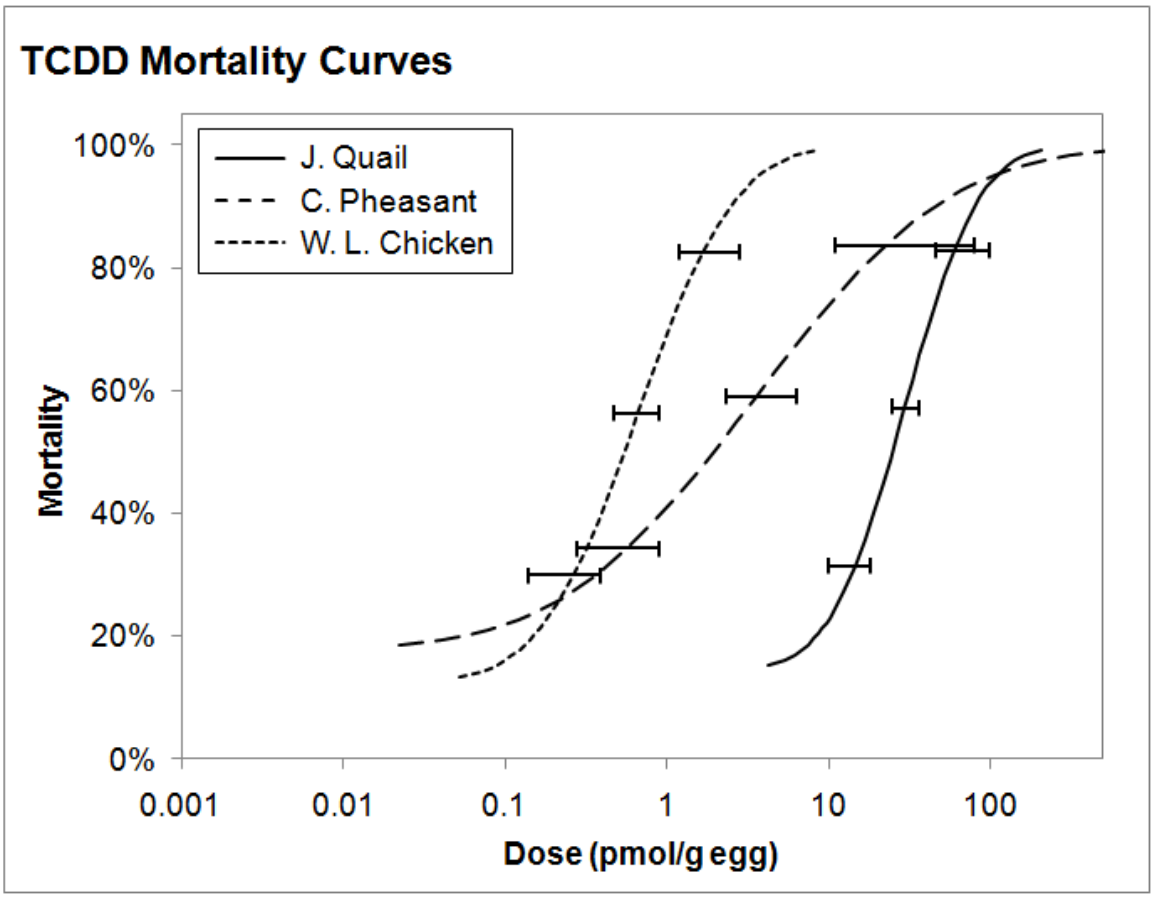


Figure 7. Mortality of Japanese quail, Common pheasant or White Leghorn chicken eggs injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to incubation. Mortality curves take into account the rate of background mortality for each species. The 95% confidence intervals for the LD 20, 50 and 80 are shown for each species.

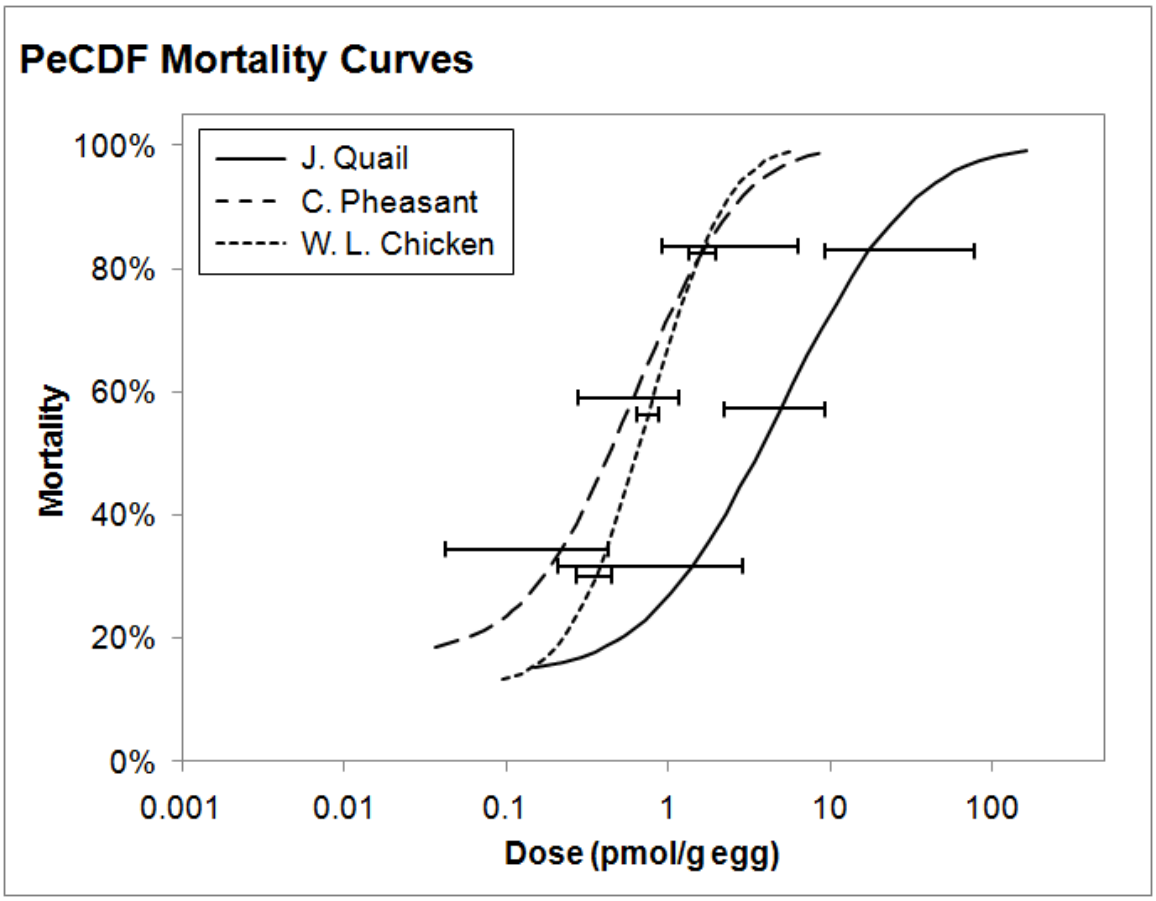


Figure 8. Mortality of Japanese quail, Common pheasant or White Leghorn chicken eggs injected with 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) prior to incubation. Mortality curves take into account the rate of background mortality for each species. The 95% confidence intervals for the LD 20, 50 and 80 are shown for each species.

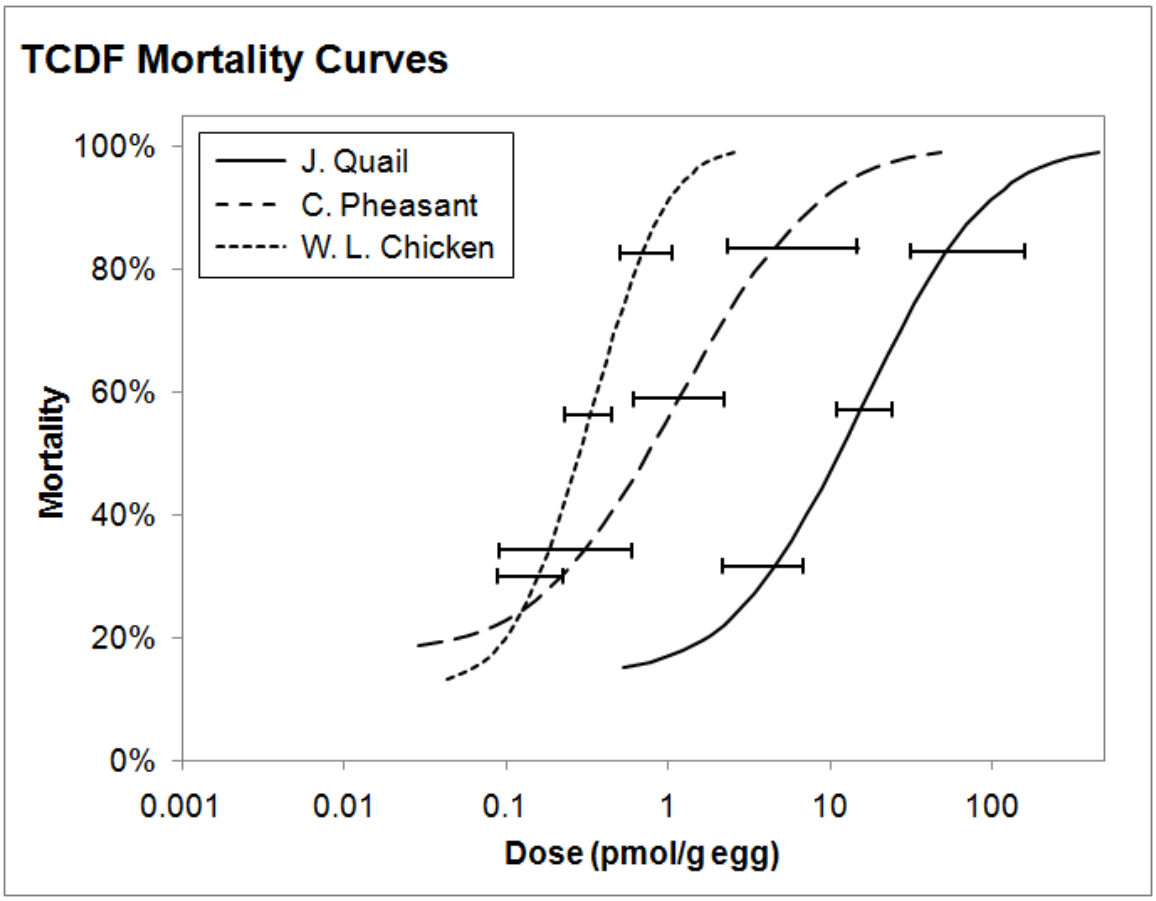


Figure 9. Mortality of Japanese quail, Common pheasant or White Leghorn chicken eggs injected with 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation. Mortality curves take into account the rate of background mortality for each species. The 95% confidence intervals for the LD 20, 50 and 80 are shown for each species.

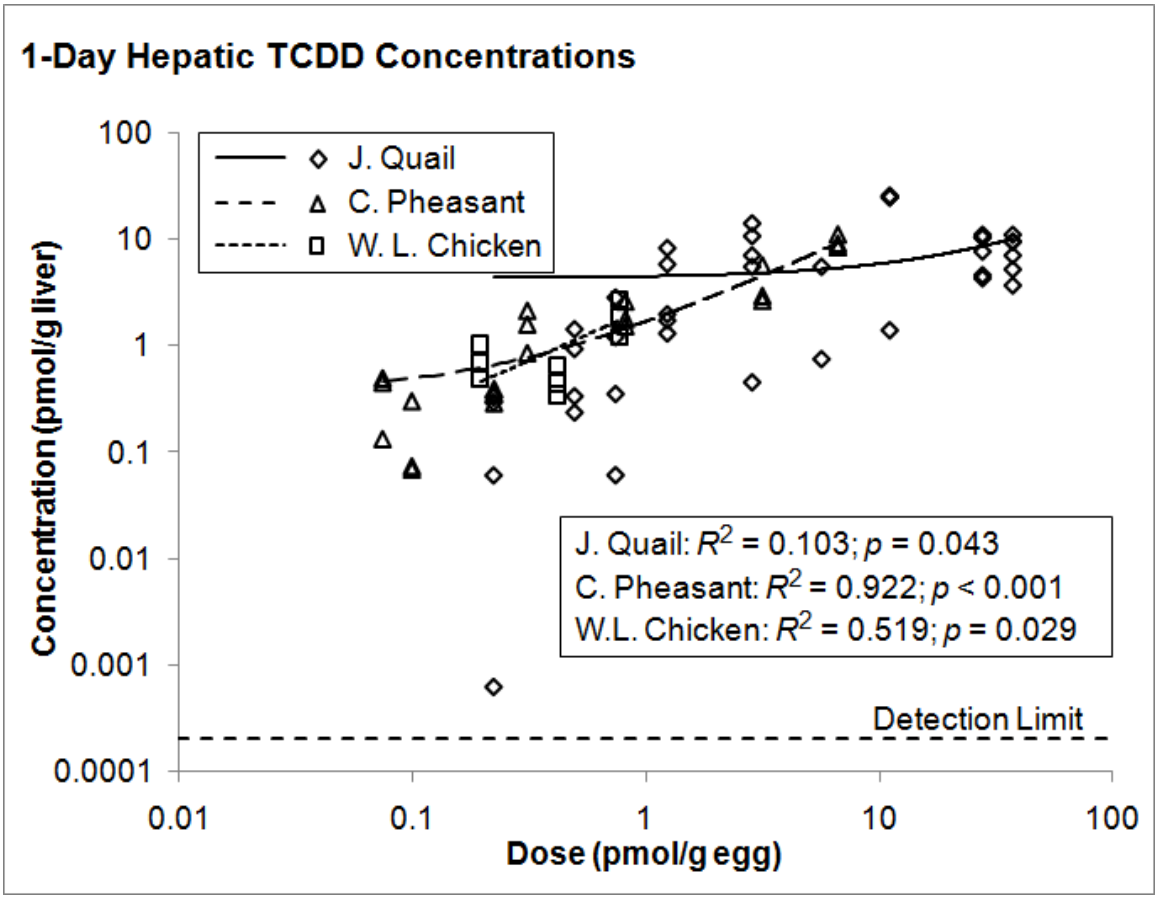


Figure 10. Concentration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the livers of 1-day-old Japanese quail, Common pheasant and White Leghorn chicken hatchlings. R-squared and associated *p*-values are presented for each species.

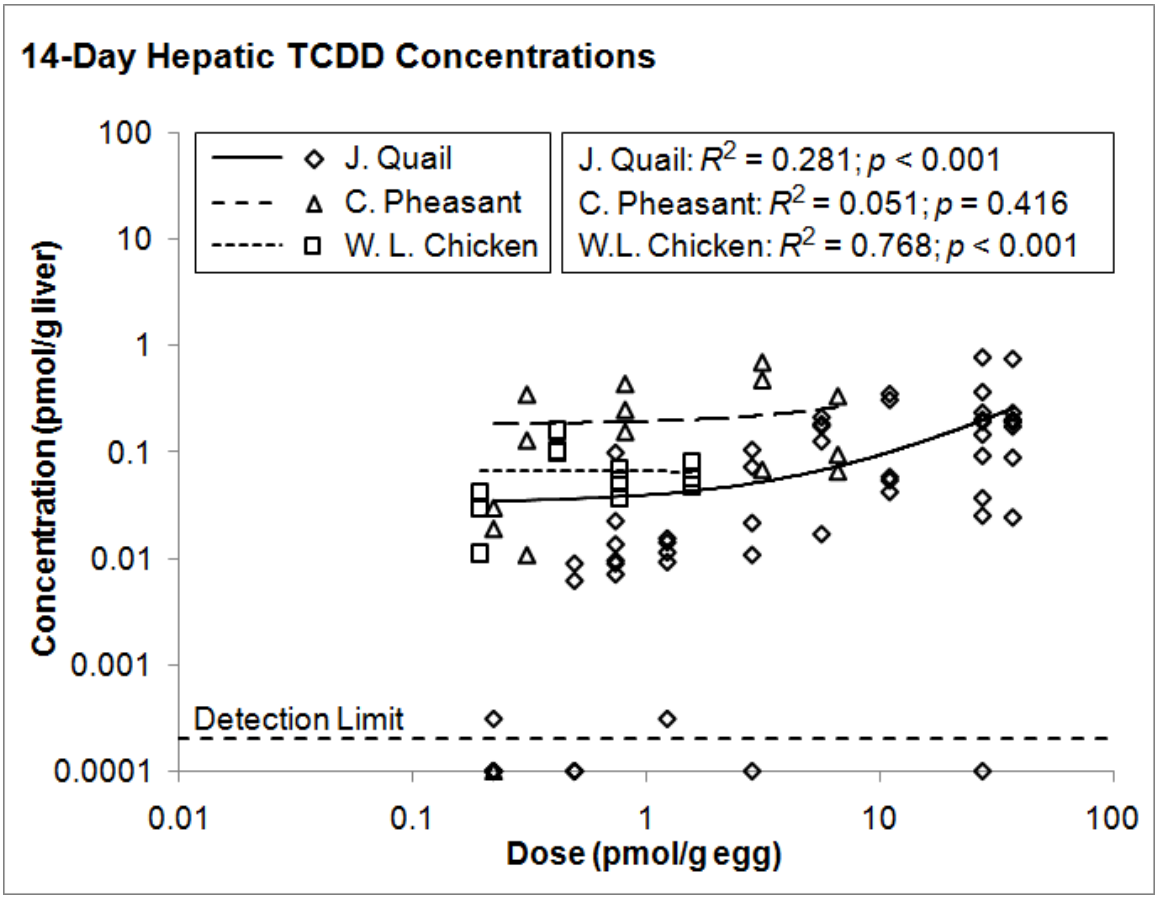


Figure 11. Concentration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in the livers of 14-day-old Japanese quail, Common pheasant and White Leghorn chicken chick livers. R-squared and associated *p*-values are presented for each species.

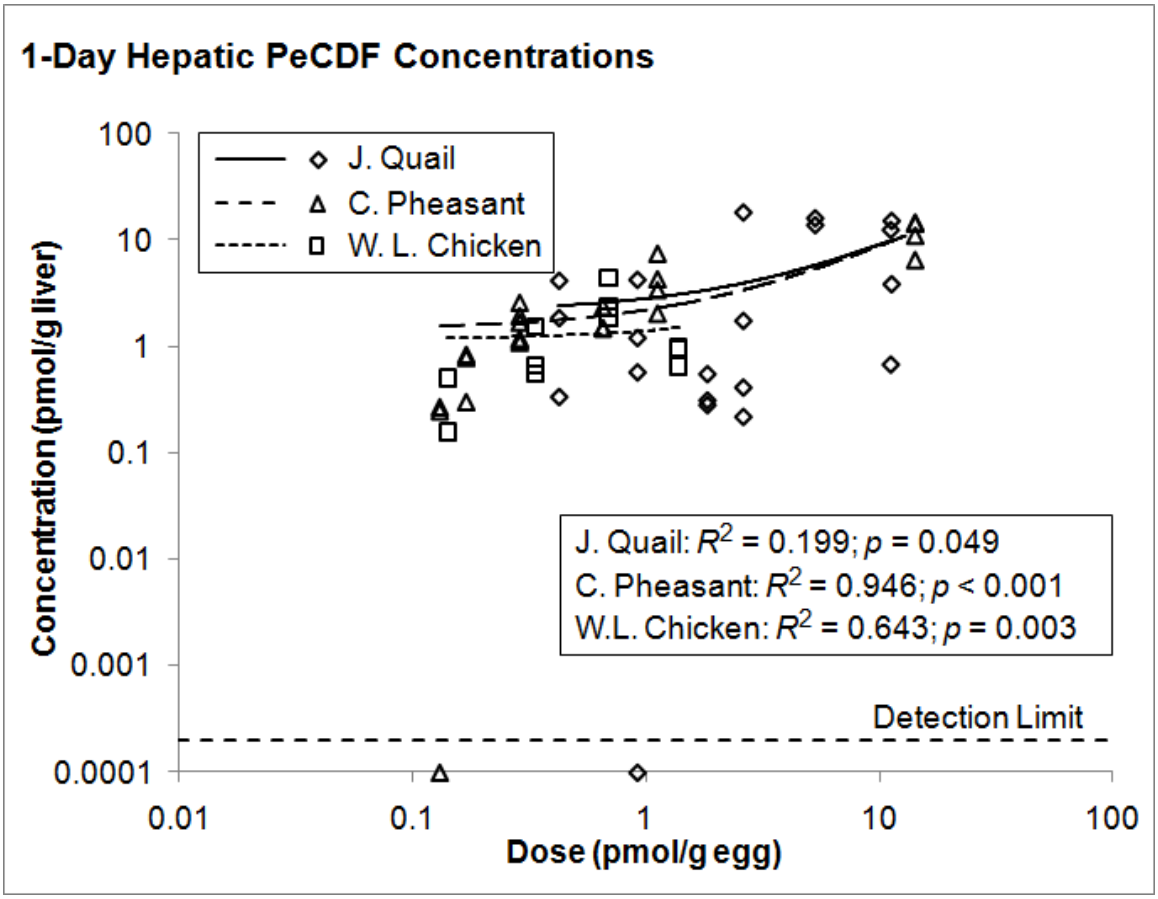


Figure 12. Concentration of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in the livers of 1-day-old Japanese quail, Common pheasant and White Leghorn chicken hatchling livers. R-squared and associated *p*-values are presented for each species.

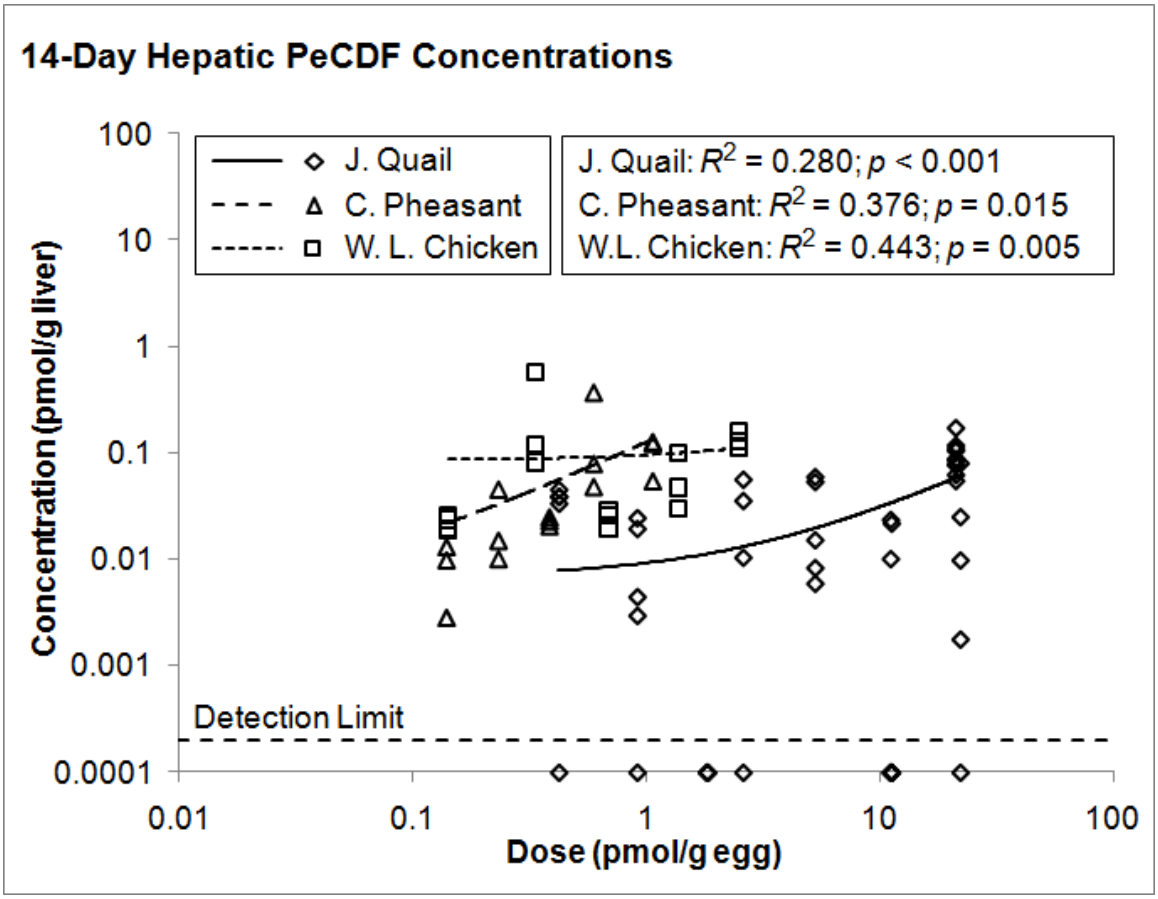


Figure 13. Concentration of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) in the livers of 14-day-old Japanese quail, Common pheasant and White Leghorn chicken chick livers. R-squared and associated *p*-values are presented for each species.

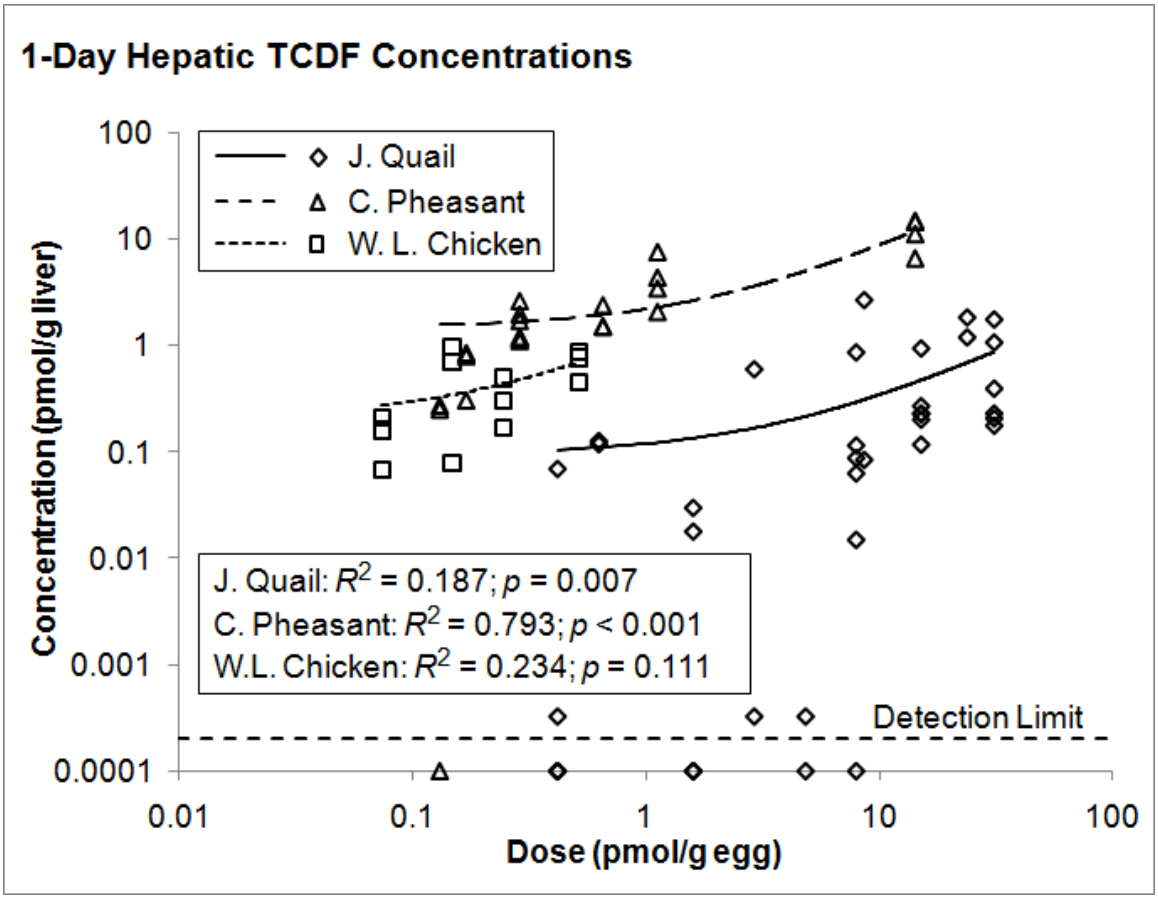


Figure 14. Concentration of 2,3,7,8-tetrachlorodibenzofuran (TCDF) in the livers of 1-day-old Japanese quail, Common pheasant and White Leghorn chicken hatchling livers. R-squared and associated *p*-values are presented for each species.

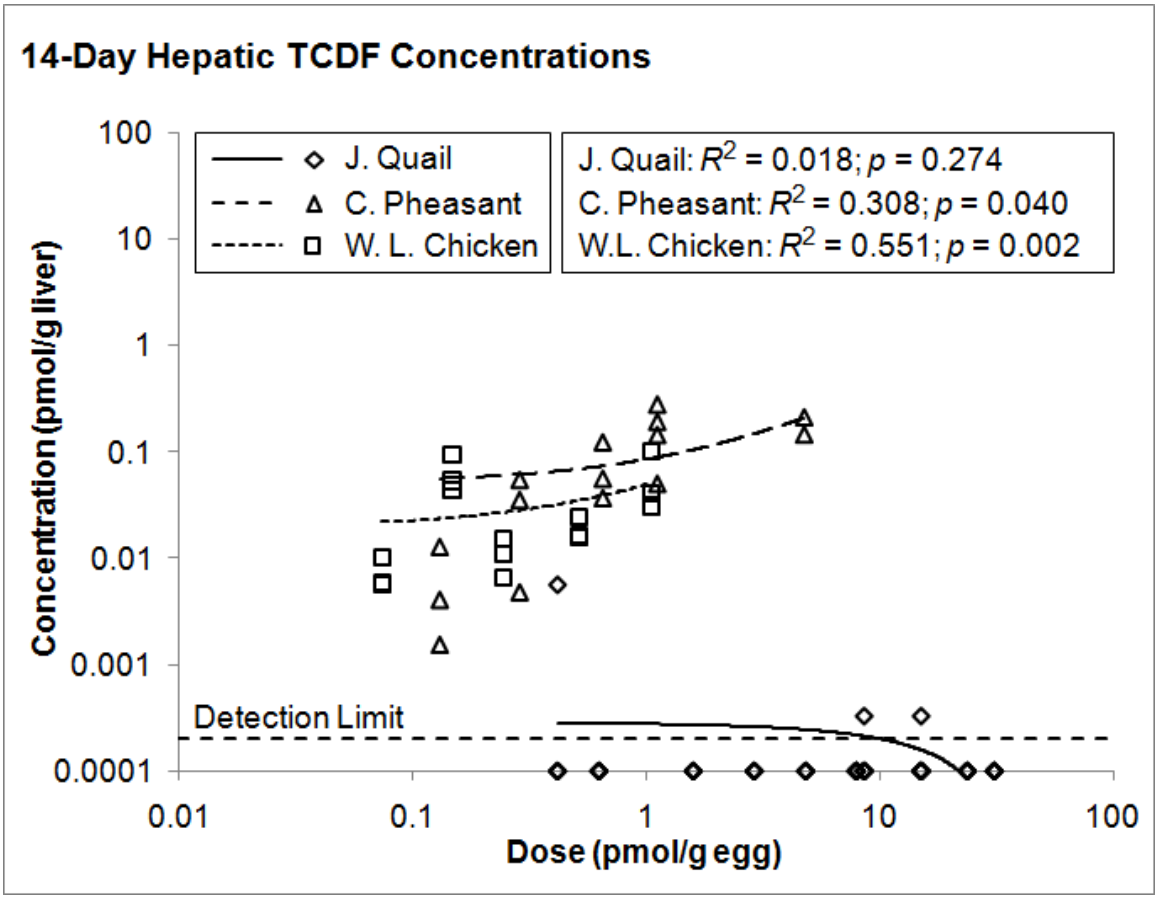


Figure 15. Concentration of 2,3,7,8-tetrachlorodibenzofuran (TCDF) in the livers of 14-day-old Japanese quail, Common pheasant and White Leghorn chicken chick livers. R-squared and associated *p*-values are presented for each species.

DISCUSSION

Initial research that assessed the induction of EROD activity in hepatocyte cultures of different avian species by TCDD suggested that this methodology might be useful for estimating the sensitivity of avian species to the embryotoxic effects of TCDD and TCDD-like chemicals that act through the aryl hydrocarbon receptor. Kennedy *et al*. (1996) demonstrated that chicken hepatocyte cultures were 5- to 10-fold more sensitive to EROD induction by TCDD than were pheasant hepatocyte cultures, which is identical to the difference in sensitivity of these species to the embryotoxic effects of TCDD after *in ovo* injection.

More recently, molecular studies provided a mechanistic basis for the hypothesis that specifically controlled hepatocyte EROD EC50 values might be useful in predicting *in vivo* TCDD sensitivity for individual species of birds. 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 to the chicken (250-fold difference) could be explained, in part, by a difference of two amino acids in the ligand-binding domain of the aryl hydrocarbon receptor (Ile324 and Ser380 in the chicken and Val325 and Ala381 in the tern). Head *et al*. (2008) extended these findings by investigating whether the identity of these two amino acid residues (Ile324 and Ser380) could predict embryonic sensitivity to TCDD-like compounds in a wide range of birds. The aryl hydrocarbon receptor sequences were determined in avian species for which sensitivity data were available. Of the species surveyed, the chicken was the only one having the Ile/Ser genotype and it was the most sensitive species. The wild turkey (*Meleagris gallopavo*), Common pheasant

and eastern bluebird (intermediate Ile/Ala genotype) were less sensitive than the chicken, but more sensitive than the American kestrel (*Falco sparverius*), common tern, double-crested cormorant (*Phalacrocorax auritis*), herring gull (*Larus argentatus*), wood duck (*Aix sponsa*), mallard (*Anas platyrhynchos*), and Japanese quail (Val/Ala genotype).

Most recently, Head and Kennedy (2010) tested the perceived association between the biochemical and toxicological measurements of TCDD sensitivity in avian species. They provided evidence that the well-characterized biochemical measure of potency of TCDD-like compounds (EROD EC50 in hepatocyte cultures) was significantly correlated with the toxicological measure of TCDD sensitivity (LD50) in birds and felt these data provided further validation of the EROD bioassay as a useful predictive tool for ecological risk assessment.

The study described herein was part of a group of collaborative studies designed to further validate this model at the molecular (Yang et al. 2010), *in vitro* (Hervé *et al*. 2010a,b) and *in ovo* levels. 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 (6-fold compared to TCDD) followed by TCDF (2- to 3-fold compared to TCDD) in terms of embryotoxicity in both the Japanese quail and the Common pheasant, while TCDF is more potent (2-fold) than TCDD and PeCDF in the chicken. Furthermore, we demonstrate the chicken to be the most sensitive species to *in ovo* TCDD and TCDF exposure, followed by the

pheasant and then quail, supporting the species sensitivity classification model. The chicken and pheasant are equally sensitive to PeCDF while the quail is approximately 7-fold less sensitive.

Control Mortality Data

Mortality of vehicle control embryos was similar that of non-injected egg values published from other studies using the Japanese quail, Common pheasant or White Leghorn chicken. In Japanese quail, vehicle control mortality in the present study was 14%. Historical hatchability of untreated Japanese quail eggs at the MSU Poultry Research and Teaching Center is 85%. Vehicle control mortality for the Common pheasant in the present study was 18%, 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). In a 1957 study, the natural rate of embryo mortality for the Common pheasant has been reported to be approximately 30% (Fant, 1957). Subsequent selection for reproductive performance or improved incubation techniques may explain differences between the historical data and our background mortality. In the present study, mortality of control White leghorn

chicken embryos was 16%. This is within the range reported in other egg injection studies of chicken where triolein was used as the vehicle. Mortalities of embryos exposed to this vehicle by yolk sac injection were of 23% (13/56) and 13%, respectively (Powell *et al*., 1996; Blankenship *et al*., 2003).

Effects of TCDD, PeCDF, and TCDF on Mortality

Prior to this study, little information was pertaining to the *in ovo* toxicity of TCDD, PeCDF, and TCDF in Galliform species other than the chicken. The LD50 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 are the first published for these compounds in this species. The LD50 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, respectively, are similar to those reported in other *in ovo* toxicity studies. For pheasants, Nosek *et al.* (1993) reported a LD50 of 4.2 pmol TCDD/g egg when injected into the albumin (within the 95% CI of the LD50 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 LD50 of 0.47 pmol/g egg, which approximates the lower 95% CI in the present study, while Allred and Strange (1977) reported an LD50 of 0.75 pmol TCDD/g egg. Injection into the air cell resulted in an LD50 value of 0.92 pmol TCDD/g egg while injection into the yolk resulted in an LD50 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 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 to 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 while TCDF was more potent than TCDD and PeCDF in the chicken. Relative potencies based on EC50 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. (2010a) 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 indicated TCDD to be less potent than TCDF (Table 5). In the pheasant, both Hervé *et al*. (2010a) 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. 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*., 2010a; 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. The *in ovo* data indicated that TCDF was almost 3-fold more potent than TCDD. In the chicken, the relative potencies among the three chemicals were similar based on EROD induction (relative potency = 0.9) (Hervé *et al.,* 2010a) or *CYP1A5* expression (relative potency = 0.6) (Yang *et al.,* 2010) in hepatocyte cultures. These results are consistent with those

reported by Bosveld *et al.* (1992) and Kennedy *et al*. (1996) who assessed EROD induction in hepatocytes. *In ovo* results indicated that TCDF was approximately 3-fold more potent than TCDD and PeCDF. The greater potency of TCDF *in ovo* compared to *in vitro* potency indicates the *in vitro* approach may not always accurately reflect the *in vivo* toxicity of the chemical.

Relative Sensitivity of Japanese Quail and Common Pheasant compared to 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 from greatest to least was chicken > pheasant > quail based on relative sensitivity values for TCDD and TCDF (Table 6). The order of species sensitivity to PeCDF, was pheasant ≈ chicken > quail.

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 5-fold less sensitive (Table 6) (Hervé *et al*. 2010a). 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.* 2010a).

Concentrations of TCDD, PeCDF and TCDF in Liver

With the exception of TCDF-exposed Japanese quail (Figures 13 and 14), concentrations of all three compounds in the livers of 1- and 14-d chicks were proportional to the dose injected (Figures 9 - 12). In 14-d quail, only 3 of the 69 samples (4.3%) had detectable concentrations of TCDF. This was in contrast to 1-d quail, where 30 of the 38 samples (79%) had detectable concentrations. These results suggest this species has the ability to metabolize and/or eliminate TCDF to a greater extent than TCDD or PeCDF. For all three compounds, differences in concentrations between 1- and 14-d chicks (with the exception of TCDF in 14-d quail and 1-d chickens and TCDD in 14-d pheasant) can be attributed to growth dilution when concentrations for both age groups are normalized for growth using the following equations:

1-d growth normalized concentration = 1-d concentration x (1-d chick mass ÷ 14-d chick mass)

14-d growth normalized concentration = 14-d concentration x (14-d chick mass ÷ 1-d 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-d chicks of 1.62 pmol/g liver is converted to 0.355 pmol/g liver and the original TCDF concentration in 14-d 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. The concentrations reported here are representative of only those embryos surviving until hatch. Thus, these values could underestimate actual accumulation of chemical within the liver as embryo mortality prevented sampling from dose groups exposed to greater concentrations.

Differences in the metabolism of TCDF and other TCDD-like compounds have been reported in other avian species as well as mammals. 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 resulted in enhanced metabolism of TCDF. In rodents, TCDF is rapidly metabolized compared to other TCDD-like compounds; a process accelerated by dose-dependent upregulation of CYP1A genes (Tai *et al*., 1993). Results similar to those reported for the cormorant suggest enhanced metabolism or elimination of TCDF compared to PeCDF in wild mink populations residing in environments with elevated concentrations of both compounds (Zwiernik *et al*., 2008).

Results of this study and companion studies indicate: (1) the potency of TCDD-like chemicals in birds varies with species and that 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 LBD of the aryl hydrocarbon receptor deserves serious consideration as a tool for ecological risk assessment. The variation in potency of TCDD-like chemicals 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 chemicals accompanied by adequate *in vitro*, and when possible, *in ovo* confirmation, should reduce

the error inherent in assigning risk associated with environmental exposure of a variety of species to TCDD-like chemicals.

SUMMARY AND CONCLUSIONS

In summary, *in ovo* exposure of TCDD, PeCDF and TCDF caused significantly greater mortality of (1) quail embryos at doses greater than 5.7 pmol TCDD/g egg, 1.8 pmol PeCDF/g egg, and 2.9 pmol TCDF/g egg, (2) pheasant embryos at doses greater than 0.31 pmol TCDD/g egg, 0.39 pmol PeCDF/g egg, and 0.29 pmol TCDF/g egg, and (3) chicken embryos at doses greater than 0.19 pmol TCDD/g egg, 0.14 pmol PeCDF/g egg, and 0.15 pmol TCDF/g egg. LD50 values (95% confidence intervals) were as follows; (1) for quail, 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, (2) for pheasants, 3.5 (2.3 – 6.3) pmol TCDD/g egg, 0.61 (0.28 – 1.2) pmol PeCDF/g egg and 1.2 (0.62 – 2.2) pmol TCDF/g egg, and (3) for chicken, 0.66 (0.47 – 0.90) pmol TCDD/g egg, 0.75 (0.64 – 0.87) pmol PeCDF/g egg and 0.33 (0.23 – 0.45) pmol TCDF/g egg. 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. Differences between 1- and 14-d hepatic concentrations of all three compounds in the quail suggest this species has the ability to metabolize and/or eliminate TCDF to a greater extent than TCDD or PeCDF.

LITERATURE CITED

Literature Cited

Allred, P.M., and Strange, J.R. (1977) The effects of 2,4,5- trichlorophenoxyacetic acid and 2,3,7,8-tetrachlorndibenzo-p-dioxin on developing chicken embryos. *Arch. Environ. Contam. Toxicol.* **6**:483-489.

Blankenship, A.L., Hilscherova, K., Nie, M., Coady, K.K., Villalobos, S.A., Kannan, K., Powell, D.C., Bursian, S.J., and Giesy, J.P. (2003) Mechanisms of TCDD-induced abnormalities and embryo lethality in white leghorn chickens. *Comp. Biochem. Physiol.* **136**(Part C):47-62.

Bosveld, A.T.C., Van den Berg, M., and Theelen, R.M.C. (1992) Assessment of the EROD inducing potency of eleven 2,3,7,8- substituted PCDD/Fs and three coplanar PCBs in the chick embryo. *Chemosphere.* **25**: 911-916.

Fant, R.J. (1957) Criteria for aging pheasant embryos. *J. Wildlife Management.* **21**:324-328.

Fredricks, T.B., Zwiernik, M.J., Seston, R.M., Coefield, S.J., Plsutz, S.C., Tazelaar, D.L., Shotwell, M.S., Bradley, P.W., Kay, D.P., and Giesy, J.P. (2010) Passerine exposure to primarily PCDFs and PCDDs in the river floodplains near Midland, Michigan, USA. *Arch. Environ. Contam. Toxicol.* **58**:1048-1064.

Giesy, J.P., and Kannan, K. (1998) Dioxin-like and non-dioxin-like toxic effects of polychlorinated biphenyls (PCBs): implications for risk assessment. *Crit. Rev. Toxicol*. **28**:511-569.

Hahn, M.E. (1998) The aryl hydrocarbon receptor: a comparative perspective. *Comp. Biochem. Physiol.* **121**(Part C):23-53.

Head, J.A., and Kennedy, S.W. (2010) Correlation between an in vitro and in vivo measure of dioxin sensitivity in birds. *Ecotoxicology* **19**:377-382.

Head, J.A., Hahn, M.E., and Kennedy, S.W. (2008) Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ. Sci. Technol.* **42**:7535-7541.

Heinz, G.H., Hoffman, D.J., Kondrad, S.L., and Erwin, C.A. (2006) Factors affecting the toxicity of methylmercury injected into eggs. *Arch. Environ. Contam. Toxicol.* **50**:264-279.

Hervé, J.C., Crump, D., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., and Kennedy, S.W. (2010b) Ethoxyresorufin *O*-deethylase induction by TCDD, PeCDF and TCDFin ring-necked pheasant and Japanese quaul hepatocytes: Time-dependent effects on concentration-response curves. *Toxicol. In Vitro.* **24**:1301-1305.

Hervé, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., and Kennedy, S.W. (2010a) Cytochrome P450A induction by 2,3,4,8-tetrachlorodibenzo-*p*-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. *Toxicol. Sci*. **113**:380-391.

Henshel, D., Hehn, B., Wagey, R., Vo, M., and Steeves, J.D. (1997) The relative sensitivity of chicken embryos to yolk- or air-cell-injected 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Environ. Toxicol. Chem*. **16**:725-732.

Huwe, J.K. (2002) Dioxins in food: A modern agricultural perspective. *J. Agric. Food Chem.* **50**:1739-1750.

Karchner, S.I., Franks, D.G., Kennedy, S.W., and Hahn, M.E. (2006) The molecular basis for differential dioxin sensitivity in birds: Role of the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci*. **103**:6252-6257.

Kennedy, S.W., Lorenzen, A., Jones, S.P., Hahn, M.E., and Stegeman, J.J. (1996) Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. AppI. Pharmacol.* **141**:214-230.

Kubota, A., Iwata, H., Tanabe, S., Yoneda, K., and Tobata, S. (2005). HepaticCYP1A induction by dioxin-like compounds, and congener-specific metabolism and sequestration in wild common cormorants from Lake Biwa, Japan. *Environ. Sci. Technol.* **39**:3611-3619.

Kubota, A., Iwata, H., Tanabe, S., Yoneda, K., and Tobata, S. (2006) Congener-specific toxicokinetics of PCDD, PCDF, and copalanar PCBs in Black-eared kites Cyt P4501A dependent hepatic sequestration. *Environ. Toxicol. Chem*. **25**(4): 1007-1016.

Kumar, K.S., Kannan, K., Giesy, J.P., and Masunaga, S. (2002) Distribution and elimination of polychlorinated dibenzo-*p*-dioxins, dibenzofurans, biphenyls, and *p,p*'-DEE in tissues of Bald Eagles from the Upper Peninsula of Michigan. *Environ. Sci. Technol.* **36**:2789-2796.

Nosek, J.A., Sullivan, J.R., Craven, S.R., Gendronfitzpatrick, A., and Peterson, R.E. (1993) Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the Ring-necked pheasant. *Environ. Toxicol. Chem.* **12**:1215-1222.

Okey, A. (2007). An aryl hydrocarbon receptor odyssey to the shore of toxicology: The Deichmann Lecture International Congress of Toxicology, XI. *Toxicol. Sci*. **98**:5-38.

Poland, A., and Glover, E. (1977) Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: a study of the structure-activity relationship. *Mol. Pharmacol.* **13**:924-938.

Powell, D.C., Aulerich, R.J., Meadows, J.C., Tillitt, D.E., Giesy, J.P., Stromborg, K.L., and Bursian, S.J. (1996) Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) injected into yolks of chicken (*Gallus domesticus*) eggs prior to incubation. *Arch. Environ. Contam. Toxicol.* **31**:404-409.

Safe, S. (1998) Limitations of the toxic equivalency factor approach for risk assessment of TCDD related compounds. *Teratog. Carcinog. Mutagen.* **17**:285-304.

Sanderson, J.T., Kennedy, S.W., and Giesy, J.P. (1998) In vitro induction of ethoxyresorufin-*O*-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. *Environ. Toxicol. Chem*. **17**:2006–2018.

Tai, H. L., McReynolds, J. H., Goldstein, J. A., Eugster, H. P., Sengstag, C., Alworthy, W. L., and Olson, J. R. (1993) Cytochrome P4501A1 mediates metabolism of 2,3,7,8-tetrachlorodibenzofuran in the rat and human. *Toxicol. Appl. Pharmacol*. **123**:34-42.

Telliard, W.A. (1994) Method 1613: Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS. U.S. Environmental Protection Agency Office of Water.

Van den Berg, M., Birnbaum, L. S., Bosveld, A. T. C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W. *et al*. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect*. **106**:775–792.

Van den Berg, M., De Jongh, J., Poiger, H., and Olson, J. R. (1994) The toxicokinetics and metabolism of polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. *Crit. Rev. Toxicol.* **24**:1-74.

Verrett, M.J. (1976) Investigation of the toxic and teratogenic effects of halogenated dibenzo-*p*-dioxins and dibenzofurans in the developing chicken embryo. Memo Rept, U.S. Food and Drug Admin, Washington, D.C., 14 pp.

Wan, Y., Jones, P.D., Holem, R.R., Khim, J.S., Chang, H., Kay, D.P., Roark, S.A., Newsted, J.L., Patterson, W.P., and Giesy, J.P. (2010) Bioaccumulation of polychlorinated dibenzo-p-dioxins, dibenzofurans, and dioxin-like polychlorinated biphenyls in fishes from the Tittabawassee and Saginaw Rivers, Michigan, USA. *Sci. Total Environ.* **408**:2394–2401.

Yang, Y., Wiseman, S., Cohen-Barnhouse, A.M., Wan, Y., Jones, P., Newsted, J.L., Kay, D.P., Kennedy, S.W., Zwiernick, M.J., Bursian, S.J., and Giesy, J.P. (2010) Effects of *in ovo* exposure of White Leghorn chicken, Common pheasant and Japanese quail to TCDD, 2,3,4,7,8-PeCDF and 2,3,7,8-TCDF on CYP1A induction. *Environ. Toxicol. Chem*. **29**:1490-1502.

Zwiernik, M.J., Bursian, S., Aylward, L.L., Kay, D.P., Moore, J., Rowlands, C., Woodburn, K., Shotwell, M., Khim, J.S., Giesy, J.P., and Budinsky, R.A. (2008). Toxicokinetics of 2,3,7,8-TCDF and 2,3,4,7,8-PeCDF in mink (*Mustela vison*) at ecologically relevant exposures. *Toxicol. Sci.* **105**:33-43.

CHAPTER 2

Post-Hatch Effects of *In Ovo* Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) and 2,3,7,8-Tetrachlorodibenzofuran (TCDF) in Japanese Quail (*Coturnix japonica*), Common Pheasant (*Phasianus colchicus*) and White Leghorn Chicken (*Gallus gallus domesticus*) Embryos

ABSTRACT

Eggs from Japanese quail (*Coturnix japonica*), Common pheasants (*Phasianus colchicus*) and White Leghorn chickens (*Gallus gallus domesticus*) were injected with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation to assess compound-related differences in embryotoxicity and post-hatching endpoints in 1- and 14-day chicks. Doses ranging from 0.044 to 37 pmol/g egg (0.015 to 12 ng/g egg) were injected into the air cell of eggs prior to incubation. Embryo mortality was categorized by stage of development that indicated similar patterns of early- and late-stage dose-related embryo lethality. Body and organ masses of 1- and 14-day chicks were unaffected at doses up to 37 pmol TCDD/g egg, 22 pmol PeCDF/g egg and 31 pmol TCDF/g egg for the quail, and 6.7 pmol TCDD/g egg, 6.8 pmol PeCDF/g egg and 14 pmol TCDF/g egg for the pheasant. Results were similar in the chicken at doses up to 3.1 pmol TCDD/g egg, 2.5 pmol PeCDF/g egg and 4.0 pmol TCDF/g egg; however, a decrease in 14-d body mass occurred above concentrations of 0.77 pmol TCDD/g egg. The percentage of deformed embryos surviving past embryonic day 6 (quail), 10 (pheasant) or 8 (pheasant) for all three compounds was greatest in the quail, followed by the pheasant, and then the chicken. TCDD was not the most teratogenic compound among those tested. No dose related effects were detected in the heart, brain, bursa and spleen tissues of the three species, while histological lesions of the liver resulting from high doses of each compound occurred in only the quail.

INTRODUCTION

Currently, elevated concentrations of polychlorinated dibenzofurans and measurable concentrations of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have been detected in several freshwater ecosystems of the Great Lakes region (Kumar *et al*. 2002; Zwiernik *et al*., 2008; Fredricks *et al*., 2010). Historically, exposure to these and other TCDD-like compounds have been linked to the impairment of reproductive performance in avian species, including; the Double-crested cormorant (*Phalacrocorax auritus*) (Fox *et al.* 1991), Herring gull (*Larus argenatatus*) (Fox *et al*., 1978, 1988), Common tern (*Sterna hirundo*) (Hoffman *et al.,* 1998), Caspian tern (*Hydroprogne caspia*) (Ludwig *et al.*, 1996) and Forster's tern (*Sterna forsteri*) (Hoffman *et al*., 1987). Unfortunately, unilateral characterization of the risk to avian species in contaminated areas remains a significant challenge. This is due, in part, to environmental concentrations that differ spatially and temporally (Giesy *et al*., 1994; Van den Berg *et al*., 1998) as well as broad differences in species-specific sensitivity (Kennedy *et al*., 1996; Hervé *et al*., 2010, Cohen-Barnhouse *et al.,* 2010).

The toxicity of TCDD and TCDD-like compounds is thought to be linked to their interactions with the aryl hydrocarbon receptor, a ligand-activated nuclear transcription factor (Hahn, 1998). Among birds, variations in the amino acid sequence of the ligand-binding domain of the aryl hydrocarbon receptor have been associated with differences in sensitivity to TCDD-like compounds (Karchner *et al.* 2006; Head *et al.*, 2008). Those species with an amino acid sequence similar to that of the White Leghorn chicken (*Gallus gallus domesticus*) are considered 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 aryl hydrocarbon receptor genotypes (Head *et al.* 2008).

Clinical signs of exposure to TCDD and TCDD-like compounds are similar across avian species. These include elevated embryonic and chick mortality, growth retardation, and developmental abnormalities such as bill deformities, club feet, missing eyes, and defective feathering (Gilbertson *et al.* 1991; Giesy *et al*., 1994; Larson *et al*., 1996). Others include subcutaneous, pericardial and peritoneal edema, liver enlargement, liver necrosis, porphyria, and the induction of several mixed-function monooxygenase enzymes (Flick *et al.,* 1965; Bronström and Anderson, 1988; Fox et al., 1988; Elliott *et al.,* 1990; Sanderson *et al*., 1998). 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); consistent with 'chick-edema disease' previously described in commercial poultry (Flick *et al.,* 1965; Gilbertson *et al.* 1991). 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 mixtures or individual congeners. Very few of these signs have been described for avian species exposed to specific members of the polychlorinated dibenzofuran family.

The current risk assessment of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) is based on the results of egg injection and hepatocyte studies using EROD-induction potential (Poland and Glover, 1977; Bosveld *et al*., 1992; Kennedy *et al*., 1996; Sanderson *et al*., 1998). More recently, a series of

collaborative studies was conducted to assess differences in relative potency and species sensitivity among these compounds, including TCDD, in the Japanese quail, Common pheasant, and White Leghorn chicken; Galliform species from each of the proposed avian sensitivity classification categories. Lethal dose (LD) estimates derived from embryo mortality after *in ovo* exposure (Cohen-Barnhouse *et al.,* 2010) and effective concentration (EC) estimates based on EROD, *CYP1A4* or *CYP1A5* induction (*in ovo:* Yang *et al.* 2010; Cohen-Barnhouse *et al.*, 2010; *in vitro*: Hervé *et al*., 2010) were used to compare species and compounds.

The present study was designed to 1) assess differences in embryotoxicity and post-hatching endpoints resulting from *in ovo* exposure to TCDD, PeCDF or TCDF, and 2) compare these endpoints between the Japanese quail, Common pheasant, and White Leghorn chicken. For each of the three species, these endpoints include; the stage at which embryo mortality occurred defined by developmental characteristics, the occurrence and types of embryo and chick deformities, 1- and 14-d chick body mass, and histology and masses of liver, heart, brain, bursa and spleen tissues.

METHODS AND MATERIALS

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. Nine doses of TCDD

and PeCDF and 10 doses of TCDF were injected into Japanese quail eggs, while seven doses of each test compound were injected into pheasant or chicken eggs (Table 1). Dose concentrations were based on previous egg injection studies (Nosek *et al.* 1993; Powell *et al.* 1996b; Henshel *et al.*, 1997), estimates regarding species sensitivity (Head *et al.*, 2008) and environmentally relevant concentrations of each test compound in wild avian species (Fredricks *et al.*, 2010).

Egg Preparation

Pheasant eggs were purchased from McFarlane Pheasants (Janesville, WI, USA) while Japanese quail and White Leghorn chicken eggs were obtained from the Michigan State University (MSU) Poultry Research and Teaching Center (East Lansing, MI, USA). 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 – 15.0 °C until 24 h prior to injection. Eggs were weighed to the nearest 0.1 g and 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. Eggs were assigned a unique identification number written in pencil on the exterior of the shell.

Preparation of Injection Solutions and Egg Injection Procedures

The preparation of injection solutions and egg injection procedures are described in Cohen-Barnhouse *et al.* (2010). Stock solutions of TCDD, TCDF and PeCDF (Sigma-

Aldrich; St. Louis, MO, USA) 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 for quail were formulated based on an injection volume of 0.2 µL/g egg using an average egg weight of 10 g, while for pheasants and chickens, an injection volume of 0.1 µL/g egg was used assuming egg weights of 30 g and 58 g, respectively. 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, Plymouth, MN, USA). A single hole was drilled through the shell into the air cell using a Dremel tool (Robert Bosch Tool Corporation, Racine, WI, USA). Quail eggs were injected with 2.0 µL of the test compound, pheasant eggs were injected with 3.0 µL of the test compound and chickens eggs with 6.0 µL. Injections were made with a positive displacement pipettor (Gilson, Middleton, WI, USA) and the sterile pipette tip was changed after each injection. The site of injection was then sealed using heated paraffin (Royal Oak Sales, Roswell, GA, USA) 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 Co., Gettysburg, OH, USA). Incubation parameters were standard for commercial operations (37.5 to 37.7°C with 50 to 60% humidity). Eggs were automatically rotated every two hours for 13 days (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, Dallas, GA, USA). The internal environment of the hatcher was maintained between 37.2 to 37.8°C at 70 to 75% 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 one day prior to the expected hatching date to two days beyond anticipated hatching.

Egg 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 which may have occurred during transport or incubation. Embryos were categorized into one of five stages of development (quail: 0-3 days, 4-6 days, 7-10 days, 11-13 days, 14 days - pipping, pheasants: 0-5 days, 6-10 days, 11-15 days, 16-20 days, or 21 days - pipping and chickens: 0-4 days, 5-8 days, 9-12 days, 13-16 days, and 17 days - pipping) based on key developmental characteristics.

1- and 14-d Chick Necropsy and Histopathology

A sub-sample of 10 chicks from each dose group from each species was randomly taken from all treatment groups and euthanized by cervical dislocation at both 1- and 14-d of age. Livers from all chicks were removed, weighed and a portion was placed in an I-Chem jar (VWR International, Chicago, IL, USA) on ice for subsequent contaminant analysis (Cohen-Barnhouse *et al.*, 2010). Additional samples of liver from 14-d chicks were placed into; a microtube containing RNAlater (Ambion, Austin, TX, USA) for

analysis of CYP1A4 and CYP1A5 mRNA expression (Yang *et al*., 2010), a microtube frozen in liquid nitrogen for analysis of ethoxyresorufin O-deethylase (EROD) activity (Yang *et al*., 2010), and a vial with 10% buffered formalin for histological evaluation. Livers from all dose groups, as well as the 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 in Cohen-Barnhouse *et al,* (2010). In general, congener concentrations were determined by isotope dilution following the US 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 PCDDs and PCDFs (Wellington Laboratories, Guelph, ON, CA). The methodology for the identification and quantification for these compounds as well as the quality assurance and quality control (QA/QC) procedures are described in Wan *et al*. (2010).

Data Analysis

All statistical analyses were performed using SAS (Version 9.2; SAS, Cary, NC, USA) with statement of significance based on $p < 0.05$. Categorical data (stage of embryo death and incidence of deformities) 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. Differences between body and organ masses were compared using a mixed linear model (Proc Mixed) and compared against control values using a Dunnett's test. Organ mass corrected by body mass, actual organ mass, and the arcsine normalized organ mass (arcsine of the square root of organ mass by body mass) were compared across dose groups. Differences between trials within species, when appropriate, were taken into account within each analysis.

RESULTS

Effects of TCDD, PeCDF and TCDF on Stage of Embryo Mortality

In the Japanese quail, Common pheasant and White Leghorn chicken vehicle control groups, the most embryonic death occurred near the beginning or end of incubation (first and last stages). This pattern remained consistent, with 15.4% of embryo mortality occurring between embryonic days 0 and 3 and 73.1% occurring between embryonic day 14 and pipping in quail; 20.0% of embryo mortality occurring between embryonic day 0 and 5 and 60.0% between embryonic day 21 and pipping in pheasants; and 18.8% of embryo mortality occurring between embryonic days 0 and 4 and 62.5% between embryonic day 19 and pipping in chickens.

The mortality of embryos varied temporally throughout incubation in all three species exposed to the three compounds of interest (Figures 16-24). In general, two

changes in embryo mortality occurred, the first being an increase in mortality at and then following the second developmental stage, and the second being an increase in mortality prior to hatching, during last developmental stage. In Japanese quail, a significant increase in the incidence of embryo mortality was observed between day 4 through 10 for all three compounds at doses greater than 11 pmol TCDD/g egg, 1.8 pmol PeCDF/g egg, and 7.9 pmol TCDF/g egg when compared to the vehicle control (Figures 16, 19 and 22). A significant increase in embryo mortality during the 14-day to pipping stage also occurred in those embryos exposed to TCDF between 7.9 and 15 pmol/g egg and in the 2.6 pmol PeCDF/g egg dose group (Figures 19 and 22). In the Common pheasant, significant increases in embryo mortality occurred between days 6 and 10 at doses greater than 0.82 pmol TCDD/g egg, 0.39 pmol PeCDF/g egg, and 0.65 pmol TCDF/g egg (Figures 17, 20 and 23). In the White Leghorn chicken, there was significantly greater embryo mortality between days 0 and 4 in the 3.1 pmol TCDD/g egg dose group and at doses greater than 1.1 pmol TCDF/ g egg. All three compounds caused significantly greater embryo mortality between days 5 and 8 at doses greater than 0.19 pmol TCDD/g egg, 0.34 pmol PeCDF/g egg, and 0.15 pmol TCDF/g egg. In addition, significantly greater mortality of stage 9 to 12 day embryos occurred at doses greater than 0.77 pmol TCDD/g egg, and between 0.25 and 4.0 pmol TCDF/g egg (Figures 18, 21 and 24). For surviving hatchlings of all three species, post-hatch mortality was not significantly different from that of the vehicle control.

TCDD-, PeCDF- and TCDF-Induced Teratogenesis

Morphological deformities observed in embryos surviving past embryonic day 6 for Japanese quail, embryonic day 10 for Common pheasants and embryonic day 8 for White Leghorn chickens were grouped into four categories: cranial, bill, trunk, and limb (Tables 9-20). 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, clubbed feet and supernumerary appendages. Of the 2,167 quail embryos surviving past embryonic-day 6, 4.25% were deformed. The majority of total deformities (n = 107) were of the bill (36%) and limbs (43%), with fewer instances of cranial (15%) and trunk (7%) deformities. Within the vehicle control group, there was one instance of curled toes and one embryo with gastroschisis. In pheasants, of the 1,099 embryos surviving past embryonic-day 10, 2.64% were deformed, and similar to the quail, the majority of total deformities (n = 29) were of the bill (30%) and limb (48%). Cranial and trunk type deformities each made up 9% of total deformities. There was one instance of curled toes within the pheasant vehicle control group. Of the 1,480 chicken embryos surviving past embryonic-day 8, only 0.88% were deformed. In contrast to quail and pheasants, the majority of total deformities (n = 17) were trunk-type (59%) compared to cranial (18%) and bill (24%) and limb (6%). There were no deformities in chicken eggs injected with the vehicle control.

Effects of TCDD, PeCDF, and TCDF on Chick Mass

Relatively few treatment-related differences in the body mass (expressed as mean ± 1 SD) of 1- and 14-d chicks were observed in Japanese quail, Common pheasants or White Leghorn chickens. In 1-d quail and pheasant hatchlings exposed to any of the three compounds, body masses were not significantly different from those injected with the vehicle only (quail, 6.9 ± 0.66 g; pheasant, 20.0 ± 1.8 g) (Tables 18 and 19). In 1-d chicken hatchlings, only those injected with 0.77 pmol TCDD/g egg had body masses significantly less than that of the vehicle control (39.5 ± 2.4 g) (Table 20).

In 14-d quail, the 28 and 37 pmol TCDD/g egg, 11.3 and 21 pmol PeCDF/g egg, and 0.63, 2.9, 15 and 31 pmol TCDF/g egg dose groups had body masses significantly greater than that of the vehicle control group (56.2 ± 7.4) (Table 18). Body masses of 14-d pheasants and 14-d 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 and 0.60 pmol PeCDF/g egg for the pheasant and 0.42, 1.6 and 3.1 pmol TCDD/g egg and 1.1 pmol TCDF/g egg for the chicken (Tables 19 and 20).

Effects of TCDD, PeCDF and TCDF on the Liver of 1- and 14-d Chicks.

Differences in relative liver mass (expressed as percent body mass, mean [95% confidence interval]) in all three species were sporadic and not associated with a dose. In 1-d quail, mean relative liver masses significantly greater than that of the vehicle control (4.20 [3.52, 4.87]) occurred at 28 pmol TCDD/g egg and 15 pmol TCDF/g egg, while those significantly less than the vehicle control occurred at doses of 1.8, 2.6 and 5.3 pmol

PeCDF/g egg. Fourteen days later, only the only the 7.9 and 31 pmol TCDF/g egg dose groups had relative liver masses significantly greater than the vehicle control (2.78 [2.52, 3.04]) (Table 21). Relative liver masses in 1-d pheasants were significantly greater than the vehicle control (3.02 [2.73, 3.32]) at doses of 0.39 and 1.1 pmol PeCDF/g egg and 14 pmol TCDF/g egg. Those dose groups resulting in significantly greater relative masses compared to the vehicle control (2.59 [2.47, 2.71]) in 14-d chicks included the 0.075 and 0.31 pmol TCDD/g egg, and 0.60 pmol PeCDF/g egg dose groups (Table 22). In 1- and 14-d chickens, relative liver masses were not significantly different that those of the vehicle control (1-d: 2.93 [2.37, 3.50], 14-d: 2.86 [2.57, 3.15]) (Table 23).

There were no significant histological lesions of the liver associated with TCDD, PeCDF or TCDF exposure in either the Common pheasant or White Leghorn chicken. An increase in hepatic vaculation due to lipid accumulation across all dose groups 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 an increase in hepatic vaculation, along with incidences of focal bile duct hyperplasia, bi-nucleation, and karyomegalic (enlarged hepatocyte nuclei) and necrotic hepatocytes, at doses greater than 11 pmol TCDD/g egg, 11.2 pmol PeCDF/g egg and 4.8 pmol TCDF/g egg.

Effects of TCDD, PeCDF and TCDF on the Heart, Brain, Bursa and Spleen of 14-d Chicks

Differences in relative organ mass (expressed as percent body mass, mean [95% confidence interval]) in 14-d chicks of all three species were sporadic and not associated

with dose. In quail, relative heart mass was significantly greater in the 0.50 pmol TCDD/g egg dose group when compared to the vehicle control (0.791 [0.712, 0.871]) (Table 24). Relative bursa mass was significantly less in the 28 pmol TCDD/g egg and 0.42 pmol PeCDF/g egg dose groups when compared to the vehicle control (0.090 [0.076, 0.105] (Table 25). Relative brain and spleen masses were not significantly different than those of the vehicle control (brain: 0.891 [0.806, 0.977], spleen: 0.038 [0.030, 0.045]) (Tables 24 and 25). In pheasants, relative bursa mass in chicks exposed to 0.22 pmol TCDD/g egg were significantly greater than that of the vehicle control (bursa: 0.163 [0.132, 0.193]). There were no significant differences between vehicle control relative heart (1.62 [1.39, 1.85]), brain (0.784 [0.755, 0.812] and spleen (0.084 [0.057, 0.111]) masses compared to treatment dose groups (Tables 26 and 27). Differences in relative organ masses of chickens included significantly greater relative heart mass in the 0.25, 0.52, and 1.1 pmol TCDF/g egg 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 dose group. There were no significant differences 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] (Tables 28 and 29). There were no significant histological lesions associated with TCDD, PeCDF or TCDF exposure in the heart, brain, bursa or spleen of all three species.

Table 9. Incidence of deformities by type found in Japanese Quail embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	175	1.14%	2	-	-	1	1
0.22	85	0.0%	0	-	-	-	-
0.50	88	2.27%	2	-	-	-	2
0.75	83	0.0%	0	-	-	-	-
1.2	83	2.41%	2	-	-	-	2
2.9	86	1.16%	1	-	-	-	1
5.7	90	4.44%	4	-	-	-	4
11	85	1.18%	1	-	-	-	1
28	73	9.59%	10	4	5	-	1
37	51	5.88%	3	-	3	-	-
Total	724	2.76%	23	4	8	0	11

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 6

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 10. Incidence of deformities by type found in Common Pheasant embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	71	1.41%	1	-	-	-	1
0.075	68	1.5%	1	-	1	-	-
0.099	64	0.0%	-	-	-	-	-
0.22	67	2.99%	3	1	1	-	1
0.31	67	0.0%	-	-	-	-	-
0.82	62	6.45%	4	1	0	1	2
3.2	38	0.0%	-	-	-	-	-
6.7	41	4.88%	2	-	1	-	1
Total	407	2.21%	10	2	3	1	4

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 10

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 11. Incidence of deformities by type found in White Leghorn Chicken embryos exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	95	0.0%	0	-	-	-	-
0.049	89	1.1%	2	1	1	-	-
0.096	91	0.0%	0	-	-	-	-
0.19	91	2.20%	2	1	-	-	1
0.42	81	1.23%	1	-	-	1	-
0.77	57	0.0%	0	-	-	-	-
1.6	52	1.92%	1	-	-	1	-
3.1	32	6.25%	2	-	2	-	-
Total	493	1.42%	7	2	3	2	1

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 8

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 12. Incidence of deformities by type found in Japanese Quail embryos exposed to 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	175	1.14%	2	-	-	1	1
0.42	93	4.30%	5	-	2	-	3
0.92	81	3.70%	3	-	-	-	3
1.8	90	5.56%	5	-	2	-	3
2.6	76	9.21%	8	-	3	1	4
5.3	50	10.0%	5	-	-	-	5
11.2	37	5.41%	2	-	-	-	2
11.3	29	3.45%	1	1	-	-	-
21	34	0.0%	0	-	-	-	-
22	24	8.33%	2	1	-	-	1
Total	514	5.64%	31	2	7	1	21

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 6

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 13. Incidence of deformities by type found in Common Pheasant embryos exposed to 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	71	1.41%	1	-	-	-	1
0.14	63	1.59%	2	-	1	1	-
0.24	59	5.08%	3	-	-	-	3
0.39	63	3.17%	2	-	-	-	2
0.60	51	1.96%	2	1	1	-	-
1.1	22	13.6%	3	-	3	-	-
4.1	12	0.0%	0	-	-	-	-
6.8	6	0.0%	0	-	-	-	-
Total	276	3.62%	12	1	5	1	5

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 10

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 14. Incidence of deformities by type found in White Leghorn Chicken embryos exposed to 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	95	0.0%	0	-	-	-	-
0.044	96	0.0%	0	-	-	-	-
0.087	90	2.22%	3	1	1	1	-
0.14	99	0.0%	0	-	-	-	-
0.33	93	0.0%	0	-	-	-	-
0.69	66	1.52%	1	-	-	1	-
1.4	44	0.0%	0	-	-	-	-
2.5	27	3.70%	1	-	-	1	-
Total	515	0.777%	7	1	1	5	0

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 8

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 15. Incidence of deformities by type found in Japanese Quail embryos exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	175	1.14%	2	-	-	1	1
0.42	87	3.45%	3	-	1	-	2
0.63	89	0.0%	0	-	-	-	-
1.6	88	3.41%	3	1	-	1	1
2.9	90	5.56%	5	1	2	-	2
4.8	84	9.52%	8	-	3	-	5
7.9	84	3.57%	4	2	1	1	-
8.6	74	9.46%	9	1	6	1	1
15	69	7.25%	8	2	4	1	1
24	44	20.5%	10	3	5	1	1
31	45	2.22%	1	-	1	-	-
Total	754	5.84%	51	10	23	5	13

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 6

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 16. Incidence of deformities by type found in Common Pheasant embryos exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	71	1.41%	1	-	-	-	1
0.13	65	1.54%	1	-	-	-	1
0.17	68	5.88%	6	-	1	1	4
0.29	70	4.29%	3	-	-	-	3
0.65	65	0.0%	0	-	-	-	-
1.1	51	0.0%	0	-	-	-	-
4.8	15	0.0%	0	-	-	-	-
14	11	9.09%	1	-	-	-	1
Total	345	2.61%	11	0	1	1	9

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 10

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 17. Incidence of deformities by type found in White Leghorn Chicken embryos exposed to 2,3,7,8-tetrachlorodibenzofuran (TCDF) prior to incubation.

Dose (pmol/g egg)	n[b]	% Deformed Embryos	Total Deformities	Cranial[c]	Bill[d]	Trunk[e]	Limb[f]
VC[a]	95	0.0%	0	-	-	-	-
0.074	93	0.0%	0	-	-	-	-
0.15	88	1.14%	1	-	-	1	-
0.25	76	0.0%	0	-	-	-	-
0.52	56	0.0%	0	-	-	-	-
1.1	41	2.44%	1	-	-	1	-
1.8	17	0.0%	0	-	-	-	-
4.0	6	16.7%	1	-	-	1	-
Total	377	0.796%	3	0	0	3	0

[a] Vehicle Control (Triolein)

[b] Sample size = number of eggs containing embryos which survived past embryonic day 8

[c] Cranial deformities include; exencephaly, anophthalmos or microphthalmos

[d] Bill deformities include; incomplete or lack of upper/lower beak or crossbill

[e] Trunk deformities include; edema, gastroschisis, or achondroplasia

[f] Limb deformities include; club foot, curled toes, or extra limb development

Table 18. Effects of TCDD on body mass of 1- and 14-d-old Japanese Quail chicks.[a]

Compound[a]	Dose (pmol/g egg)	1-d		14-d	
		n[b]	BM (g)[c]	n[b]	BM (g)[c]
Vehicle Control	0.0	154	6.87 ± 0.66	61	56.2 ± 7.4
TCDD	0.22	74	6.83 ± 0.66	61	58.4 ± 5.8
	0.50	80	6.80 ± 0.61	58	55.2 ± 6.5
	0.75	72	6.96 ± 0.72	62	58.1 ± 5.6
	1.2	76	6.92 ± 0.77	63	57.4 ± 9.3
	2.9	76	6.93 ± 0.60	62	58.2 ± 5.6
	5.7	79	6.82 ± 0.59	58	58.9 ± 8.2
	11	67	6.91 ± 0.71	43	57.2 ± 6.6
	28	40	7.00 ± 0.75	29	68.9 ± 13A
	37	26	7.24 ± 0.73	16	66.7 ± 12A

Note. d, day; BM, body mass

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean ± standard deviation. Means significantly different than the appropriate control value are designated with 'A'

Table 19. Effects of PeCDF or TCDF on body mass of 1- and 14-d-old Japanese Quail chicks.[a]

Compound[a]	Dose (pmol/g egg)	1-d		14-d	
		n[b]	BM (g)[c]	n[b]	BM (g)[c]
Vehicle Control	0.0	154	6.87 ± 0.66	61	56.2 ± 7.4
PeCDF	0.42	84	6.65 ± 0.63	71	57.4 ± 6.5
	0.92	72	6.70 ± 0.60	54	59.2 ± 6.8
	1.8	75	6.62 ± 0.68	55	59.7 ± 8.1
	2.6	32	6.71 ± 0.58	25	56.6 ± 6.4
	5.3	27	6.69 ± 0.67	16	58.2 ± 7.2
	11.2	23	6.85 ± 0.55	15	59.6 ± 6.7
	11.3	7	6.76 ± 0.97	6	72.9 ± 4.4A
	21	23	7.01 ± 0.78	15	67.4 ± 7.2A
	22	13	6.76 ± 0.57	8	56.0 ± 5.9
TCDF	0.42	74	6.83 ± 0.70	49	59.7 ± 9.2
	0.63	81	6.81 ± 0.60	64	60.8 ± 6.9
	1.6	78	6.66 ± 0.58	64	59.0 ± 8.2A
	2.9	74	6.81 ± 0.58	60	60.3 ± 8.8
	4.8	63	6.65 ± 1.07	41	60.6 ± 7.7A
	7.9	50	6.97 ± 0.56	36	58.9 ± 8.5
	8.6	34	6.71 ± 0.64	18	54.6 ± 10
	15	42	6.93 ± 0.47	26	62.2 ± 8.6
	24	25	6.80 ± 0.78	17	59.4 ± 6.0
	31	32	7.18 ± 0.75	24	72.0 ± 15

Note. d, day; BM, body mass

[a] 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean ± standard deviation. Means significantly different than the appropriate control value are designated with 'A'

Table 20. Effect of TCDD, PeCDF or TCDF on body mass of 1- and 14-d-old Common Pheasant chicks.[a]

Compound[a]	Dose (pmol/g egg)	1-d		14-d	
		n[b]	BM (g)[c]	n[b]	BM (g)[c]
Vehicle Control	0.0	59	20.0 ± 1.8	43	88.5 ± 12
TCDD	0.024	54	19.9 ± 1.5	32	86.0 ± 10
	0.032	54	20.4 ± 1.7	33	82.8 ± 12
	0.072	54	19.7 ± 1.9	33	88.6 ± 15
	0.10	51	20.3 ± 1.8	29	72.0 ± 9.0A
	0.26	47	19.6 ± 1.8	32	90.7 ± 15
	1.0	27	19.6 ± 1.6	19	86.4 ± 16
	2.2	25	19.9 ± 1.8	15	89.5 ± 20
PeCDF	0.048	55	19.5 ± 1.5	32	87.7 ± 14
	0.080	44	19.4 ± 1.9	34	93.6 ± 13
	0.13	47	19.4 ± 1.9	31	93.0 ± 12
	0.20	23	19.7 ± 2.0	12	74.1 ± 14A
	0.36	7	20.0 ± 1.7	4	90.8 ± 5.0
	1.4	6	19.5 ± 1.0	4	79.9 ± 6.6
	2.3	4	18.9 ± 2.2	4	89.8 ± 12
TCDF	0.040	53	19.6 ± 1.5	29	83.7 ± 10
	0.052	52	19.5 ± 1.7	33	84.0 ± 11
	0.088	51	19.6 ± 1.6	32	89.2 ± 14
	0.20	45	20.1 ± 1.6	29	87.6 ± 11
	0.34	22	19.9 ± 1.9	15	90.7 ± 12
	1.5	6	19.1 ± 1.5	4	75.5 ± 4.6
	4.3	8	19.2 ± 1.8	5	87.9 ± 12

Note. d, day; BM, body mass

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean ± standard deviation. Means significantly different than the appropriate control value are designated with 'A'

Table 21. Effects of TCDD, PeCDF and TCDF on body mass of 1-and 14-d-old White Leghorn Chicken chicks.[a]

Compound[a]	Dose (pmol/g egg)	1-d		14-d	
		n[b]	BM (g)[c]	n[b]	BM (g)[c]
Vehicle Control	0.0	83	39.5 ± 2.4	60	115 ± 13
TCDD	0.016	84	39.4 ± 3.1	41	111 ± 11
	0.031	81	39.5 ± 2.5	36	116 ± 20
	0.063	81	38.5 ± 2.7A	44	112 ± 13
	0.13	52	38.6 ± 3.1	38	110 ± 14A
	0.25	29	37.9 ± 2.6A	15	110 ± 12
	0.51	27	38.2 ± 2.8A	16	104 ± 15A
	0.99	5	39.3 ± 3.7	4	95 ± 15A
PeCDF	0.015	87	39.6 ± 2.7	42	114 ± 11
	0.030	83	39.4 ± 2.6	40	115 ± 13
	0.048	86	39.1 ± 2.6	40	117 ± 11
	0.11	70	39.7 ± 5.5	40	114 ± 12
	0.24	48	39.3 ± 2.2	33	111 ± 11
	0.47	19	38.7 ± 3.1	10	108 ± 15
	0.85	8	40.1 ± 2.7	5	112 ± 12
TCDF	0.023	87	38.9 ± 2.8	42	116 ± 11
	0.045	72	38.5 ± 3.5	44	117 ± 10
	0.075	51	38.7 ± 3.0	37	114 ± 12
	0.16	15	39.1 ± 3.0	6	109 ± 13
	0.32	10	39.5 ± 2.4	7	101 ± 10A
	0.56	4	39.4 ± 1.4	4	113 ± 10
	1.2	1	41.0	1	110

Note. d, day; BM, body mass

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean ± standard deviation. Means significantly different than the appropriate control value are designated with 'A'

Table 22. Effect of TCDD on relative liver mass (expressed as % body mass) of 1- and 14-d-old Japanese Quail.[a]

Compound[a]	Dose (pmol/g egg)	n[b]	1-d Liver[c]	n[b]	14-d Liver[c]
Vehicle Control	0	12	4.20 [3.52 , 4.87]	10	2.78 [2.52 , 3.04]
TCDD	0.22	6	3.68 [2.91 , 4.45]	10	2.72 [2.56 , 2.88]
	0.5	6	3.12 [2.71 , 3.53]	10	2.74 [2.53 , 2.94]
	0.75	6	3.22 [2.68 , 3.75]	10	3.11 [2.89 , 3.32]
	1.2	6	3.49 [2.26 , 4.73]	10	2.31 [1.74 , 2.88]
	2.9	6	3.44 [3.18 , 3.69]	10	2.71 [2.46 , 2.97]
	5.7	6	3.78 [3.38 , 4.17]	10	2.61 [2.35 , 2.86]
	11	6	3.43 [2.40 , 4.46]	10	2.99 [2.73 , 3.25]
	28	6	5.83 [4.84 , 6.82]AC	10	3.04 [2.57 , 3.52]B
	37	6	4.55 [3.76 , 5.34]	10	3.10 [2.80 , 3.39]B

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass

Table 23. Effect of PeCDF or TCDF on relative liver mass (expressed as % body mass) of 1- and 14-d-old Japanese Quail.[a]

Compound[a]	Dose (pmol/g egg)	n[b]	1-d Liver[c]	n[b]	14-d Liver[c]
Vehicle Control	0	12	4.20 [3.52 , 4.87]	10	2.78 [2.52 , 3.04]
PeCDF	0.42	6	3.73 [3.31 , 4.16]	10	2.82 [2.48 , 3.15]
	0.92	6	3.83 [3.29 , 4.38]	10	2.96 [2.75 , 3.17]
	1.8	6	3.28 [2.89 , 3.66]AC	10	2.80 [2.56 , 3.03]
	2.6	6	3.17 [2.76 , 3.58]AC	10	2.75 [2.54 , 2.95]
	5.3	6	3.23 [2.90 , 3.55]AC	10	2.65 [2.28 , 3.02]
	11.2	6	3.78 [3.53 , 4.03]	9	2.66 [2.47 , 2.86]
	11.3		n/a	6	2.66 [1.97 , 3.35]
	21		n/a	10	2.49 [2.28 , 2.71]
	22	5	3.45 [2.89 , 4.02]	8	2.96 [2.75 , 3.17]
TCDF	0.42	6	3.64 [3.28 , 3.99]	10	2.89 [2.70 , 3.08]
	0.63	6	3.63 [3.15 , 4.12]	10	2.91 [2.71 , 3.11]
	1.6	6	3.54 [2.98 , 4.10]	10	2.69 [2.35 , 3.02]
	2.9	6	3.39 [2.89 , 3.89]	10	2.91 [2.59 , 3.23]
	4.8	6	3.49 [2.76 , 4.23]	11	2.84 [2.66 , 3.03]
	7.9	6	5.65 [5.23 , 6.06]C	10	4.02 [3.76 , 4.28]ABC
	8.6	6	3.26 [2.72 , 3.80]	10	3.07 [2.37 , 3.78]
	15	6	6.02 [3.76 , 8.28]	10	3.21 [3.00 , 3.42]B
	24	6	3.61 [2.76 , 4.47]AC	10	3.00 [2.76 , 3.24]
	31	6	5.52 [3.41 , 7.63]	10	3.64 [3.36 , 3.92]AB

Note. d, day; n/a, not available

[a] 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass

Table 24. Effect of TCDD, PeCDF or TCDF on relative liver mass (expressed as % body mass) of 1- and 14-d-old Common Pheasants.[a]

Compound[a]	Dose (pmol/g egg)	n[b]	1-d Liver[c]	n[b]	14-d Liver[c]
Vehicle Control	0.0	6	3.02 [2.73 , 3.32]	10	2.59 [2.47 , 2.71]
TCDD	0.075	6	3.33 [3.00 , 3.66]	10	3.13 [2.87 , 3.39]AC
	0.099	6	3.44 [3.15 , 3.73]	10	2.91 [2.52 , 3.30]
	0.22	6	3.24 [2.66 , 3.83]	10	2.63 [2.48 , 2.78]
	0.31	6	3.03 [2.49 , 3.57]	10	3.60 [3.06 , 4.14]AC
	0.82	6	3.38 [3.24 , 3.52]	10	2.60 [2.37 , 2.83]
	3.2	6	3.62 [3.40 , 3.84]	10	2.70 [2.54 , 2.86]
	6.7	6	3.40 [3.05 , 3.75]	10	2.96 [2.71 , 3.22]
PeCDF	0.14	6	3.29 [2.89 , 3.69]	10	2.76 [2.53 , 2.98]
	0.24	6	3.24 [3.03 , 3.46]	10	2.74 [2.53 , 2.95]
	0.39	6	3.30 [3.09 , 3.51]AC	10	2.99 [2.69 , 3.28]
	0.60	6	3.58 [3.32 , 3.84]C	10	3.14 [2.78 , 3.50]AC
	1.1	3	3.59 [2.57 , 4.61]AC	4	2.79 [2.30 , 3.29]
	4.1	2	4.13 [0.63 , 8.90]	4	2.86 [2.06 , 3.67]
	6.8		n/a	4	2.99 [2.37 , 3.60]
TCDF	0.13	6	3.30 [3.02 , 3.57]	10	2.84 [2.52 , 3.16]
	0.17	6	3.54 [3.21 , 3.87]	10	2.82 [2.54 , 3.10]
	0.29	6	3.19 [2.68 , 3.69]	10	2.80 [2.18 , 3.42]
	0.65	6	3.27 [2.81 , 3.74]	10	2.88 [2.53 , 3.22]
	1.1	6	3.34 [2.91 , 3.77]	10	2.82 [2.44 , 3.21]
	4.8	3	3.98 [1.01 , 5.20]	4	2.72 [2.10 , 3.34]
	14	2	3.10 [2.56 , 5.41]A	5	2.95 [2.69 , 3.22]

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass

Table 25. Effect of TCDD, PeCDF or TCDF on relative liver mass (expressed as % body mass) of 1- and 14-d-old White Leghorn Chickens.[a]

Compound[a]	Dose (pmol/g egg)	n[b]	1-d Liver[c]	n[b]	14-d Liver[c]
Vehicle Control	0.0	6	2.93 [2.37, 3.50]	10	2.86 [2.57, 3.15]
TCDD	0.049	6	3.32 [2.79, 3.84]	10	3.04 [2.81, 3.27]
	0.096	6	3.18 [2.88, 3.48]	10	2.99 [2.74, 3.23]
	0.19	7	3.04 [2.44, 3.64]	10	2.87 [2.64, 3.10]
	0.42	6	3.31 [2.65, 3.97]	10	3.02 [2.79, 3.25]
	0.77	6	3.08 [2.54, 3.62]	10	3.03 [2.82, 3.25]
	1.6	6	3.14 [2.69, 3.60]	10	3.01 [2.73, 3.29]
	3.1		n/a	5	3.25 [2.50, 4.01]
PeCDF	0.044	6	3.10 [2.78, 3.42]	10	2.78 [2.53, 3.02]
	0.087	6	3.23 [2.59, 3.87]	10	3.02 [2.86, 3.17]
	0.14	6	3.16 [2.94, 3.39]	10	3.09 [2.94, 3.25]
	0.33	6	3.23 [2.84, 3.62]	10	2.99 [2.65, 3.32]
	0.69	6	3.59 [3.28, 3.90]	10	3.07 [2.82, 3.32]
	1.4	6	3.00 [2.30, 3.69]	9	2.99 [2.82, 3.16]
	2.5	2	2.84 [1.96, 3.73]	6	3.17 [3.04, 3.29]
TCDF	0.074	6	3.36 [2.60, 4.12]	5	2.87 [2.55, 3.20]
	0.15	6	3.13 [2.71, 3.55]	10	3.01 [2.85, 3.17]
	0.25	6	3.21 [2.75, 3.67]	10	2.98 [2.77, 3.20]
	0.52	4	3.14 [2.80, 3.48]	9	2.99 [2.77, 3.20]
	1.1	3	2.87 [1.92, 3.82]	6	3.19 [2.94, 3.45]
	1.8		n/a	4	2.85 [2.33, 3.36]
	4.0		n/a	1	2.16

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass

Table 26. Effect of TCDD on 14-d Japanese Quail relative heart and brain mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Heart[c]	Brain[c]
Vehicle Control	0.0	10	0.791 [0.712, 0.871]	0.891 [0.806, 0.977]
TCDD	0.22	10	0.870 [0.835, 0.905]	0.835 [0.772, 0.898]
	0.50	10	0.903 [0.826, 0.981]AC	0.902 [0.839, 0.966]
	0.75	10	0.865 [0.813, 0.917]	0.912 [0.768, 1.056]
	1.2	10	0.847 [0.778, 0.916]	0.882 [0.822, 0.941]
	2.9	10	0.820 [0.738, 0.901]	0.886 [0.815, 0.958]
	5.7	10	0.881 [0.823, 0.940]B	0.897 [0.825, 0.970]
	11	10	0.877 [0.818, 0.936]	0.939 [0.820, 1.058]
	28	10	0.773 [0.726, 0.821]B	0.932 [0.808, 1.056]
	37	10	0.781 [0.713, 0.848]B	0.876 [0.790, 0.961]

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 27. Effect of PeCDF or TCDF on 14-d Japanese Quail relative heart and brain mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Heart[c]	Brain[c]
Vehicle Control	0.0	10	0.791 [0.712 , 0.871]	0.891 [0.806 , 0.977]
PeCDF	0.42	10	0.793 [0.742 , 0.845]	0.952 [0.837 , 1.066]
	0.92	10	0.843 [0.786 , 0.900]	0.933 [0.838 , 1.028]
	1.8	10	0.865 [0.796 , 0.935]	0.924 [0.850 , 0.998]
	2.6	10	0.817 [0.685 , 0.949]	0.931 [0.865 , 0.997]
	5.3	10	0.834 [0.701 , 0.967]	0.907 [0.862 , 0.952]
	11.2	8	0.849 [0.765 , 0.934]	0.951 [0.813 , 1.090]
	11.3	6	0.776 [0.708 , 0.844]B	0.861 [0.785 , 0.936]
	21	10	0.789 [0.763 , 0.816]B	0.870 [0.779 , 0.962]
	22	8	0.869 [0.793 , 0.945]	0.961 [0.826 , 1.095]
TCDF	0.42	10	0.821 [0.733 , 0.909]	0.894 [0.818 , 0.970]
	0.63	10	0.839 [0.769 , 0.909]	0.931 [0.884 , 0.978]
	1.6	10	0.895 [0.790 , 1.000]	0.915 [0.821 , 1.009]
	2.9	10	0.824 [0.735 , 0.912]	0.937 [0.796 , 1.077]
	4.8	11	0.882 [0.816 , 0.947]	0.982 [0.868 , 1.096]
	7.9	10	0.910 [0.850 , 0.970]B	0.864 [0.827 , 0.901]
	8.6	10	0.969 [0.782 , 1.157]	1.118 [0.833 , 1.402]BC
	15	10	0.826 [0.777 , 0.875]B	0.896 [0.806 , 0.986]
	24	10	0.857 [0.769 , 0.944]	1.047 [0.904 , 1.190]
	31	10	0.784 [0.718 , 0.850]B	0.904 [0.840 , 0.968]B

Note. d, day; n/a, not available

[a] 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 28. Effect of TCDD on 14-d-old Japanese Quail relative bursa and spleen mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Bursa[c]	Spleen[c]
Vehicle Control	0.0	10	[0.090 0.076, 0.105]	0.038 [0.030, 0.045]
TCDD	0.22	10	[0.088 0.075, 0.101]	0.041 [0.035, 0.046]
	0.50	10	[0.091 0.075, 0.107]	0.038 [0.032, 0.045]
	0.75	10	[0.080 0.073, 0.088]	0.038 [0.030, 0.045]
	1.2	10	[0.104 0.084, 0.123]	0.043 [0.034, 0.053]
	2.9	10	[0.087 0.069, 0.105]	0.040 [0.032, 0.048]
	5.7	10	[0.106 0.087, 0.125]	0.046 [0.037, 0.055]
	11	10	[0.078 0.061, 0.094]	0.035 [0.027, 0.043]
	28	10	[0.059 0.038, 0.079]AB	0.043 [0.029, 0.058]B
	37	10	[0.064 0.048, 0.080]B	0.045 [0.034, 0.056]

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 29. Effect of PeCDF or TCDF on 14-d-old Japanese Quail relative bursa and spleen mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Bursa[c]	Spleen[c]
Vehicle Control	0.0	10	0.090 [0.076, 0.105]	0.038 [0.030, 0.045]
PeCDF	0.42	10	0.063 [0.056, 0.070]AC	0.040 [0.032, 0.048]
	0.92	10	0.081 [0.069, 0.093]	0.033 [0.029, 0.038]
	1.8	10	0.091 [0.073, 0.110]	0.040 [0.034, 0.046]
	2.6	10	0.096 [0.087, 0.105]	0.044 [0.034, 0.054]
	5.3	10	0.073 [0.058, 0.089]	0.039 [0.030, 0.049]
	11.2	8	0.087 [0.061, 0.113]	0.037 [0.030, 0.045]
	11.3	6	0.109 [0.076, 0.142]B	0.043 [0.035, 0.051]
	21	10	0.074 [0.062, 0.085]	0.046 [0.038, 0.054]B
	22	8	0.085 [0.071, 0.099]	0.042 [0.030, 0.054]
TCDF	0.42	10	0.081 [0.065, 0.097]	0.037 [0.031, 0.042]
	0.63	10	0.092 [0.078, 0.106]	0.037 [0.030, 0.044]
	1.6	10	0.090 [0.061, 0.118]	0.038 [0.030, 0.047]
	2.9	10	0.075 [0.060, 0.089]	0.041 [0.033, 0.050]
	4.8	9	0.078 [0.058, 0.097]	0.049 [0.038, 0.059]
	7.9	10	0.071 [0.060, 0.083]	0.049 [0.038, 0.061]
	8.6	10	0.096 [0.060, 0.131]	0.044 [0.035, 0.052]
	15	9	0.087 [0.064, 0.110]	0.045 [0.033, 0.057]
	24	10	0.092 [0.069, 0.114]	0.050 [0.040, 0.061]
	31	10	0.069 [0.054, 0.083]	0.038 [0.033, 0.043]

Note. d, day; n/a, not available

[a] 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 30. Effect of TCDD, PeCDF or TCDF on 14-d-old Common Pheasant relative heart and brain mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Heart[c]	Brain[c]
Vehicle Control	0.0	10	1.621 [1.393 , 1.850]	0.784 [0.755 , 0.812]
TCDD	0.075	10	1.673 [1.551 , 1.795]	0.848 [0.765 , 0.932]
	0.099	10	1.669 [1.584 , 1.753]	0.824 [0.748 , 0.899]
	0.22	10	1.582 [1.431 , 1.734]	0.730 [0.671 , 0.788]
	0.31	10	1.873 [1.744 , 2.002]C	0.778 [0.681 , 0.876]
	0.82	10	1.589 [1.388 , 1.791]	0.835 [0.758 , 0.912]
	3.2	10	1.517 [1.350 , 1.683]	0.778 [0.690 , 0.867]
	6.7	10	1.522 [1.402 , 1.643]	0.814 [0.678 , 0.949]
PeCDF	0.14	10	1.580 [1.429 , 1.731]	0.751 [0.668 , 0.835]
	0.24	10	1.475 [1.324 , 1.627]	0.713 [0.640 , 0.785]
	0.39	10	1.549 [1.453 , 1.644]	0.732 [0.664 , 0.800]
	0.60	10	1.745 [1.528 , 1.962]	0.799 [0.692 , 0.907]
	1.1	4	1.567 [1.494 , 1.639]	0.863 [0.713 , 1.013]
	4.1	4	1.741 [1.645 , 1.838]	0.744 [0.643 , 0.844]
	6.8	4	1.556 [1.427 , 1.685]	0.758 [0.643 , 0.872]
TCDF	0.13	10	1.604 [1.440 , 1.768]	0.832 [0.704 , 0.960]
	0.17	10	1.593 [1.441 , 1.744]	0.761 [0.706 , 0.817]
	0.29	10	1.657 [1.253 , 2.061]	0.806 [0.630 , 0.983]
	0.65	10	1.538 [1.349 , 1.727]	0.823 [0.700 , 0.946]
	1.1	10	1.558 [1.437 , 1.679]	0.866 [0.774 , 0.959]
	4.8	4	1.720 [1.547 , 1.893]	0.810 [0.411 , 1.209]
	14	5	1.547 [1.408 , 1.685]	0.799 [0.616 , 0.981]

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 31. Effect of TCDD, PeCDF or TCDF on 14-d-old Common Pheasant relative bursa and spleen mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Heart[c]	Brain[c]
Vehicle Control	0.0	10	0.163 [0.132 , 0.193]	0.084 [0.057 , 0.111]
TCDD	0.075	10	0.188 [0.160 , 0.217]	0.062 [0.050 , 0.074]
	0.099	10	0.158 [0.125 , 0.191]	0.080 [0.068 , 0.092]
	0.22	10	0.210 [0.189 , 0.232]A	0.087 [0.065 , 0.110]
	0.31	10	0.119 [0.088 , 0.149]BC	0.064 [0.048 , 0.080]
	0.82	10	0.158 [0.138 , 0.178]	0.086 [0.061 , 0.112]
	3.2	10	0.164 [0.133 , 0.195]	0.098 [0.068 , 0.128]
	6.7	10	0.181 [0.148 , 0.215]	0.074 [0.061 , 0.088]
PeCDF	0.14	10	0.196 [0.158 , 0.234]	0.084 [0.057 , 0.112]
	0.24	10	0.207 [0.173 , 0.242]	0.076 [0.062 , 0.091]
	0.39	10	0.174 [0.134 , 0.213]	0.080 [0.062 , 0.097]
	0.60	10	0.171 [0.121 , 0.220]	0.083 [0.058 , 0.107]
	1.1	4	0.185 [0.108 , 0.261]	0.062 [0.031 , 0.094]
	4.1	4	0.151 [0.099 , 0.203]	0.071 [0.053 , 0.089]
	6.8	4	0.208 [0.103 , 0.312]	0.060 [0.051 , 0.070]
TCDF	0.13	10	0.188 [0.147 , 0.230]	0.086 [0.067 , 0.104]
	0.17	10	0.188 [0.153 , 0.223]	0.061 [0.045 , 0.076]
	0.29	10	0.190 [0.165 , 0.214]	0.081 [0.059 , 0.104]
	0.65	10	0.180 [0.147 , 0.213]	0.076 [0.056 , 0.096]
	1.1	10	0.190 [0.161 , 0.220]	0.096 [0.060 , 0.132]
	4.8	4	0.176 [0.122 , 0.231]	0.093 [0.048 , 0.137]
	14	5	0.169 [0.130 , 0.209]	0.071 [0.061 , 0.081]

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 32. Effect of TCDD, PeCDF or TCDF on 14-d-old White Leghorn Chicken relative heart and brain mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Heart[c]	Brain[c]
Vehicle Control	0.0	10	1.147 [1.075 , 1.219]	0.653 [0.570 , 0.736]
TCDD	0.049	10	1.183 [1.119 , 1.247]	0.703 [0.643 , 0.764]
	0.096	10	1.203 [1.135 , 1.272]	0.714 [0.661 , 0.767]
	0.19	10	1.130 [1.062 , 1.197]	0.731 [0.666 , 0.797]
	0.42	10	1.262 [1.173 , 1.350]	0.693 [0.656 , 0.730]
	0.77	10	1.117 [1.037 , 1.196]	0.698 [0.598 , 0.799]
	1.6	10	1.236 [1.144 , 1.327]	0.688 [0.645 , 0.731]
	3.1	5	1.355 [1.085 , 1.624]	0.800 [0.628 , 0.972]AC
PeCDF	0.044	10	1.205 [1.140 , 1.270]	0.706 [0.619 , 0.793]
	0.087	10	1.135 [1.064 , 1.205]	0.700 [0.613 , 0.787]
	0.14	10	1.088 [1.019 , 1.156]	0.718 [0.670 , 0.765]
	0.33	10	1.151 [1.106 , 1.196]	0.733 [0.662 , 0.804]
	0.69	10	1.144 [1.069 , 1.219]	0.690 [0.639 , 0.741]
	1.4	9	1.195 [1.126 , 1.264]	0.698 [0.625 , 0.772]
	2.5	6	1.167 [1.080 , 1.253]	0.798 [0.689 , 0.908]
TCDF	0.074	5	1.168 [1.006 , 1.331]	0.711 [0.632 , 0.790]
	0.15	10	1.101 [1.012 , 1.190]	0.746 [0.679 , 0.813]
	0.25	10	1.166 [1.073 , 1.259]AC	0.813 [0.715 , 0.911]
	0.52	9	1.168 [1.109 , 1.227]AC	0.822 [0.727 , 0.918]
	1.1	6	1.210 [1.130 , 1.289]AC	0.854 [0.715 , 0.992]B
	1.8	4	1.184 [1.145 , 1.223]	0.707 [0.510 , 0.904]
	4.0	1	1.182	1.052

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

Table 33. Effect of TCDD, PeCDF or TCDF on 14-d-old White Leghorn Chicken relative bursa and spleen mass (expressed as % body mass).[a]

Compound[a]	Dose (pmol/g egg)	n[b]	Heart[c]	Brain[c]
Vehicle Control	0.0	10	0.449 [0.381 , 0.517]	0.107 [0.092 , 0.122]
TCDD	0.049	10	0.450 [0.384 , 0.517]	0.112 [0.095 , 0.129]
	0.096	10	0.521 [0.421 , 0.620]	0.106 [0.090 , 0.121]
	0.19	10	0.524 [0.458 , 0.590]	0.122 [0.094 , 0.149]
	0.42	10	0.409 [0.364 , 0.455]	0.105 [0.081 , 0.129]
	0.77	10	0.473 [0.358 , 0.589]	0.116 [0.102 , 0.130]
	1.6	10	0.413 [0.351 , 0.474]	0.125 [0.111 , 0.138]
	3.1	5	0.350 [0.211 , 0.488]	0.145 [0.111 , 0.179]
PeCDF	0.044	10	0.498 [0.418 , 0.578]	0.121 [0.100 , 0.142]
	0.087	10	0.513 [0.448 , 0.578]	0.112 [0.088 , 0.136]
	0.14	10	0.529 [0.467 , 0.592]	0.123 [0.108 , 0.139]
	0.33	10	0.426 [0.362 , 0.489]	0.109 [0.094 , 0.125]
	0.69	10	0.416 [0.349 , 0.484]	0.105 [0.089 , 0.121]
	1.4	9	0.439 [0.395 , 0.484]	0.119 [0.099 , 0.139]
	2.5	6	0.394 [0.330 , 0.459]	0.117 [0.101 , 0.134]
TCDF	0.074	5	0.460 [0.407 , 0.513]	0.106 [0.074 , 0.137]
	0.15	10	0.505 [0.435 , 0.576]	0.110 [0.095 , 0.126]
	0.25	10	0.508 [0.382 , 0.635]	0.124 [0.111 , 0.137]
	0.52	9	0.432 [0.366 , 0.498]	0.122 [0.097 , 0.148]
	1.1	6	0.448 [0.359 , 0.537]	0.112 [0.093 , 0.132]
	1.8	4	0.509 [0.372 , 0.647]	0.126 [0.081 , 0.172]
	4.0	1	0.488	0.126

Note. d, day; n/a, not available

[a] 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and vehicle control (triolein)

[b] Sample size

[c] Data expressed as mean % relative organ mass compared to body mass [95% confidence interval]. Means significantly different than the appropriate control value are designated with 'A' (relative organ mass), 'B' (actual organ mass) and 'C' (arcsine transformed relative organ mass)

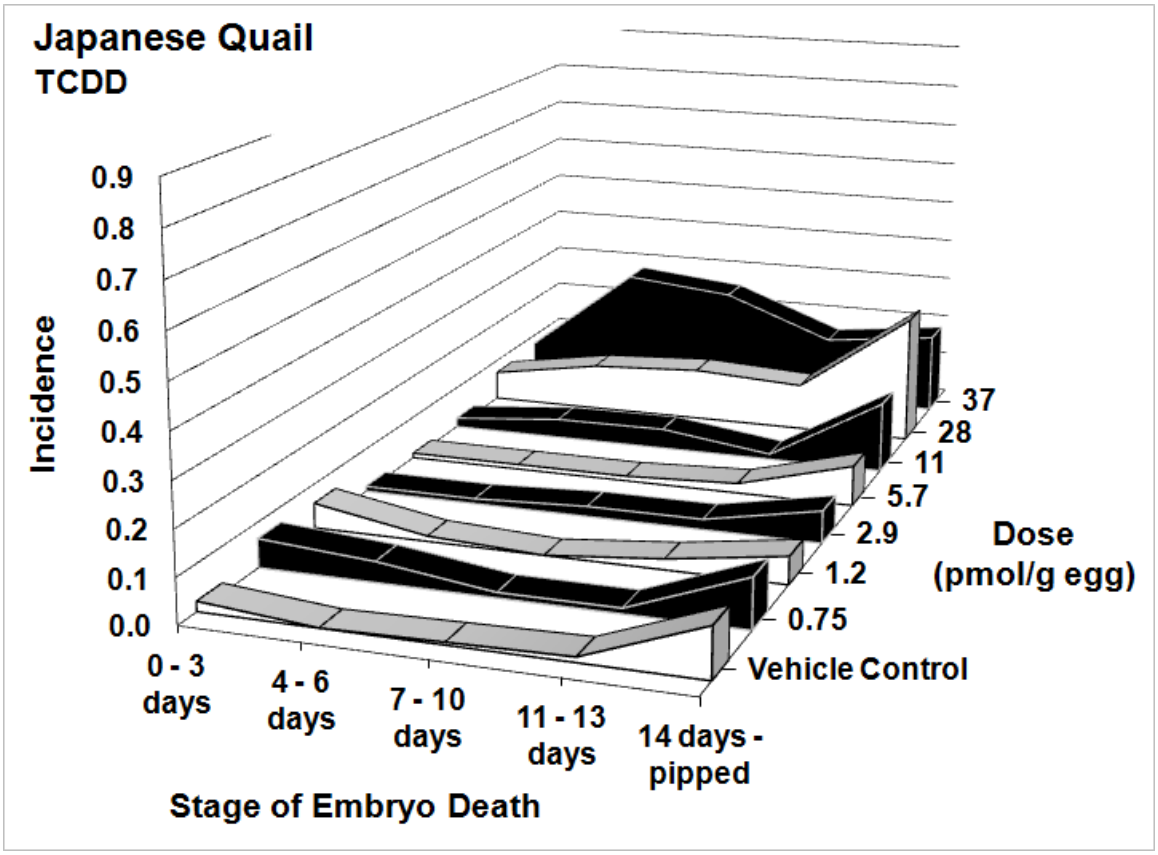


Figure 16. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on stage of Japanese quail embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

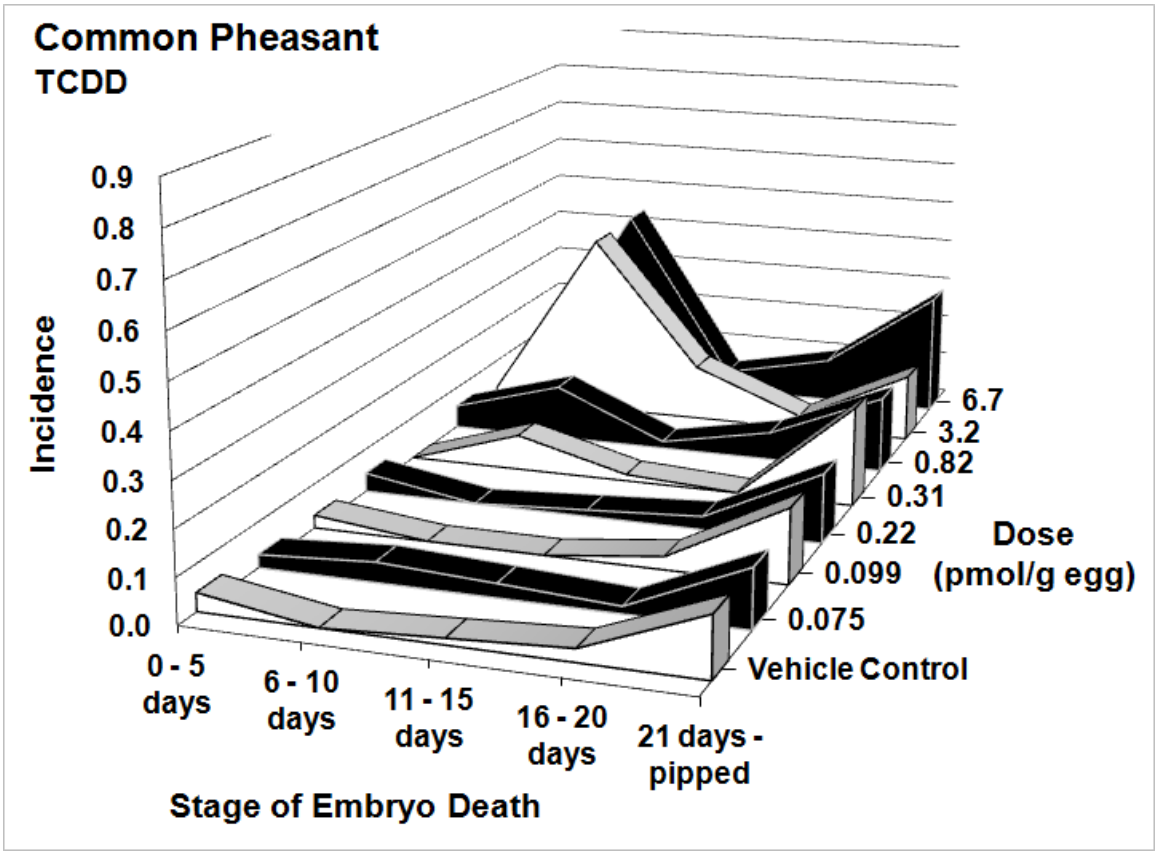


Figure 17. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on stage of Common pheasant embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

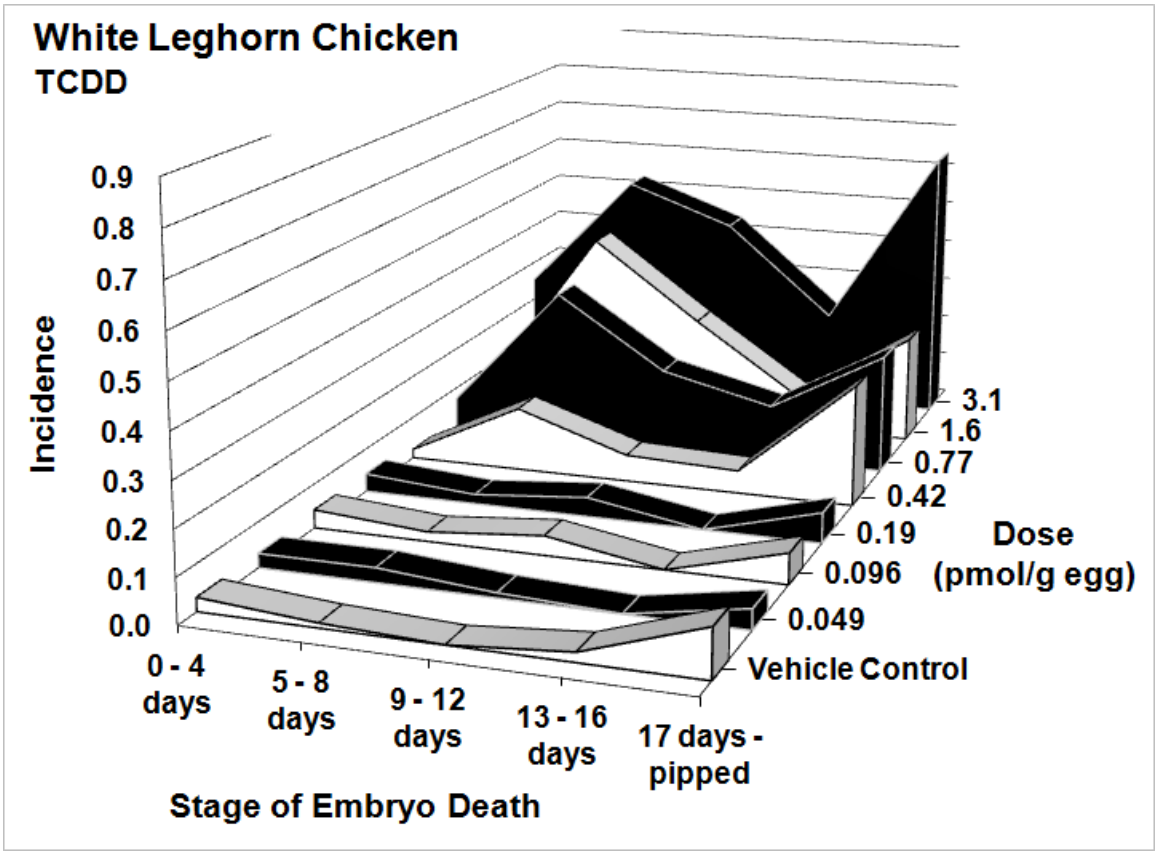


Figure 18. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on stage of White Leghorn chicken embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

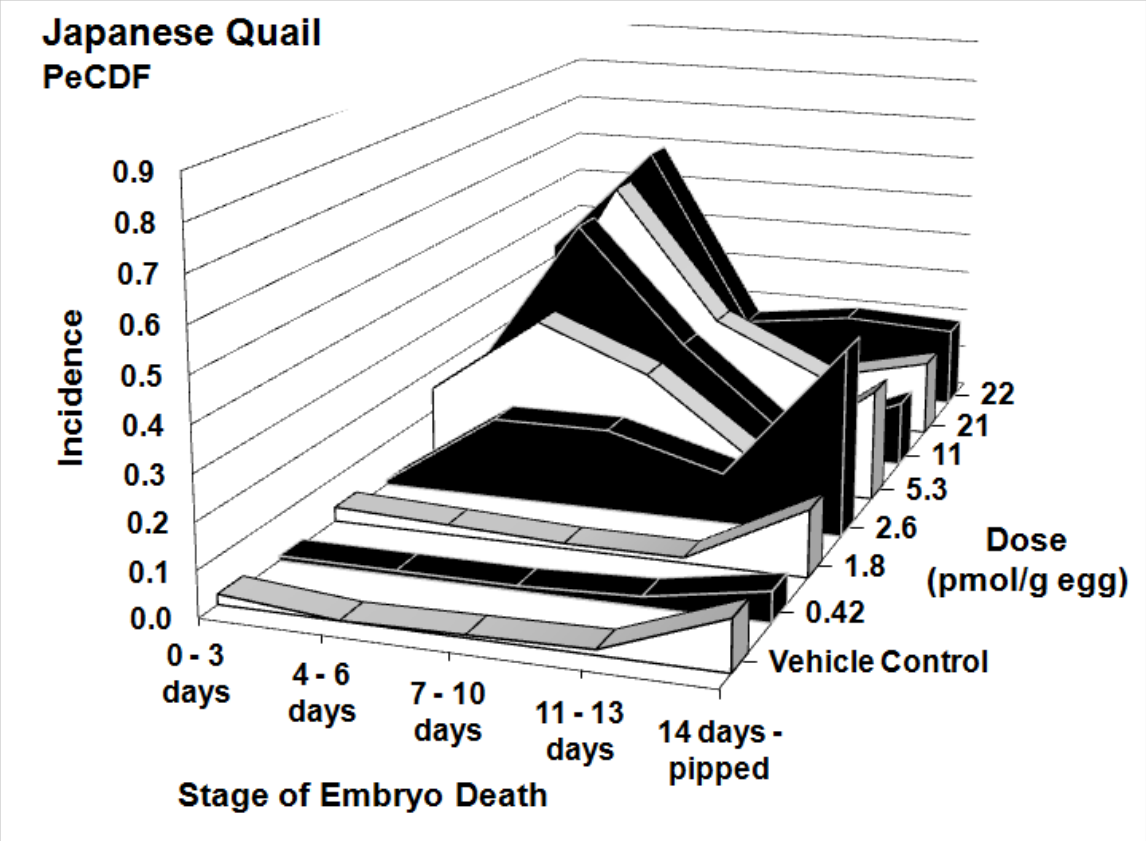


Figure 19. Effect of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) on stage of Japanese quail embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

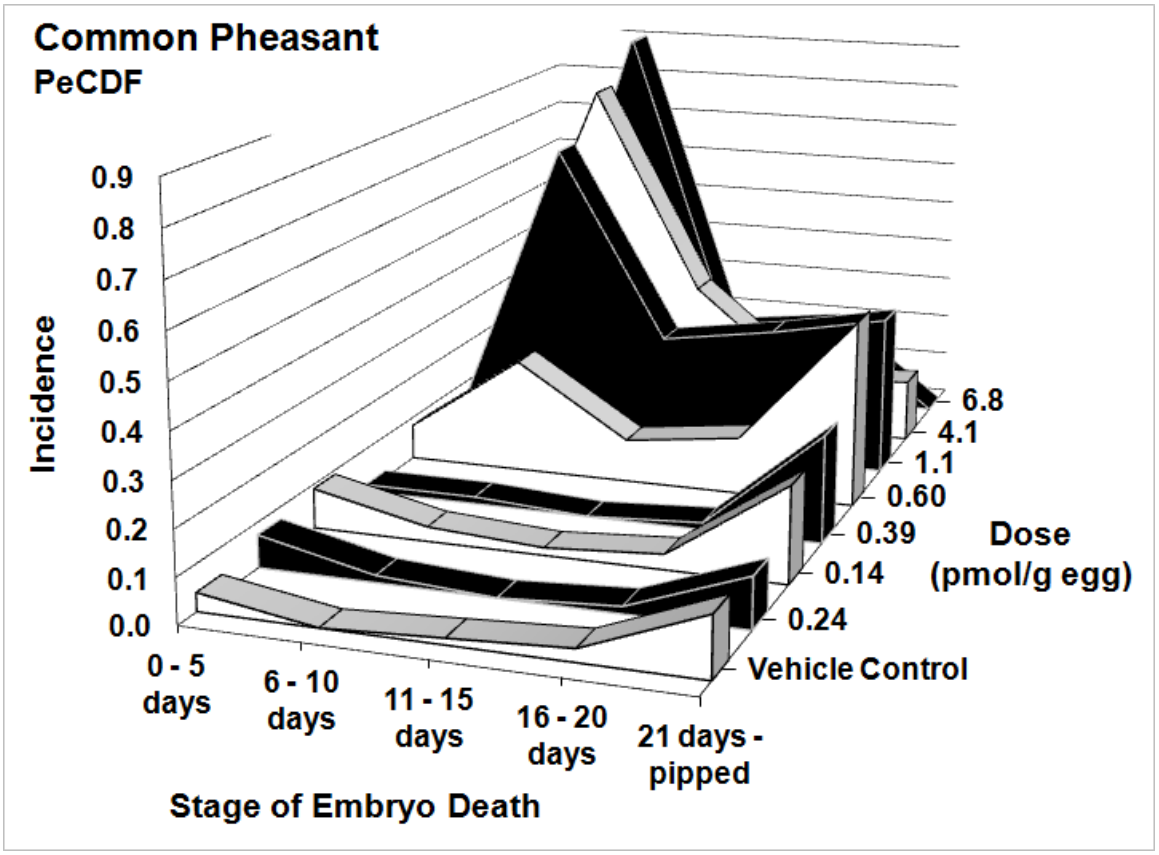


Figure 20. Effect of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) on stage of Common pheasant embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

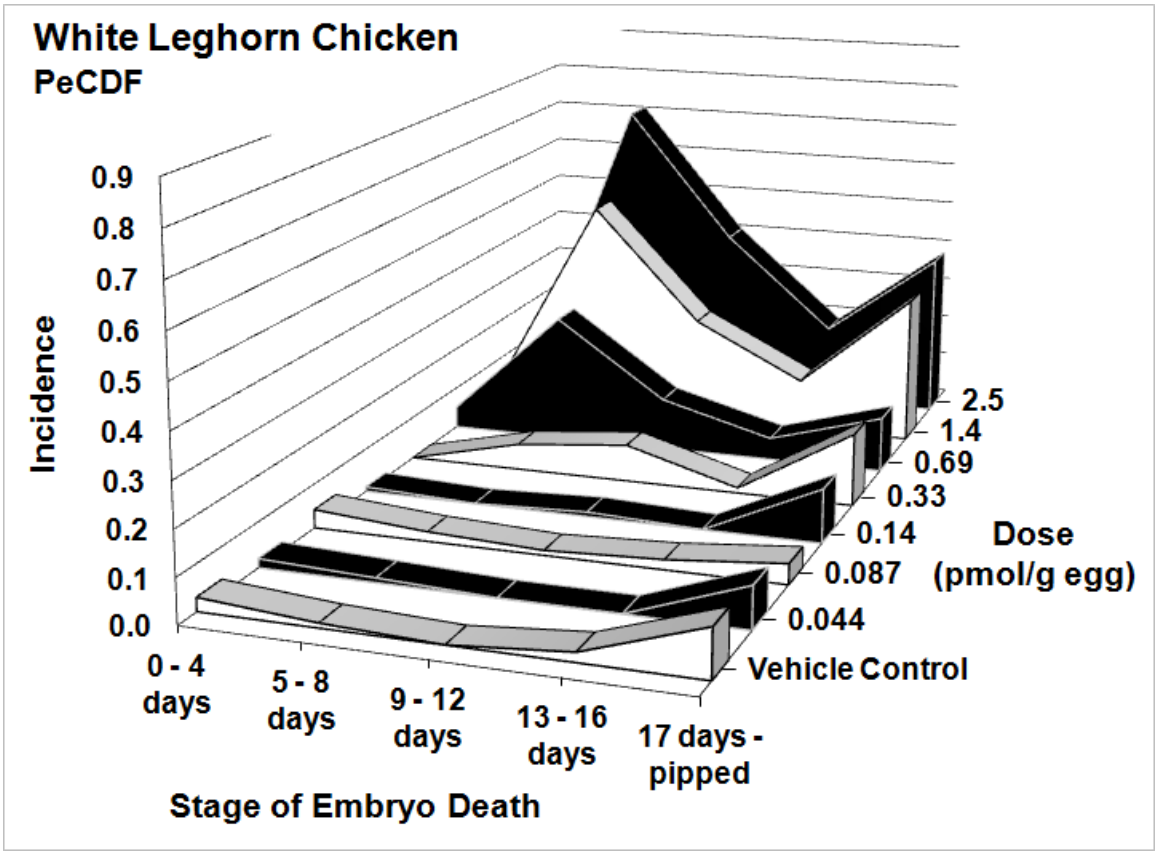


Figure 21. Effect of 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) on stage of White Leghorn chicken embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

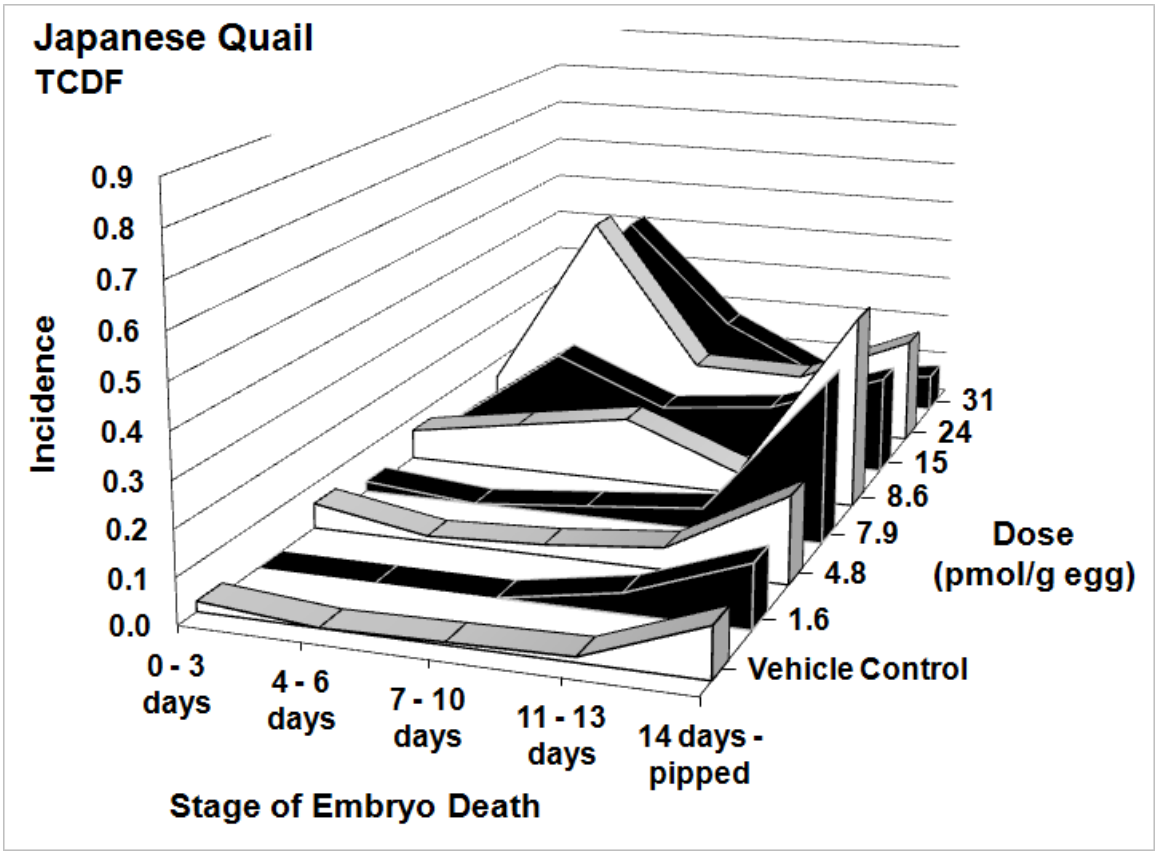


Figure 22. Effect of 2,3,7,8-tetrachlorodibenzofuran (TCDF) on stage of Common pheasant embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

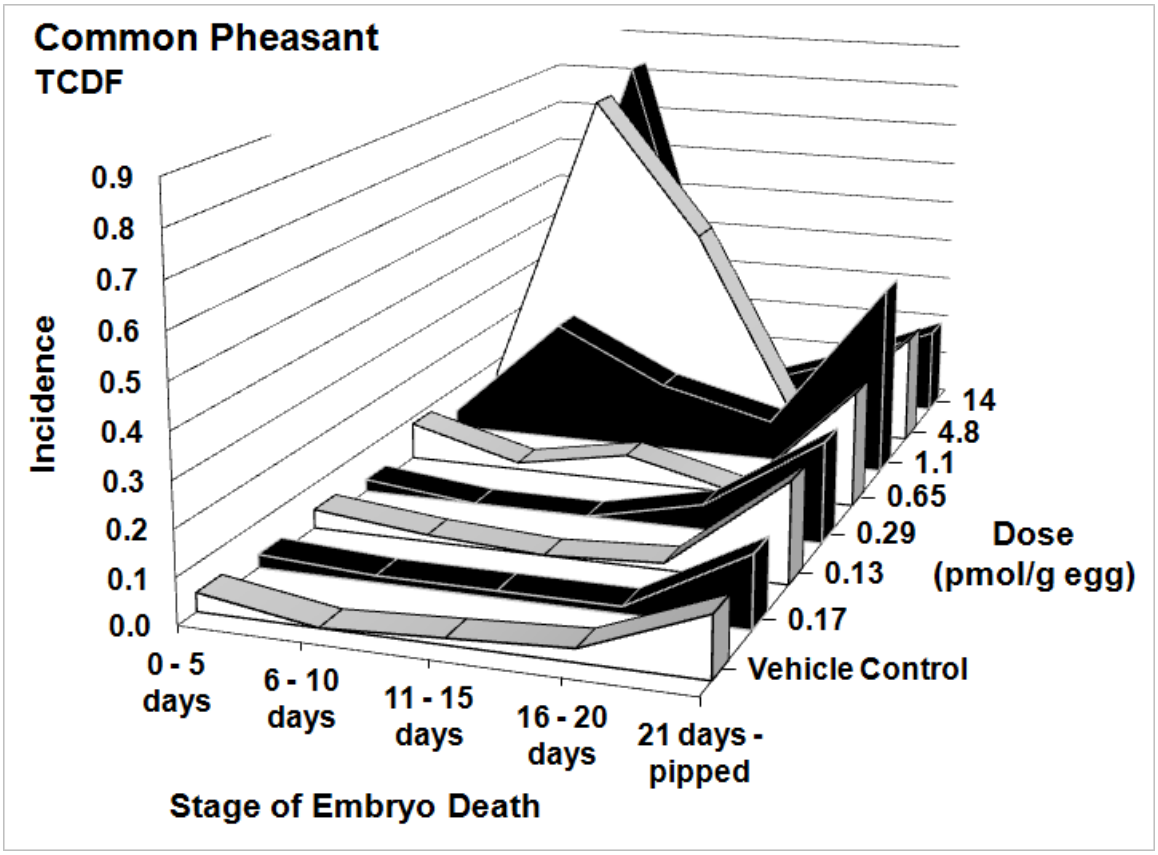


Figure 23. Effect of 2,3,7,8-tetrachlorodibenzofuran (TCDF) on stage of Common pheasant embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

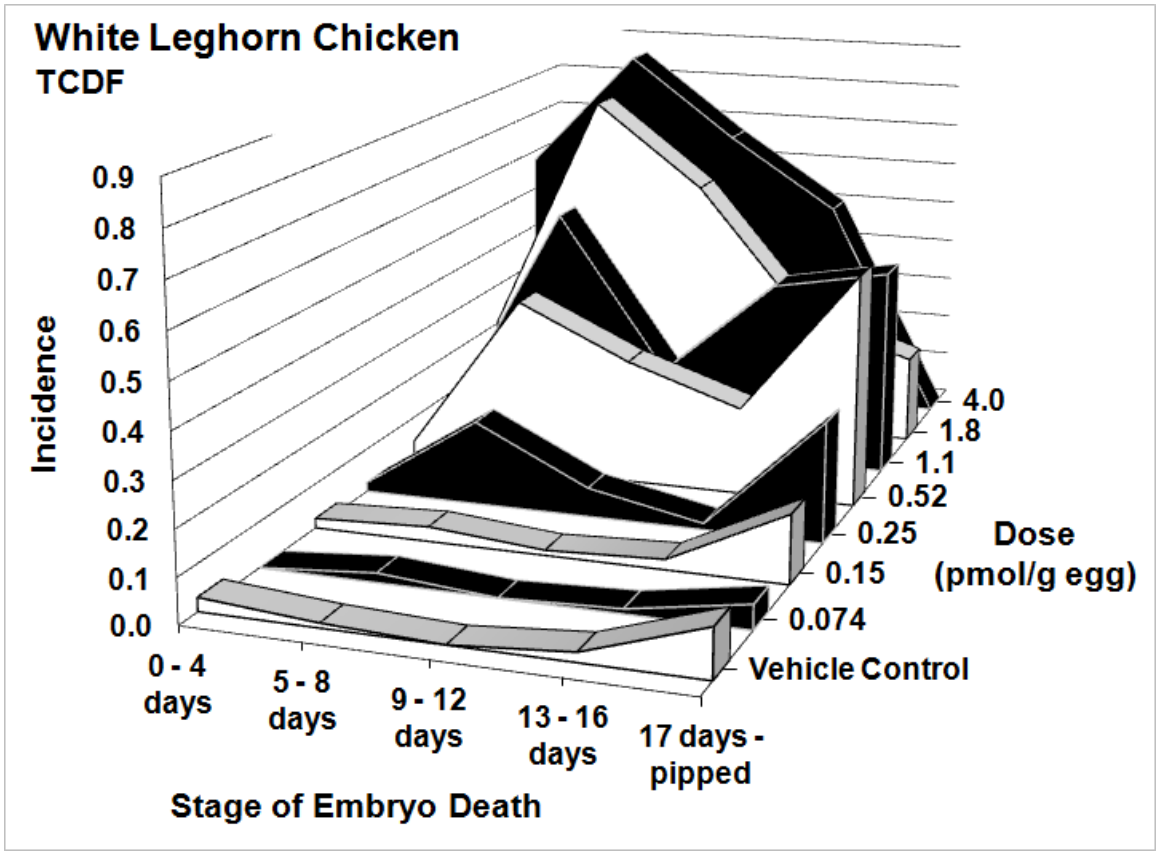


Figure 24. Effect of 2,3,7,8-tetrachlorodibenzofuran (TCDF) on stage of White Leghorn chicken embryo mortality. Five stages were identified based on appearance of key developmental endpoints. Triolein was used as the vehicle control. Incidence = the number of observed mortalities in a stage / (total number of fertile eggs – embryo mortalities from previous stages).

DISCUSSION

Stage of Embryonic Mortality

Galliform birds experience embryonic mortality both in wild and commercial populations. While the reason for such mortality may not be readily apparent, it can be influenced by a number of factors including inherited weakness, nutritional deficiencies in the breeding hens, and egg damage caused by motion during transport. Additionally, late-stage embryo mortality can be due to malpositioning of embryos prior to hatch or to developmental deformities interfering with normal pipping behavior (Romanoff, 1949, 1972; Fant, 1957). The pattern of embryonic mortality observed in eggs injected with the vehicle for all three species is characteristic of that described under normal incubation practices (Fant, 1957), therefore comparisons against such would seem valid.

Several studies have shown embryo mortality in avian species to be perhaps the most predominant result of exposure to TCDD or TCDD-like compounds (reviewed in Gilbertson *et al*., 1991); however, few have looked at the particular stage of development during which mortality occurs. Dose-dependent increases in embryo mortality during the 4 - 6 day stage in quail, 6 - 10 day stage in pheasants, and 5 - 9 day stage in chickens suggest exposure to TCDD, PeCDF or TCDF results in an embryo mortality pattern unique to compounds of this type (Figures 15-23). These results indicate the toxicity of these compounds was apparent only after the avian embryo had competed organogenesis, developing most organ primordia, particularly of the liver (Kingsbury *et al*, 1956; Fukuda and Mizuno, 1978). For all three species, several late stage embryos failed to hatch upon pipping during the 2-day post hatch examination. Although few treatment groups had a significant increase in the incidence of mortality during this stage (those which included

pipping), the effect of dose was significant in all but quail and pheasant TCDD and chicken PeCDF treatment groups (data not shown). Similar findings were observed in wild Herring gull and Foster's tern populations contaminated with TCDD-like compounds (Gilbertson and Fox, 1977; Hoffman *et al*., 1987); however, mortality paralleled occurrences of edema, the incidence of which was insignificant in the present study.

TCDD-, PeCDF-, and TCDF-Induced Teratogenesis

Predominant deformities included microphthalmos and anophthalmos (deformed or absent eyes), anencephaly or exencephaly (absence or partial exposure of the brain), abnormal limbs, and partial or incomplete development of the bill. Less common deformities included acephalia (absence of the head), gastromelus (having supernumerary legs attached to the abdomen), dwarfism, gastroschisis (exposed abdominal cavity), cyclopia (fusion of both eyes medially into one) and subcutaneous edema. Deformities observed in the embryos of all three species exposed to PeCDF and TCDF in this study were similar to those reported in other studies assessing the effects of TCDD and other TCDD-like compounds using chickens (Vos, 1973; Powell *et al*., 1996ab; Blankenship *et al*., 2003), pheasants (Nosek *et al*., 1992) or other wild avian species (reviewed in Gilbertson *et al*., 1991; Giesy *et al*., 1994).

Of all chicken embryos surviving past embryonic-day 8, 0.14% had subcutaneous edema of any type; isolated to the wing, neck or eyelids of two 1-d hatchlings in the 0.69 pmol PeCDF/g egg and 0.15 pmol TCDF/g egg dose groups. In addition, several early-stage chicken embryos (before embryonic day 5) had excessive amounts of fluid in the

amnion, a condition not described in similar exposure studies. Only one 14-d quail (0.05% of the embryos surviving past embryonic-day 6) was noted to have abdominal edema. As edema was absent in the TCDD-exposed chickens and quail and in all of the pheasant treatment groups, it may not be the most accurate predictor of TCDD-like exposure in these species when compared to embryo mortality.

Overall, the percentage of deformed embryos was greatest in the quail, followed by the pheasant, and then chicken, for all three compounds (Tables 9-17). And rather than TCDD, PeCDF was the most potent teratogen in all species tested. Limb- and bill-type deformities made up the majority of deformities in both the quail and pheasant, whereas, trunk-type deformities were more prevalent in the chicken. The relatively high rate of early-stage embryo mortality in three species at higher doses may have prevented the occurrence of teratogenic effects that were grossly apparent.

Compared to the results of this study, others have shown differences in the occurrence of TCDD-induced teratogenic effects among species tested. Nosek *et al.* (1993) reported 1- and 28-d pheasant chicks exposed *in ovo* to doses as high as 3.1 pmol/g egg failed to exhibit signs of "chick edema disease". In contrast, Blankenship *et al*. (2003) reported TCDD to be highly teratogenic in chickens, where 25.6% of all embryos and hatchlings exposed to 0.47 pmol/g egg *in ovo* had some type of developmental abnormality.

Effects of TCDD, PeCDF and TCDF on Chick Mass

There were no consistent dose-related changes in 1-d body mass of all three species exposed to all three compounds or 14-d body masses of quail or pheasants

exposed to any of the three compounds (Tables 18, 19 and 20). Only in chickens exposed to concentrations of TCDD greater than 0.77 pmol/g egg did a decrease in 14-d body mass occur (Table 20). This effect mimics wasting syndrome, a condition described as a characteristic delayed onset of loss of body mass over time, and has been observed in chickens (Flick *et al.*, 1965; Greig *et al.*, 1973) and pheasants (Nosek *et al*., 1992) administered single or multiple doses of TCDD or TCDD-like compounds. Other studies have shown decreases in embryonic body mass occuring in chickens exposed to TCDD at doses greater than 0.31 pmol/g egg (Henshel *et al*., 1997); while, in contrast, doses as great as 2.0 pmol TCDD/g egg caused no significant effects on mass of hatchlings (Powell *et al*., 1996b).

Effects of TCDD, PeCDF and TCDF on the Liver, Heart, Brain, Bursa and Spleen

Several studies have described either no effect or inconsistent changes in organ mass associated with exposure to TCDD or TCDD-like compounds. In the liver, while no changes in relative mass were detected in 1- or 14-d chicks of all three species, there were dose-related histological lesions associated with exposure to all three compounds in the quail similar to those described in herring gulls. Related to TCDD exposure, these included; lipid accumulation, swollen hepatocytes, compressed sinusoids, and occasional necrotic hepatocytes characterized by pycnotic nuclei (Gilbertson and Fox, 1977; Gilbertson *et al*., 1991). In chicken hatchlings, Powell *et al*. (1997) found an increase in relative heart mass in association with exposure to TCDD-like compounds as well as a decrease in relative bursa mass in chicks exposed to 0.25 pmol TCDD/g egg *in ovo*. Decreases in bursa mass have also been described by Nikolaidis *et al*. (1990) following

exposure to compounds similar to TCDD. The relatively high incidence of embryo mortality at equivalent doses in the present study accounting for fewer hatchlings in greater dose groups (for sample sizes see Tables 21-29) could explain the lack of consistent alterations in organ mass and absence of histopathology.

SUMMARY AND CONCLUSIONS

Embryo mortality categorized by stage of development indicated similar patterns of early- and late-stage embryo lethality induced by dose-related TCDD, PeCDF and TCDF exposure in each species. These included increases in embryo mortality during the 4 - 6 day stage in quail, 6 - 10 day stage in pheasants, and 5 - 9 day stage in chickens suggesting the toxic effects of these compounds greatly increase after organogenesis occurs. Body and organ masses of 1- and 14-day hatchlings were unaffected at doses up to 37 pmol TCDD/g egg, 22 pmol PeCDF/g egg and 31 pmol TCDF/g egg for the quail, and 6.7 pmol TCDD/g egg, 6.8 pmol PeCDF/g egg and 14 pmol TCDF/g egg for the pheasant. In the chicken, body mass of 1-d hatchlings were not significantly different than those of the vehicle control group for all three compounds at doses up to 3.1 pmol TCDD/g egg, 2.5 pmol PeCDF/g egg and 4.0 pmol TCDF/g egg. While, in 14-d chickens exposed to concentrations of TCDD greater than 0.77 pmol/g egg, a significant decrease in body mass occurred. There were no significant changes in organ mass associated with dose in the chicken. The percentage of deformed embryos surviving past embryonic day 6 (quail), 10 (pheasant) or 8 (pheasant) for all three compounds was greatest in the quail, followed by the pheasant, then the chicken. PeCDF, rather than TCDD, was the most

teratogenic compound among those tested. No effects were detected in the heart, brain, bursa and spleen tissues of all three species, while histological lesions of the liver resulting from compound exposure occurred in the quail only; at doses greater than 11 pmol TCDD/g egg, 11.2 pmol PeCDF/g egg and 4.8 pmol TCDF/g egg.

LITERATURE CITED

Literature Cited

Blankenship, A.L., Hilscherova, K., Nie, M., Coady, K.K., Villalobos, S.A., Kannan, K., Powell, D.C., Bursian, S.J., Giesy, J.P. (2003). Mechanisms of TCDD-induced abnormalities and embryo lethality in White Leghorn chickens. *Comp. Biochem. Physiol.* **136**(C):47-62.

Bosveld, A.T.C., Van den Berg, M., Theelen, R.M.C. (1992). Assessment of the EROD inducing potency of eleven 2,3,7,8- substituted PCDD/Fs and three coplanar PCBs in the chick embryo. *Chemosphere* **25**: 911-916.

Bronström, B., and Anderson, L. (1988). Toxicity and 7-ethoxyresorufin-*O*-deethylase-inducing potency of coplanar polychlorinated biphenyls (PCBs) in chick embryos. *Arch. Toxicol.* **62**:263-266.

Cheung, M.O., Gilbert, E.F., Peterson, R.E. (1981). Cardiovascular teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the chick embryo. *Tox. Appl. Pharm*. **61**:197-204.

Cohen-Barnhouse, A.M. Bursian, S.J., Link, J.E., Fitzgerald, S.D., Kennedy, S.W., Hervé , J., Giesy, J.P., Wiseman, S.,Yang, Y., Jones, P.D., Wan, Y., Collins, B., Newsted, J.L., Kay, D,. Zwiernik, M.J. (2010). Sensitivity of Japanese Quail (*Coturnix japonica*), Common Pheasant (*Phasianus colchicus*) and White Leghorn Chicken (*Gallus gallus domesticus*) Embryos to *In Ovo* Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-Pentachlorodibenzofuran (PeCDF) and 2,3,7,8-Tetrachlorodibenzofuran (TCDF). *Toxicol. Sci.* (*in press)*

Elliott, J.E., Kennedy, S.W., Peakall, D.B., Won, H. (1990). Polychlorinated biphenyl (PCB) effects on hepatic mixed function oxidases and porphyria in birds. I. Japanese quail. *Comp. Biochem. Physiol.* **96C**(1):205-210.

Fant, R.J. (1957) Criteria for aging pheasant embryos. *J. Wildlife Manage.* **21**:324-328.

Flick, D.F., O'Dell, R.G., and Childs, V.A. (1965). Studies of the chick-edema disease. 3. Similarity of symptoms produced by feeding chlorinated biphenyl. *Poult. Sci.* **44**:1460-1465.

Fox, G.A., Collins, B., Hayakawa, E., Weseloh, D.V., Ludwig, J.P., Kubiak, T.J., Erdman, T.C. (1991). Reproductive outcomes in colonial fish-eating birds: a biomarker for developmental toxins in Great Lakes food chains. II. Spatial variation in the occurrrencs and prevalence of bill defects in young double-crested cormorants in the Great Lakes, 1979-1987. *J. Great Lakes Res.* **17**:158-167.

Fox, G.A., Gilman, A.P., Peakall, D.B., Anderka, F.W. (1978). Behavioral abnormalities of nestling Lake Ontario herring gulls. *J. Wildlife Manage.* **42**:477-483.

Fox, G.A., Kennedy, S.W., Norstrom, R.J., Wigfield, D.C. (1988). Porphyria in herring gulls: a biochemical response to chemical contamination of Great Lakes food chains. *Enviorn. Toxicol. Chem*. **7**:831-839.

Fredricks, T.B., Zwiernik, M.J., Seston, R.M., Coefield, S.J., Plsutz, S.C., Tazelaar, D.L., Shotwell, M.S., Bradley, P.W., Kay, D.P., and Giesy, J.P. (2010). Passerine exposure to primarily PCDFs and PCDDs in the river floodplains near Midland, Michigan, USA. *Arch. Environ. Contam. Toxicol.* **58**:1048-1064.

Fukuda , S. and Mizuno, T. (1978) Hepatic parenchyma, biliary ducts and gall bladder forming potency in the hepatic primordium in the Quail Embryo. *Anat. Embryol*. **155**:15-21.

Giesy, J. P., Ludwig, J. P., and Tillitt, D. E. (1994). Deformities in birds of the Great Lakes region. *Environ. Sci. Technol.* **28**:128A-135A.

Gilbertson, M., and Fox, G.A. (1977). Pollutant-associated embryonic mortality of Great lakes herring gulls. *Environ. Pollut*. **12**:211-216.

Gilbertson, M., Kubiak, T., Ludwig, J., Fox, G. (1991). Great Lakes embryo mortality, edema, and deformities syndrome (GLEMEDS) in colonial fish-eating birds: similarity to chick-edema disease. *J. Toxicol. Env. Health.* **33**:455–520.

Greig, J.B., Jones, G., Butler, W.H., Barnes, J.M. (1973). Toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Food. Cosmet. Tox*. **11**:585-595.

Hahn, M.E. (1998). The aryl hydrocarbon receptor: a comparative perspective. *Comp. Biochem. Physiol.* **121**(Part C):23-53.

Head, J.A., Hahn, M.E., and Kennedy, S.W. (2008) Key amino acids in the aryl hydrocarbon receptor predict dioxin sensitivity in avian species. *Environ. Sci. Technol.* **42**:7535-7541.

Henshel, D., Hehn, B.; Wagey, R., V.M.; Steeves, J.D. (1997). The relative sensitivity of chicken embryos to yolk- or air-cell-injected 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Environ. Toxicol. Chem*. **16**:725-732.

Hervé, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., Kennedy, S.W. (2009). Cytochrome P450A induction by 2,3,4,8-Tetrachlorodibenzo-*p*-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. *Toxicol. Sci*. **113**:380-391.

Hoffman, D.J., Melancon, M.J., Klein, P.N., Eisemann, J.D., Spann, J.W. (1998). Comparative developmental toxicity of planar polychlorinated biphenyl congeners in chickens, American kestrels, and common terns. *Environ. Toxicol. Chem.* **17**:747-757.

Hoffman, D.J., Rattner, G.A., Sileo, L., Docherty, D., Kubiak, T.J. (1987). Embryo toxicity, teratogenicity and aryl hydrocarbon hydroxylase activity in Forster's terns on Green Bay, Lake Michigan. *Environ. Res*. **42**:176-184.

Kay, D.P., Blankenship, A.L., Coady, K.K., Neigh, A.M., Zwiernik, M.J., Millsap, S.D., Strause, K., Park, C., Bradley, P., Newsted, J.L., Jones, P.D., Giesy, J.P. (2005) Differential accumulation of polychlorinated biphenyl congeners in the aquatic food web at the Kalamazoo River superfund side, Michigan. *Environ. Sci. Technol.* **39**(16):5964-5974.

Karchner, S.I., Franks, D.G., Kennedy, S.W., Hahn, M.E. (2006). The molecular basis for differential dioxin sensitivity in birds: Role of the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci*.**103**:6252-6257.

Kennedy, S.W., Lorenzen, A., Jones, S.P., Hahn, M.E., Stegeman, J.J. (1996). Cytochrome P4501A induction in avian hepatocyte cultures: a promising approach for predicting the sensitivity of avian species to toxic effects of halogenated aromatic hydrocarbons. *Toxicol. AppI. Pharm.* **141**:214-230.

Kingsbury, J.W., Alexanderson, M., Kornstein, E.S. (1956). The development of the liver in the chick. *Anat. Rec.* **124**:165–187.

Larson, J.M., Karasov, W.H., Stromborg, K.L., Hanbidge, B.A., Giesy, J.P., Jones, P.D., Tillitt, D.E., Verbrugge, D.A. (1995). Reproductive success, developmental abnormalities, and environmental contaminants in double-crested cormorants (*Phalacrocorax autitus*). *Environ. Toxicol. Chem.* **15**(4):553-559.

Ludwig, J.P., KuritaMatsuba, H., Ludwig, M.E., Summer, C.L., Giesy, J.P., Tillitt, D.E., Jones, P.D. (1996). Deformities, PCBs, and TCDD-Equivalents in double-crested cormorants (*Phalacrocorax auritus*) and Caspian terns (*Hydroprogne caspia*) of the upper Great Lakes 1986-1991: Testing a cause-effect hypothesis. *J. Great Lakes Res*. **22**(2):172-197.

Nikolaidis, E., Brunström. B., Dencker, L, Veromaa, T. (1990). TCDD inhibits the support of B-cell development by the bursa of Fabricius. *Pharm. Toxicol.* **67**:22-26.

Nosek, J.A., Craven, S.R., Sullivan, J.R., Hurley, S.S., Peterson, R.E. (1992). Toxicity and reproductive effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Ring-necked pheasant hens. *J. Toxicol. Envrion. Health.* **35**(3):187-198.

Nosek, J.A., Sullivan, J.R., Craven, S.R., Gendronfitzpatrick, A., and Peterson, R.E. (1993). Embryotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in the Ring-necked pheasant. *Environ. Toxicol. Chem.* **12**:1215-1222.

Poland, A. and Glover, E. (1977). Chlorinated biphenyl induction of aryl hydrocarbon hydroxylase activity: a study of the structure-activity relationship. *Mol. Pharmoacol.* **13:**924-938.

Powell, D.C., Aulerich, R.J., Meadows, J.C., Tillitt, D.E., Giesy, J.P., Stromborg, K.L., and Bursian, S.J. (1996b). Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB126) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) injected into yolks of chicken (*Gallus domesticus*) eggs prior to incubation. *Arch. Environ. Contam. Toxicol.* **31**:404-409.

Powell, D.C., Aulerich, R.J., Meadows, J.C., Tillitt, D.E., Powell, J.F., Restum, J.C., Stromborg, K.L., Giesy, J.P., and Bursian, S.J. (1997). Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB126), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), or an extract derived from field-collected cormorant eggs injected into double-crested cormorant (*Phalacrocorax auritus*) eggs. *Environ. Toxcol. Chem.* **16**:1450-1455.

Powell, D.C., Aulerich, R.J., Stromborg, K.L., Bursian, S.J. (1996a). Effects of 3,3',4,4'-tetrachlorobiphenyl, 2,3,3',4,4',-pentachlorobiphenyl and 3,3',4,4',5-pentachlorobiphenyl on the developing chicken embryo when injected prior to incubation. *J. Toxicol. Environ. Health.* **49**:319-338.

Romanoff, A.L. (1949). Periods and Causes of Death in Avain Embryonic Development. *The Auk*. **66**(3): 264-270.

Romanoff, A.L. (1972). Pathogenesis of the Avian Embryo: An Analysis of Causes of Malformation and Prenatal Death. New York; John Wiley & Sons, Inc. 5-35 p.

Sanderson, J.T., Kennedy, S.W., Giesy, J.P. (1998). In vitro induction of ethoxyresorufin-*O*-deethylase and porphyrins by halogenated aromatic hydrocarbons in avian primary hepatocytes. *Environ. Toxicol. Chem*. **17**:2006–2018.

Telliard, W.A. (1994). Method 1613: Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS. U.S. Environmental Protection Agency Office of Water.

Van den Berg, M., Birnbaum, L.S., Bosveld, A.T.C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W. *et al*. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Persp*. **106**:775–792.

Vos, J.G. (1978). 2,3,7,8-tetrachlorodibenzo-*para*-dioxin: effects and mechanisms. *Ecol. Bull.* (Stockholm) **27**:165-176.

Yi Wan, P. D. Jones, R.R. Holem, J. S. Khim, H.Chang, D. P. Kay, S. A. Roark, J. L. Newsted, W. P. Patterson, J. P. Giesy. (2010). Bioaccumulation of polychlorinated dibenzo-p-dioxins, dibenzofurans, and dioxin-like polychlorinated biphenyls in fishes from the Tittabawassee and Saginaw Rivers, Michigan, USA. *Sci. Total Environ.* **408**:2394–2401.

Yang, Y., Wiseman, S., Cohen-Barnhouse, A.M., Wan, Y., Jones, P., Newsted, J.L., Kay, D.P., Kennedy, S.W., Zwiernick, M.J., Bursian, S.J., Giesy, J.P. (2010) Effects of *in ovo* exposure of White-1 leghorn Chicken, Common pheasant and Japanese quail to TCDD, 2,3,4,7,8-PeCDF and 2,3,7,8-TCDF on CYP1A induction. *Environ. Toxicol. Chem.* **29**:1490-1502.

Zwiernik, M.J., Kay, D.P., Moore, J., Beckett, K.J., Khim, J.S., Newsted, J.L., Roark, S.A., Giesy, J.P. (2008). Exposure and effects assessment of resident mink (*Mustela vison*) exposed to polychlorinated dibenzofurans and other dioxin-like compounds in the Tittabawassee River basin, Midland, Michigan, USA. *Environ. Toxicol. Chem*. **27**(10): 2076-2087.

CHAPTER 3

Summary and Conclusions

The current methodology to assess the risk associated with exposure to TCDD or TCDD-like compounds in vertebrate species assumes toxic effects are mediated through their interaction with the aryl hydrocarbon receptor (AhR) (Van den Berg *et al*., 1998). This risk assessment approach utilizes toxic equivalency factors (TEFs) or relative potency (ReP) values, depending on the availability of data, to estimate the toxicity of TCDD-like compounds. A TEF is a consensus value based on multiple endpoints from different species belonging to a class of animals, such as birds or mammals. Unlike TEFs, RePs are based on species-specific endpoints and are simply the ratio of potency of a TCDD-like compound relative to a reference compound, normally TCDD, which is often assumed to be the most potent or toxic of compounds in its class. While a TEF expresses the relative toxicity of a TCDD-like compound, it is meant to be 'protective' in a risk assessment rather than being 'predictive'. Nevertheless, the rank order of TEFs and RePs are generally similar (Blankenship *et al*., 2008).

Present World Health Organization TEF values for PeCDF and TCDF are based on relatively limited research that does not encompass many toxic-responses at an *in vivo* level. Based on limited *in vitro* data, the toxic potencies of these two TCDD-like compounds are assumed to be equivalent to that of TCDD (TEF = 1.0). However, in terms of embryotoxicity, the studies herein have indicated otherwise. Embryo mortality derived LD50 values show PeCDF to be the most potent compound (6-fold more potent compared to TCDD) followed by TCDF (2- to 3-fold more potent compared to TCDD) in both the Japanese quail and Common pheasant. TCDF is only 2-fold more potent than TCDD in the White Leghorn chicken based on ReP values presented in Table 5. Similar *in vitro* results have been reported based on EROD induction and *CYP1A4*/*5* mRNA

expression in primary hepatocyte cultures from the quail and pheasant. For example, PeCDF was found to be 13-fold more potent than TCDD in inducing EROD activity in quail (Hervé *et al*., 2010) and 15-fold more potent than TCDD in its induction of *CYP1A4* in the pheasant (Yang *et al*., 2010). In the chicken, EROD or mRNA based toxic endpoints failed to distinguish differences between the three compounds; in contrast to this study, where TCDF was demonstrated to be more potent.

Aside from clear differences in species-specific potencies among TCDD, PeCDF and TCDF, variations in compound-specific sensitivity were shown to occur between the quail, pheasant and chicken. In agreement with the avian sensitivity classification scheme based on amino acid substitutions in the LBD of the AhR (Karchner *et al*. 2006; Head *et al*. 2008), the results of this study demonstrate the chicken to be the most sensitive species to *in ovo* TCDD and TCDF exposure, followed by the pheasant and then quail (Table 4; Figures 6 and 8). This conclusion is somewhat equivocal for PeCDF, in that the 95% confidence intervals for chicken and pheasant LD50 estimates overlap. However, clearly both species are more sensitive than the quail (Table 4; Figure 7).

Comparisons between *in ovo* and post-hatch endpoints reported in this study suggest differences between compound-induced effects are better reflected by *in ovo* measurements (hatchability or embryo mortality). Similarities between the stages of embryo lethality and types of deformities among species provide evidence for similar modes of toxicity for these compounds. However, post-hatch endpoints such as organ mass, histopathology, and concentrations of each compound within the liver are only representative of those embryos in which mortality does not occur *in ovo*. Thus,

measurements of these post-hatch endpoints could underestimate actual effects caused by these compounds on such organs or chemical bioaccumulation.

Overall, the results of this study indicate the potency of TCDD-like compounds in birds varies with species and that TCDD is not necessarily the more potent compound among TCDD-like compounds. This variation in potency among TCDD-like compounds within species highlights the uncertainty associated with the use of TEFs in risk assessment. This study also provides further support for the avian sensitivity classification model based on the AhR. For that reason, the categorization of a greater number of avian species based on their AhR LBD amino acid sequences could serve as a useful tool in risk assessment in relation to exposure to TCDD or TCDD-like compounds. As these and similar pollutants continue to pose a threat to wild avian species, the importance of accurately quantifying their harmful effects remains a necessity in ecological risk assessment.

LITERATURE CITED

Literature Cited

Blankenship, A.L., Hilscherova, K., Nie, M., Coady, K.K., Villalobos, S.A., Kannan, K., Powell, D.C., Bursian, S.J., Giesy, J.P. (2003). Mechanisms of TCDD-induced abnormalities and embryo lethality in White Leghorn chickens. *Comp. Biochem. Physiol.* **136**(C):47-62.

Hervé, J.C., Crump, D., Jones, S.P., Mundy, L.J., Giesy, J.P., Zwiernik, M.J., Bursian, S.J., Jones, P.D., Wiseman, S.B., Wan, Y., Kennedy, S.W. (2009). Cytochrome P450A induction by 2,3,4,8-Tetrachlorodibenzo-*p*-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species. *Toxicol. Sci*. **113**:380-391.

Karchner, S.I., Franks, D.G., Kennedy, S.W., Hahn, M.E. (2006). The molecular basis for differential dioxin sensitivity in birds: Role of the aryl hydrocarbon receptor. *Proc. Natl. Acad. Sci*.**103**:6252-6257.

Van den Berg, M., Birnbaum, L.S., Bosveld, A.T.C., Brunstrom, B., Cook, P., Feeley, M., Giesy, J.P., Hanberg, A., Hasegawa, R., Kennedy, S.W. *et al*. (1998). Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Persp*. **106**:775–792.

Yang, Y., Wiseman, S., Cohen-Barnhouse, A.M., Wan, Y., Jones, P., Newsted, J.L., Kay, D.P., Kennedy, S.W., Zwiernick, M.J., Bursian, S.J., Giesy, J.P. (2010) Effects of *in ovo* exposure of White-1 leghorn Chicken, Common pheasant and Japanese quail to TCDD, 2,3,4,7,8-PeCDF and 2,3,7,8-TCDF on CYP1A induction. *Environ. Toxicol. Chem.* **29**:1490-1502.

APPENDIX

A-1. FUNDING

This research was supported by an unrestricted grant from the Dow Chemical Company to Michigan State University. Portions of the research were supported by Environment Canada and by a Discovery Grant from the National Science and Engineering Research Council of Canada (Project # 326415-07) and a grant from the Western Economic Diversification Canada (Project # 6578 and 6807). The authors wish to acknowledge the support of an instrumentation grant from the Canada Foundation for Infrastructure. Professor Giesy was supported by the Canada Research Chair program and an at-large Chair Professorship at the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong.