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EFFECTS OF CHLORINATED DIOXINS AND FURANS ON AVIAN SPECIES: INSIGHTS FROM *IN OVO* STUDIES

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Saskatoon, SK, Canada

By

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Keywords: dioxin, furan, avian, aryl hydrocarbon receptor, CYP1A

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PREFACE

The Chapter 2 of this thesis has been organized as a manuscript that will be submitted for publication in scientific journals. Some repetition of introductory and methodological material is unavoidable.

ABSTRACT

Many physiological responses to dioxin-like compounds (DLCs), including polychlorinated dibenzodioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are mediated by the aryl-hydrocarbon receptor (AhR). In birds, activation of the AhR stimulates the transcription of cytochrome P4501A (CYP1A) genes, including CYP1A4 and CYP1A5, and ultimately leads to expression of biotransformation enzymes, including ethoxyresorufin-*O*-deethylase (EROD). It is well established that potencies of different DLCs range over several orders of magnitude. There is also a wide variation among birds in their responsiveness to DLCs both in efficacy and threshold for effects. A molecular basis for this differential sensitivity has been suggested. Specifically, a comparison of the AhR ligand-binding domain (LBD) indicated that key amino acid residues are predictive of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) sensitivity. Based on sequencing of the AhR LBD from numerous avian species a sensitive classification scheme has been proposed (in order of decreasing sensitivity, chicken (type I; sensitive) > Common pheasant (type II; moderately sensitive) > Japanese quail (type III; insensitive)). A series of egg injection studies with White-leghorn chicken (*Gallus gallus domesticus*), Common pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) were performed to determine whether molecular and biochemical markers of exposure to DLCs are predictive of the proposed classification scheme. In addition, I was interested in determining whether this classification scheme applies to other DLCs, specifically dibenzofurans. Determining which species are "chicken- like", "pheasant-like" and "quail-like" in their responses to DLCs should allow more refined risk assessments to be conducted as there would

be less uncertainty about the potential effects of DLCs in those species for which population-level studies do not exist.

Several concentrations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), or 2,3,7,8-tetrachlorodibenzofuran (TCDF) (triolein vehicle) were injected into the air cells of Japanese quail, Common pheasant and chicken eggs. Liver from 14 d post-hatch chicks was harvested for analysis of CYP1A4 and CYP1A5 mRNA abundance by quantitative real-time PCR (Q-PCR), and EROD activity. Lowest observed effective concentration (LOEC) and relative potency (ReP) values for CYP1A mRNA abundance and EROD activity were determined and used to make comparisons of sensitivity between each species and DLC potency within each species.

The TCDD is widely considered to be the most potent DLC and this is supported by the rank order of LOEC values for CYP1A5 mRNA abundance in White-leghorn chicken (TCDD > PeCDF > TCDF). CYP1A4 mRNA abundance and EROD activity in White-leghorn chicken were significantly increased in the lowest dose exposure groups of each of the three DLCs, so the potency of these compounds based on these endpoints was not established. Interestingly, TCDD was not the most potent DLC in Common pheasant and Japanese quail. In Common pheasant, PeCDF is the most potent as a CYP1A4 mRNA inducer, followed by TCDD and TCDF. However, TCDF was the most potent EROD activity inducer for Common pheasant, followed by PeCDF, and then TCDD. No significant increases were found in CYP1A5 mRNA abundance in pheasant within the tested dose ranges for all the three DLCs. No significant increases in either CYP1A5 mRNA abundance or EROD activity were found in Japanese quail. In addition, PeCDF and TCDF, but not TCDD, significantly increased CYP1A4 mRNA abundance.

According to the predicted relative sensitivity by comparing the AhR LBD amino acid sequences, the White-leghorn chicken is more responsive to DLCs than the Common pheasant which is more responsive than the Japanese quail. By comparing the relative sensitivity calculated based on the LOEC values from my study, the sensitivity order to TCDD and TCDF support the proposed molecular based species sensitivity classification scheme (chicken > pheasant > quail), while pheasant is almost as sensitive as chicken to PeCDF (pheasant ≥ chicken > quail).

Taken together, the data suggest that TCDD is the most potent DLC in White-leghorn chicken, but not in Common pheasant, or or Japanese quail. The data suggest that in type II avian species PeCDF may be more potent than TCDD. In addition, I found in my study that different biomarkers have different responses, which depends on species and chemicals as well. These data provide further insight into avian sensitivities to DLCs.

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LIST OF ABBREVIATIONS

AhR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH-PAS	basic-helix-loop-helix Per-ARNT-Sim
cDNA	complementary DNA
Ct	threshold cycle
CYP1A	cytochrome P4501A
CYP1A4	cytochrome P4501A4
CYP1A5	cytochrome P4501A5
DLC	dioxin-like compound
DR	dioxin receptor
DRE	dioxin response element
EC50	median effective concentration
EROD	7-ethoxyresorufin-*O*-deethylase activity
HAH	halogenated aromatic hydrocarbons
hrs	hours
LBD	ligand binding domain
LD50	lethal dose for 50% of the population
LOEC	lowest observable effect concentration
min	minutes
mRNA	messenger RNA

MROD	7-methoxyresorufin O-demethylation (MROD)
NOEL	No observable effect level
NRC	National Research Council Canada
PAH	polyaromatic hydrocarbon
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PeCDF	2,3,4,7,8-pentachlorodibenzofuran
PCB	polychlorinated biphenyls
Q-PCR	quantitative Real-time polymerase chain reaction
QSAR	quantitative structure-activity relationship
ReP	relative potency
ReS	relative sensitivity
RT-PCR	reverse transcriptase-polymerase chain reaction
sec	seconds
t1/2	half-life
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzo-furan
TEF	toxic equivalency factor
TEQ	TCDD equivalent
UROX	uroporphyrinogen oxidation
WHO	World Health Organization
XE	xenobiotic receptor
XRE	xenobiotic response element

CHAPTER 1

GENERAL INTRODUCTION

1.1 Dioxin-like Compounds (DLCs)

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and some polychlorinated biphenyls (PCBs) belong to a class of persistent organic pollutants known as “dioxin-like” compounds (DLCs). The most widely studied of this general class of compounds is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Although TCDD is sometimes simply called "dioxin", the term "dioxin" is also commonly used to refer to the complex mixtures of TCDD and related compounds emitted from sources, or found in the environment, or in biological samples. Only seven out of the 75 possible PCDD congeners and ten out of the 135 possible PCDF congeners have chlorine substitutions in the 2,3,7,8 positions, and only these congeners have dioxin-like toxicity (USEPA, 1994a; 1994b).

The physical and chemical properties of DLCs vary according to the degree and position of chlorine substitution. They are semi-volatile and chemically stable, particularly the tetra- and higher chlorinated congeners, and are extremely stable under most environmental conditions (Tysklind et al., 1993; Webster and Commoner, 2003). These environmental contaminants have a high degree of biological activity and are resistant towards metabolism, which contribute to their extreme biological effects. Due to their lipophilic properties, DLCs bioaccumulate in the adipose tissue of humans, birds

and other wildlife resulting in elevated concentrations at higher tropic levels (Atkinson, 1991).

The presence of DLCs was first reported in incinerator fly ash samples in 1977 and 1978 (Buser et al., 1978; Olie et al., 1992), and then came to public attention in 1982 following an explosion at ICMESA factory in Seveso, Italy (Wilson, 1982). Neither PCDDs nor PCDFs have been intentionally produced other than on a laboratory scale for use in scientific analysis. Generally, they are generated during high temperature incineration of municipal waste, and as unintended by-products in industrial and biological processes (Dyke et al., 1997; Webster and Commoner, 2003).

Numerous adverse toxic effects associated with exposure to TCDD and related compounds have been reported in avian species, both in laboratory and field studies. These effects include endocrine disruption, reduced egg production and hatching success, developmental abnormalities, hepatotoxicity, immunotoxicity, and mortality (Birnbaum and Tuomisto, 2000; Gilbertson et al., 1991; Pohjanvirta and Tuomisto, 1994; Peterson et al., 1993; Kennedy et al., 1996; Giesy et al., 1994).

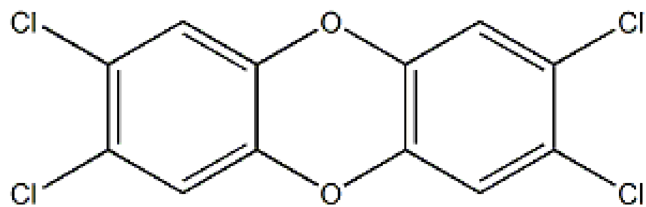
1.1.1 2,3,7,8-Tetrachlorodibenzo-*p*-dixoin (2,3,7,8-TCDD or TCDD)

The PCDDs, including TCDD, are unintentionally produced by such process as paper and pulp bleaching, incineration of municipal, toxic, and hospital wastes, in smelters, and during production of chlorophenoxy herbicides, for instance, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), which was widely used in the agricultural industry to defoliate broad-leafed plants in the 1960s and 1970s (Silkworth and Brown, 1996; Schecter, 1994; IARC, 1997; Schecter et al., 1997). TCDD has molecular weight of 322

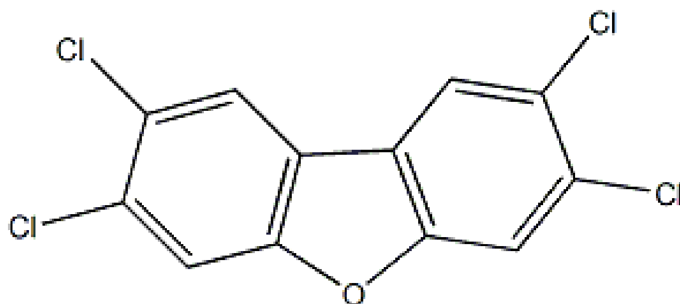
g.mol^{-1} and is insoluble in water. It is very persistent in the environment, but can be slowly degraded by sunlight (ATSDR, 1998; HSDB, 2003). TCDD is well known as a human carcinogen. There is sufficient evidence of the carcinogenicity of TCDD from human studies and from a combination of epidemiological and mechanistic information that indicates a causal relationship between exposure to TCDD and human cancer (NTP, 2005).

1.1.2 Polychlorinated dibenzofurans (PCDFs)

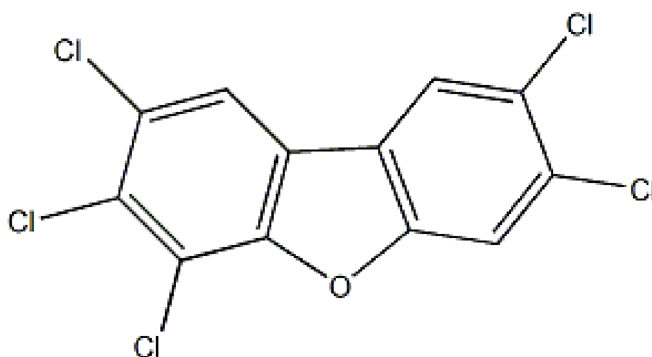
2,3,7,8-tetrachlorodibenzofuran (TCDF; Figure 1-1) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF; Figure 1-1) are members of the chlorinated dibenzofurans (PCDFs). PCDFs are structural analogs and usual co-contaminants of PCDDs. They are also highly persistent and widespread environmental contaminants that are inadvertently produced by industry. The greatest unintentional production of 2,3,7,8-TCDF is as by-products of processes such as PVC production, industrial bleaching, and incineration. The adverse health effects related to TCDF exposure include birth or developmental effects, cancer, and effects on the immune system. The clinical signs of toxicity of PeCDF are especially persistent. The long duration of toxicity was believed to be related to the very long biological half-life and minimal excretion from the body (Brewster et al., 1988; Kashimoto et al., 1981; Masuda and Yoshimura, 1984; Rappe et al., 1979).



(A) 2,3,7,8-Tetrachlorodibenzo-*p*-dixoin
(TCDD)



(B) 2,3,7,8-tetrachlorodibenzofuran (TCDF)



(C) 2,3,4,7,8-pentachlorodibenzofuran (PeCDF)

Figure 1-1. Chemical structures of (A) TCDD, (B) TCDF, and (C) PeCDF.

1.2 Physiological Responses to Dioxin-Like Compounds

1.2.1 The Aryl Hydrocarbon Receptor (AhR)

Many of the physiological responses to DLCs are mediated through an interaction with the aryl hydrocarbon receptor (also as known as AhR, dioxin receptors (DR), or xenobiotic receptor (XR)) (Masuda et al., 1998). The AhR is a member of the basic-helix-loop-helix (bHLH) superfamily of transcription factors (Burbach et al., 1992; Fukunaga et al., 1995). The AhR is a cytosolic transcription factor that is normally inactive, bound to several co-chaperones, including two molecules of heat shock protein 90 (Hsp90). Upon ligand binding the chaperones dissociate resulting in nuclear translocation and formation of a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Ema et al., 1992; Hahn, 2002; Hahn et al., 2006). The AhR: ARNT heterocomplex interacts with dioxin response elements (DREs; also termed xenobiotic response elements; XREs) in the upstream regulatory regions of target genes thereby altering gene transcription (Figure 1-2). It has been postulated that toxicity most likely occurs through some of the many alterations in gene transcription effected by ligand-activated AhR (Okey et al. 2005). The AhR protein domain structure is as shown in Figure 1-3. The N-terminal half of the protein contains the well-conserved bHLH and Per-ARNT-Sim (PAS) domains involved in ligand binding, ARNT dimerization, and DNA binding; while the C-terminal half of the AhR contains a Q-rich domain and other regions involved in transcriptional activation.

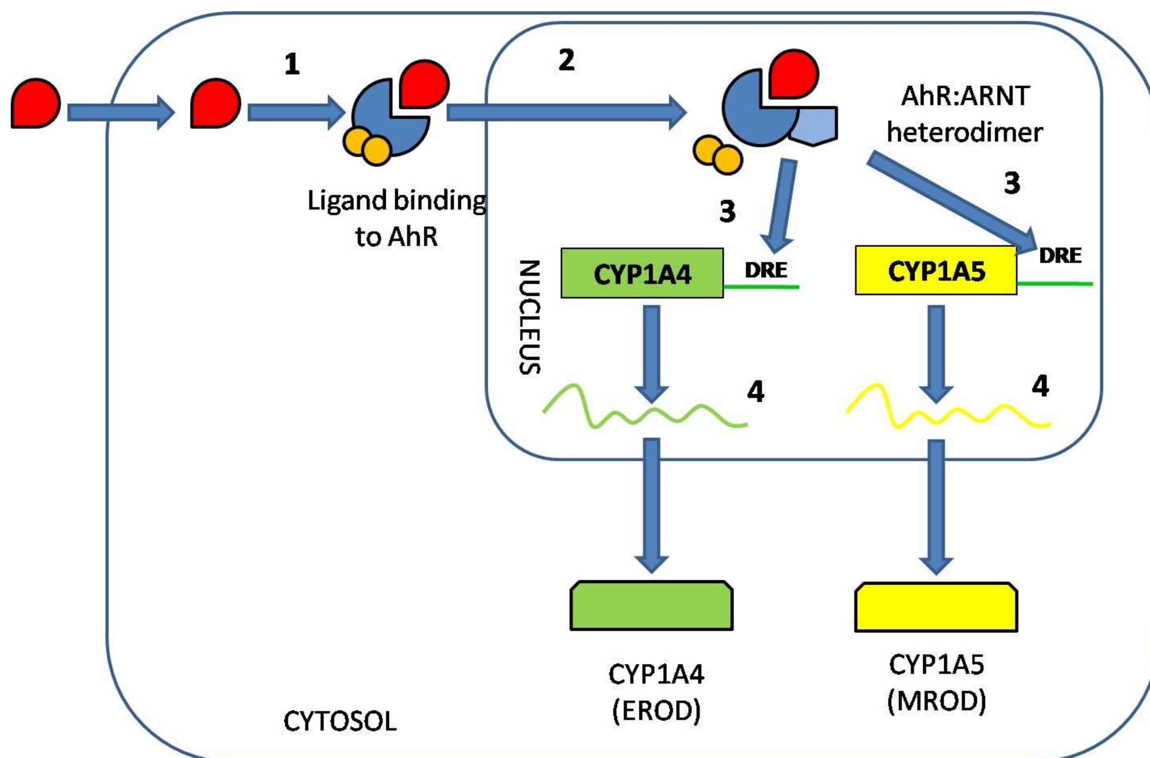


Figure 1-2. Schematic illustration of the AhR signalling pathway. (1) Dioxins (i.e. TCDD) bind to the AhR complex. (2) AhR-associated proteins dissociate from the AhR and then the Dioxin:AhR complex translocates into the nucleus. Dioxin:AhR complex dimerizes with AhR nuclear translocator protein (ARNT). (3) The heterodimers are capable of recognizing and binding DNA at the consensus sequence, GCGTG, of dioxin responsive elements (DREs). (4) This action either increases or decreases the transcription of a number of potential target genes in the Ah gene battery, including cytochrome P4501A4 (CYP1A4) and cytochrome P4501A5 (CYP1A5).

*The round shaped circles colored in yellow indicate the associate proteins.

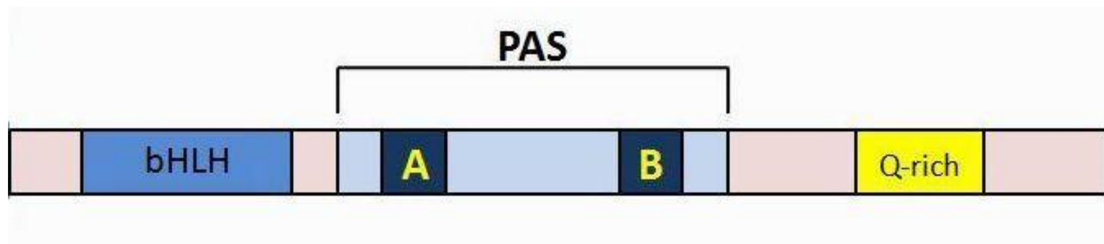


Figure 1-3. Domain structure of Aryl hydrocarbon receptor (AhR) protein. The N-terminal half of the AhR contains the well-conserved bHLH and PAS domains involved in ligand binding, ARNT dimerization, and DNA binding. The C-terminal half of the AhR contains a Q-rich domain and other regions involved in transcriptional activation.

Recent studies have demonstrated that there are two AhR forms in avian species, referred to as AhR1 and AhR2 (Yasui et al., 2004; Hahn et al., 2006). The AhR2 has poor binding affinity to TCDD, and as such it is assumed that AhR2 does not play a role in physiological responses to DLCs (Yasui et al., 2004). There are multiple lines of evidence that suggest that the AhR plays a central role in the dioxin toxicity responses. Polymorphisms in several regions of the AhR, including the ligand binding domain and the transactivation domain, have been associated with altered sensitivity to DLCs in rodents (Poland et al., 1994; Pohjanvirta et al., 1998). AhR-null mice generated by three independent laboratories were found to be highly resistant to toxic and biochemical effects to TCDD (Fernandez-Salguero et al., 1995; Mimura et al., 1997; Schmidt et al., 1996). More recently, mutation of the AhR nuclear localization sequence was shown to induce resistance to the toxic effects of TCDD in mice (Bunger et al., 2003).

1.2.2 The Cytochrome P4501A (CYP1A) Response

As illustrated in Figure 1-2, upon entry into a cell, DLCs bind to and activate the AhR, ultimately modulating transcription of DRE containing target genes. The most intensely studied and best-understood consequence of AhR activation is the upregulation of phase I xenobiotic metabolizing enzymes, most notably the CYP1A proteins. The Cytochrome P4501A (CYP1A) is a subfamily of proteins that belongs to the cytochrome P450 enzyme superfamily of proteins. They are membrane bound monooxygenases capable of transforming lipophilic compounds into more water soluble derivatives (Murray and Reidy, 1990). Members of the CYP family act on a wide range of substrates,

for instance, CYP1, CYP2 and CYP3 are responsible for most of the metabolism of xenobiotic compounds (Lewis, 2000).

Two CYP1A isoforms, CYP1A4 and CYP1A5, are constitutively expressed in liver tissue from avian species (Gilday et al., 1996; Rifkind et al., 1994), and both are inducible by TCDD (Mahajan and Rifkind, 1999). Chicken CYP1A4 and CYP1A5 are similar to mammalian CYP1A1 in sequence, but they are different from the mammalian isoforms in some important metabolic ways, and were therefore given distinct classifications (Gilday et al. 1998). In addition, previous studies indicate that the avian CYP1A5 is orthologous to mammalian CYP1A2 (Goldstone and Stegeman, 2006; Kubota et al., 2006; 2008). The CYP1A4 enzyme exhibits specificity for ethoxyresorufin-*O*-deethylase (EROD), while the CYP1A5 isoform preferentially catalyzes arachidonic acid metabolism and uroporphyrinogen oxidation (UROX) (Rifkind et al., 1994; Sinclair et al., 1997). Dose-response effects of TCDD on each of these CYP1A-related endpoints have been reported in the literature (Gilday et al., 1998; Sanderson et al., 1998), and they are now being widely used as biomarkers for DLCs in environmental samples (Head and Kennedy, 2007a; Jin et al., 2000; Kennedy et al., 1996; Zhang et al., 2009).

1.2.3 Potential *In Ovo* Avian Exposure

Most DLCs are globally distributed (Ballschmiter et al., 2002), consequently, the eggs of species inhabiting contaminated areas are at risk of being exposed. There is evidence that levels of some DLCs remain sufficiently high in some areas of North America to elicit biochemical and/or embryotoxic effects in wild birds (Elliott et al., 1996;

Fox et al., 1988; Giesy et al., 1994; Sanderson and Bellward, 1995). Possible routes of egg exposure to DLCs include transfer of chemical residues from contaminated feathers of breeding parents, or direct exposure of eggs and young birds in the nests to contaminated water or via precipitation (Hoffman, 2001). It is highly probable that large numbers of waterfowl and upland game bird eggs are directly or indirectly exposed to DLCs.

1.3 Variation in Dioxin Sensitivity

There are great variations of sensitivities to DLCs among species. One of the most often mentioned is that guinea pigs (*Cavia porcellus*) are approximately 1000-times more sensitive to TCDD than hamsters (Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). Dioxin sensitivity is also extremely variable among avian species. For instance, herring gulls (*Larus argentatus*), common terns (*Sterna hirundo*), American kestrels (*Falco sparverius*), wood ducks (*Aix sponsa*), and Eastern bluebirds (*Sialia sialis*) are 25 - 330 times less sensitive to the embryotoxic effects of DLCs than chicken (*Gallus gallus*) (Head, 2006). Based on calculated LD50 values the pheasant embryo is approximately 10 times less sensitive than the chicken embryo to the lethal effects of TCDD exposure by egg injection into egg albumin and egg yolk (Nosek et al., 1993). Similar degrees of variation among species are reported for the EROD and CYP1A mRNA inducing potency of TCDD in avian embryo hepatocytes (Head et al., 2007; Kennedy et al., 1996; 2003b; Sanderson et al., 1998). Pheasant embryo hepatocytes are approximately 5- to 10-times less sensitive to EROD induction by TCDD than White-leghorn chicken hepatocytes in cell culture studies (Kennedy et al., 1996), while Japanese

quail hepatocytes are reported to be 10-times less sensitive to EROD induction by TCDD than chicken (Brunström and Halldin, 1998).

A comparison of the amino acid sequence of the AhR Ligand Binding Domain (LBD) for those species with toxicity data indicates that several key amino acid residues are predictive of TCDD sensitivity within the order. The sequences of the AhR LBD in over 70 avian species have been determined, and where comparisons can be made to toxicity data in the literature (Table 1-1), species may be classified as Type 1 (Chicken-like, very sensitive), Type 2 (Wild Turkey-like, moderately sensitive) or Type 3 (Japanese quail-like, insensitive) (Head, 2006).

Table 1-1. Dioxin sensitivity classification for avian species *in ovo* studies (Head, 2006).

Order	Common Name	LD50* (µg/kg TEQ)	EC50** (nM TCDD)	Sensitivity LD50	Sensitivity EC50	Sensitivity LBD***
Galliformes	Chicken	0.15 - 0.3 [a]	0.004 - 0.006 [b, c, d, e]	1	1	1
	Ring-necked pheasant	1.3-2.2 [f]	0.14 [b]	2	2	2
	Japanese Quail	> 24 [g]	--	3	--	3

* Lethal dose for 50% of the population (LD50) expressed in terms of ug/kg TCDD equivalents (TEQs). Studies cited involved injection of TCDD or other dioxin-like congeners into the yolk sac, albumin or air cell on day 0 or 4 of incubation. Values reported for dioxin-like congeners other than TCDD were converted to TEQs using avian toxic equivalency factors (TEFs) of 0.05 and 0.1 respectively as suggested by Van den Berg et al. (1998). A no observable effect level (NOEL), or the range from the NOEL to 100% mortality, is reported where an LD50 value was not available. In all cases the NOEL refers to mortality.
** The effective median concentration (EC50) refers to the dose of TCDD causing a half-maximal EROD response in cultured avian hepatocytes.
*** Sensitivity classifications based on LD50 and EC50 values as follows:

Sensitivity Classification	LD50 (µg/kg TEQ)	EC50 (nM TCDD)
1	< 1	< 0.05
2	1 - 10	0.05 - 0.5
3	> 10	> 0.5

****Sensitivity classifications based on AhR LBD amino acid sequences (Head et al., 2008).
a. reviewed in Powell et al., 1996.
b. Kennedy et al., 1996.
c. Bosveld, 1995.
d. Sanderson et al., 1998.
e. Lorenzen et al., 1997b.
f. Nosek et al., 1993. The range for Common pheasant is obtained from the LD50 values from the study by injection of TCDD into the yolk and albumin.
g. Brunström 1988; Brunström and Halldin 1998. No observable effect levels (NOEL) are reported for herring gull and Japanese quail.

1.4 Variations in Biomarker Responses to Dioxin-like Compound Exposure in Avian Species

Given the global distribution of DLCs and the potential impact(s) of exposure on avian species, one of the most important goals of avian toxicology is the identification of species at risk. While determination of exposure may be accomplished by measuring tissue levels of these compounds, the mere presence of these compounds does not imply that these compounds are biologically active. Consequently, biomarkers of exposure are routinely employed to determine whether species are exposed to DLCs.

A biomarker is a biological response to a chemical or chemicals that can be detected and measured as an indicator of exposure or effect. Biomarkers are commonly used in risk assessment and monitoring of wildlife in contaminated environments (Peakall and Walker, 1994). A biomarker can refer to the overt toxic outcomes such as mortality and reproductive dysfunction, or to biochemical effects that can be predictive of these outcomes. There are a large number of biomarkers that can be and have already been used in risk assessments of DLCs exposure in avian species, including induction of CYP1A mRNA expression, alterations in mixed function oxygenase activity (EROD, MROD, etc.), heme biosynthesis, porphyrin biosynthesis, vitamin A homeostasis, immune and endocrine related endpoints, and DNA integrity (Fox 1993; Head, 2006; Head et al., 2007; Lorenzen et al., 1997a).

1.4.1 Ethoxyresorufin-*O*-deethylase (EROD) activity

One of most commonly used biomarkers of dioxin-like compound exposure in avian species is the induction of EROD activity (Figure 1-2). Induction of EROD activity by either environmental exposure or laboratory exposure to DLCs has been

observed positively in many avian species, including black-crowned night herons (*Nycticorax nycticorax*), common terns, bald eagles (*Haliaeetus leucocephalus*), ospreys (*Pandion haliaetus*), and European starlings (*Sturnus vulgaris*) (Arenal et al., 2004; Bosveld, 1995; Elliott et al., 1996, 2001; Kennedy et al., 1996, 2003b; Lorenzen et al., 1997b; Rattner et al., 2000). Although elevated EROD activity is not considered as an overtly toxic response itself, it is used as an indicator of AhR activation, which can be indicative of overt toxicity.

The induction of EROD activity has also been used as a biomarker of sensitivity to DLCs. For instance, pheasants are approximately 10 times less sensitive than chicken to EROD induction by TCDD in cultured hepatocytes, and are also approximately 10 times less sensitive to the lethal effects of TCDD via egg injection (Kennedy et al., 1996; Nosek et al., 1993). It has also been reported in wood duck (*Aix sponsa*) embryos that the basal levels of hepatic EROD activity in day 26 duck embryos were three times higher than day 19 chicken embryos (Jin et al., 2001). A statistically significant correlation has been reviewed by Head and Kennedy (2009) between induction of EROD activity in cultured hepatocytes and embryo mortality.

1.4.2 CYP1A mRNA expression

Continued advances in the area of molecular biology, notably advances in real-time quantitative PCR (Q-PCR) analysis of gene expression, have resulted in an increase in the analysis of molecular biomarkers of exposure. The CYP1A mRNA expression may be used as a direct measure of AhR activation because it is the measurement of the expression of the genes encoding for the CYP1A enzymes (Kawajir and Fujii-Kuriyama,

2007; Whitlock, 1999). Statistically significant induction of mRNA expression has been observed in several avian species, including Domestic chicken, Herring gull (*Larus argentatus*), Jungle crow (*Corvus macrorhynchos*), and Common tern (*Sterna hirundo*) (Head and Kennedy, 2007a; 2007b; Kobuta et al., 2006; Nakayama et al., 2006; Watanabe et al., 2005).

Although the induction of CYP1A4 enzyme activity (EROD) is an adaptive response to the activation of AhR by DLCs, there are some uncertainties associated with its use as a biomarker of exposure to DLCs. Most notably, many EROD substrates are in fact suicide substrates as they inhibit enzyme activity. Consequently, the overall induction of CYP1A enzyme activities is not necessarily proportional to concentrations of AhR-active DLCs in the tissues (Hestermann et al., 2000). Concentration-dependent effects of TCDD on EROD activity have been reported in primary hepatocytes from many avian species, as well as by environmental exposure in some avian species (Kennedy et al., 1996; 2003b; Lorenzen et al., 1997a; 1997b; Sanderson et al., 1998), but not in herring gulls, juvenile double-crested cormorants, jungle crows, or glaucous gulls (Custer et al., 2001; Henriksen et al., 1998; Kennedy et al., 2003a; Watanabe et al. 2005). For instance, it was found in jungle crow liver, TEQs were positively correlated with CYP1A5 mRNA expression (CYP1A4 mRNA expression was not measured), but not with EROD activity (Watanabe et al., 2005). The utility of both CYP1A4 and CYP1A5 mRNA expression as indicators of exposure to DLCs was demonstrated by several correlative studies with field-collected. For instance, In common cormorant liver, TEQs and individual congener concentrations were positively correlated with both CYP1A4 and CYP1A5 mRNA expression (Kutoba et al., 2006).

The regulation of CYP1A4 and CYP1A5 mRNA expression and stability differs across species. For instance, CYP1A4 is preferentially induced in chickens, while CYP1A5 is preferentially induced in herring gulls. Therefore, CYP1A4 mRNA expression may be a sensitive biomarker of exposure to DLCs in some avian species, while CYP1A5 mRNA expression may be more sensitive in some other avian species (Head and Kennedy, 2007a). There are also studies showing that in other species, CYP1A1 and CYP1A2 (referring to the two CYP1A isoforms in species other than avian) act differently (Nebert and Gonzalez, 1987; Quattrochi and Tukey, 1989).

The combination of the hepatic CYP1A mRNA expression together with hepatic enzyme activity data can help characterize the chemically induced mechanisms by differentiating between pretranscriptional and post-transcriptional inhibition (Zhang et al., 2009). Combining CYP1A mRNA expression with EROD activity can exploit advantages associated with each biomarker response, and at the same time, minimize tissue requirements.

1.5 Toxic equivalency factors (TEFs)

Although DLCs act via stimulation of AhR signaling, individual dioxin-like congeners differ dramatically in their potency for eliciting biological effects. DLCs in environmental and biological samples are always found to be complex mixtures of various congeners whose relative concentrations differ across trophic levels. The complex nature of dioxin-like compound mixtures complicates the risk evaluation for humans, fish, and wildlife. For this purpose, the World Health Organization (WHO)

convened a panel of experts in 1997 and the concept of toxic equivalency factors (TEFs) was developed and introduced to facilitate risk assessment and regulatory control of exposure to these mixtures (Van den Berg et al., 1998). The toxicities of dioxin congeners are expressed as TEFs, where the most toxic congener TCDD is rated as 1.0 and other dioxin-like congeners are expressed as fractions in terms of their relative potencies to TCDD. Assuming that the toxic effects are additive, TEFs, in combination with chemical residue data, can be used to calculate the toxic equivalent quantities (TEQs) concentrations in various environmental samples, including animal tissues, soil, sediment, and water. The purpose of this methodology is to provide a 'common currency' for DLCs by expressing concentrations of individual congeners or environmental mixtures in terms of TCDD-TEQs, or the potency of the mixtures relative to TCDD (Van den Berg et al., 1998).

Although there is experimental evidence supporting the utility of the TEF approach for risk assessment (reviewed in Safe, 1998), there are also limitations to this approach. For example, there is uncertainty surrounding certain derived TEFs because they were derived either from *in vitro* studies and that do not account for potential differences in bioaccumulation, tissue distribution and metabolism in different species, or from quantitative structure-activity relationships (QSAR) that are based on structural analogies among compounds (Blankenship et al., 2008). In addition organisms are most often exposed to complex chemical mixtures contain DLCs, other halogenated aromatic hydrocarbons, and naturally occurring compounds. The TEF methodology works on the premise that all chemicals in a mixture act via the same pathway and all effects are additive, while non-dioxin-like chemicals do not necessarily act via the same mechanism.

However, it has been reported that certain PCBs exhibit antagonism towards the AhR (Aarts et al., 1995; Bannister et al., 1987; Biegel et al., 1989; Davis and Safe, 1989; Sanderson et al., 1996; Zhao et al., 1997). Moreover, several classes of natural AhR agonists have been identified. The presence of these antagonistic compounds, as well as naturally occurring AhR agonists, are significant limitations for the application of the TEF approach to risk assessment (reviewed in Safe, 1998).

A major challenge to the TEF approach to risk assessment is the discovery that not all species are equally sensitive to the effects of DLCs, and that the rank order of dioxin-like compound potency may not be consistent from species to species. For example, as outlined in sections 1.3 and 1.4, avian species differ at both the lethality level and molecular level in their sensitivity to DLCs. Moreover, in some avian species the PCDFs may be more toxic or potent AhR agonists than TCDD (Herve et al., 2009; Sanderson et al., 1998). Consequently, TEF methodology is not yet accurate enough for predicting when multiple mechanisms are present from a variety of contaminants.

1.6 Thesis Overview

The Tittabawassee River, which flows through mid-Michigan into Saginaw Bay, contains elevated concentrations of DLCs, including PCDDs, PCDFs and PCBs, which are structurally related to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Fairbrother, 2003). The level of furans was reported to be as high as 110,000 parts per trillion TEQ (Michigan Department of Environmental Quality, 2003). There is concern that avian species inhabiting this site may be exposed to these chemicals through either aquatic or terrestrial exposure pathways. Based on the toxicity data for chicken, which is the most

commonly used avian model, all the avian species inhabitating at the Tittabawasse contaminated site would be in severe risk, even death could be occurred. Therefore, an investigation was carried out by sampling a variety of avian species, but no significant changes were found either on individual health (nestling weight, nestling growth, clutch size, productivity, general health, etc.) or population health (abundance, productivity, nestling return rates, adult return rates, etc.), comparing to the avian species at the reference site. One hypothesis for this contradiction is that avian species have wide variations on sensitivities of responses to DLCs. Several studies have investigated the impact of DLCs on different avian species (Karchner et al., 2006). However, it is impossible to study all the avian species which are possibly exposed to dioxin-like toxicants in different feeding guilds. Therefore, risk assessments need to be conservative and among-species safety factors need to be applied. However, different avian species vary in their responses to dioxins and DLCs (Head, 2006; Head et al., 2007). For example, if the exposed species are more sensitive to dioxins it will be more of an issue than if the exposed species are less sensitive. Therefore, if the application of these safety factors is not appropriate, results from risk assessment may lead to unwarranted remedial actions. For this reason, in order to avoid either under- or over-protection, it is necessary to know the relative sensitivities of all the species of this site, and the relative responses to DLCs of different sensitivity groups.

1.6.1 Avian Models

The three avian models for this study were selected from the order Galliformes, one from each sensitivity group, referred to as White-leghorn chicken (*Gallus gallus*

domesticus) from type 1: very sensitive, Common pheasant (*Phasianus colchicus*) from type 2: moderately sensitive, and Japanese quail (*Coturnix japonica*) from type 3: insensitive. These three species are all commonly used as model species for avian toxicity studies, among which, chicken is the most commonly used one. All of the three species are readily available year around, inexpensive, easy to maintain, and already well understood with regard to normal physiology (Hill and Hoffman, 1984; Poynter et al., 2009). Nucleotide sequences of many of the AhR battery related genes for chicken are available through the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov). These properties make chicken a commonly used surrogate for the risk assessment of exposure and potential effects of environmentally persistent organic chemicals, including DLCs. Therefore, chicken is commonly used as a model to evaluate the hazard of xenobiotic exposure, including exposure during different periods of development, for instance, *in ovo* exposure to chicken embryos (Hoffman, 1990; DeWitt et al., 2005).

The fertilized avian egg, unlike viviparous animals, is a contained system in which the embryo develops without maternal interactions via the placenta (Bloom, 1980; Tazawa and Whittow, 2000). This property allows evaluating the toxic effects of concentration-dependent studies of certain chemicals to the developing bird through external application, topical application or injection. Therefore, the avian embryo is a useful model for the study of developmental effects of xenobiotic exposure, the toxicokinetic, and the correlated responses of biomarkers during embryo development. Egg injection can be applied by injecting chemicals either into the yolk or into the air cell. The chick embryo was significantly more sensitive to TCDD when injected into the yolk

than when injected into the air cell (Henshel et al., 1997), while injection into the air cell is more relevant to environmental exposure.

1.6.2 Rationale

Although *in vitro* studies have previously been conducted in a variety of avian species, a more complete *in ovo* exposure study for avian species from all three sensitivity groups as well as characterization of the expression of CYP1A mRNA in relationship to the levels of CYP1A and associated enzymatic activities (EROD) has not been done previously. cDNA sequencing of the AhR ligand binding domain for greater than 70 avian species, in combination with existing toxicity data for several of these species has led to the hypothesis that the sequence of the AhR LBD can be used to classify avian species as either sensitive, moderately sensitive or insensitive to DLCs (Head et al., 2008). As a test of these this hypothesis, a series of egg injection studies employing a representative species from each sensitivity classification has been designed. Experiments were carried out using three different species, including Japanese quail (Type 3), Common pheasant (Type 2), and White-leghorn chicken (Type 1). These three species were selected based on both relative species sensitivities (based on species specific sequencing of the ligand-binding domain; Yasui et al., 2004) and feasibility of obtaining the necessary number of viable eggs (1,300/species) in a short timeframe. Eggs were exposed to various doses of three different DLCs, including TCDD, TCDF, and PeCDF, by egg injection. Liver samples were collected 10-14 d after hatch, and CYP1A4 and CYP1A5 transcript abundance, EROD activity, as well as AhR transcript abundance and protein expression were analyzed.

The objectives of these studies were to investigate the CYP1A response to DLCs, specifically TCDD, TCDF, PeCDF, in differentially sensitive avian species within the Order Galliformes. This research will answer two main questions:

1) Do differences in the CYP1A response to DLC exposure exist across species that are predicted to be differentially sensitive to DLCs?
2) Do differences among the effects of three different DLCs, namely TCDD, TCDF and PeCDF on the CYP1A response exist within or across each species?

The null hypotheses for this study are:

1) Within each species, there is no difference among the potencies of TCDD, TCDF and PeCDF.
2) Within each chemical, there is no difference among the sensitivities of White-leghorn chicken, Common pheasant and Japanese quail.

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

Abstract

In birds, activation of the aryl hydrocarbon receptor (AhR) by some polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) results in induction of cytochrome P4501A (CYP1A) expression. This response has been useful for predicting relative avian sensitivity to DLCs. To further investigate species-sensitivity to dioxins and DLCs induction of cytochrome P450 1A4 and 1A5 (CYP1A4 and CYP1A5) mRNA and ethoxyresorufin-*O*-deethylase (EROD) activity were quantified in liver of post-hatch White-leghorn chicken, Common pheasant and Japanese quail exposed to 2,3,7,8-tetrachlorodibenzo-*p*-doxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or 2,3,7,8-tetrachlorodibenzofuran (TCDF) via air cell injection. The rank-order of sensitivity of TCDD- and TCDF-exposed birds, based on CYP1A, was chicken > pheasant > quail. Based on CYP1A5 mRNA expression and EROD induction, the order of sensitivity of PeCDF exposed birds was identical to that for TCDD and TCDF. However, based on CYP1A4 mRNA expression the rank-order was pheasant > chicken > quail. When comparing the potency of the three compounds in each species, based on CYP1A4 mRNA expression, TCDD was the most potent compound in chicken. However, PeCDF was equally potent to TCDD in Japanese quail

and was more potent than TCDD in Common pheasant. These results suggest that quantitative real-time PCR (Q-PCR) analysis of CYP1A expression, particularly CYP1A4 mRNA expression, may be a more sensitive biomarker of exposure than analysis of EROD induction, especially in less responsive avian species. Based on these findings future risk assessments should consider the sensitivity of the species inhabiting a site and the congeners of concern that are present.

Keywords: Dioxin, Furan, Cytochrome P4501A, EROD, Avian, Sensitivity, Potency

2.1 Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and structurally related polychlorinated dibenzofurans (PCDFs) as well as some polychlorinated biphenyls (PCBs) and other structurally similar compounds, are a group of environmental contaminants referred to as DLCs. Exposure to these chemicals has been shown to cause a range of effects, including endocrine disruption, immunological effects, developmental abnormalities, reduced egg production and hatchability, and lethality in avian species (Birnbaum and Tuomisto, 2000; Elliott et al., 1996; Fox et al., 1988; Gilbertson et al., 1991; Giesy et al., 1994; Kennedy et al., 1996; Peterson et al., 1993; Sanderson and Bellward, 1995).

Toxic and adaptive responses to TCDD and structurally related DLCs are largely mediated by the aryl hydrocarbon receptor (AhR) (Fernandez-Salguero, et al., 1995, Mimura et al., 1997; Schmidt et al., 1996). The AhR is a ligand activated transcription factor that regulates expression of a suite of biotransformation enzymes, one group of which is the mixed function monooxygenase (MFO) enzymes, including the cytochrome P450 1A (CYP1A) genes (Kawajir and Fujii-Kuriyama, 2007; Whitlock, 1999). Two CYP1A genes, CYP1A4 and CYP1A5, inducible by TCDD, are constitutively expressed in avian liver (Gilday et al., 1998; Head and Kennedy, 2007a, 2007b; Mahajan et al., 1999). The CYP1A4 enzyme exhibits ethoxyresorufin-*O*-deethylase (EROD) activity while the CYP1A5 isoform preferentially catalyzes arachidonic acid metabolism and uroporphyrinogen oxidation (UROX) (Rifkind et al., 1994; Sinclair et al., 1997).

The AhR signaling pathway, including induction of CYP1A activity, is conserved in vertebrates (Schmidt et al., 1996; Hahn, 2002). Despite this conservation there are

differences in both sensitivity and efficacy of responses to TCDD and DLCs among vertebrates (Head and Kennedy, 2007a; Pohjanvirta and Tuomisto, 1994; Poland et al., 1994). Differences in sensitivity and efficacy of responses to DLCs at the whole organism, biochemical, and molecular levels have been observed in birds, and both relative sensitivity and efficacy of responses could contribute to differential toxicity of DLCs among species of birds. Here we will distinguish between sensitivity and efficacy. Sensitivity of a species is defined as the threshold concentration of a chemical to cause a statistically significant response in a species. A species exhibiting a lesser threshold concentration would be more sensitive. For instance, when exposed via egg injection, the Ring-necked pheasant (*Phasianus colchicus*) is 10-fold less sensitive to the embryotoxic effects of TCDD than the Domestic chicken (*Gallus domesticus*) (Brunström and Halldin, 1998; Head et al., 2008; Nosek et al. 1993). The efficacy of a response refers to the magnitude of responsiveness and is measured by the maximum response observed. A species exhibiting a greater magnitude of induction of an enzyme or up-regulation of gene expression when exposed to a compound of interest would be more responsive and the compound would have greater efficacy in that species. The greater responsiveness of the chicken to TCDD compared to the pheasant is demonstrated by the observation that EROD activity is 10-fold greater in chicken hepatocytes compared to ring-necked pheasant hepatocytes (Kennedy et al., 1996). Thus, the chicken is more sensitive and more responsive. A further example of differential responsiveness among species is the fact that CYP1A4 and CYP1A5 mRNA expression are 27.7- and 5.8-fold greater in chicken than in herring gull (*Larus argentatus*) hepatocytes exposed to 100 nM TCDD (Head and Kennedy, 2007a), respectively.

Although the mechanism(s) responsible for differential sensitivity and responsiveness among avian species is not completely understood, evidence of a molecular basis has been suggested (Karchner et al., 2006). Specifically, amino acid substitutions in the AhR ligand binding domain (LBD) of the AhR appear to account for differences in affinities of ligands for the AhR. The greater occupancy rates of the DLCs on the AhR lead to differences in TCDD-dependent trans-activation (Karchner et al., 2006). Based on these findings and existing toxicity data, a sensitivity classification scheme has been developed for members of the Order *Galliforms*. Specifically, based on embryotoxicity data the chicken is classified as Type 1 (very sensitive), the Common pheasant is classified as Type 2 (moderately sensitive) and Japanese quail (*Coturnix japonica*) is classified as Type 3 (insensitive) (Head et al., 2008).

Differences in sensitivity and responsiveness among species present a significant challenge in avian ecological risk assessments that are currently based on responses of the White-leghorn chicken. An implicit assumption in these assessments is that the chicken is a sensitive surrogate that would be equally or more sensitive/responsive to DLCs. Thus, risk assessments based on the chicken would be protective of other species. However, due to its sensitivity to TCDD and DLCs this species may not be representative of any wild species and therefore lead to inaccurate assessments and unnecessary remediation and the associated loss of habitat. However, risk assessors need to consider hundreds of species that might occupy a site being assessed. Since comprehensive toxicity profiles are not available for all of these species, the ability to accurately predict avian sensitivities and responsiveness to TCDD and structurally related DLCs would be advantageous. Such a predictive classification scheme, based on the amino acid sequence

of the AhR LBD (Karchner et al., 2006; Head et al., 2008) could greatly enhance avian risk assessment. However, there is a need to validate this classification scheme for a range of endpoints, such as molecular and biochemical responses as well as lethality. This study was conducted to quantify responses of commonly used functional indicators of exposure relative to the classification of species based on AhR LBD. Specifically, CYP1A4 and CYP1A5 mRNA expression and EROD activity were determined in liver of post-hatch White Leghorn chicken, Common pheasant and Japanese quail exposed to TCDD, PeCDF or TCDF injected into the air cell of fertilized eggs.

2.2 Materials and Methods

2.2.1 Source of Eggs

Fertilized eggs of Japanese quail, Common pheasant and White-leghorn chicken were obtained from the Michigan State University Poultry Research and Teaching Center and stored in a cooler at 13.5-15.0 ℃ until 24 h prior to injection. Eggs were weighed and grouped so that each treatment group received an equal distribution of eggs weighing from 52.0 to 64.0 g for chicken, 25.0 to 34.0 g for pheasant and 6.8 to 13.8 g for quail.

2.2.2 Egg Injection and Incubation Conditions

Eggs were injected at the Avian Research Center of the Department of Animal Science, Michigan State University, East Lansing, Michigan, USA. The chemicals of interest, including TCDD, PeCDF and TCDF (Wellington Laboratories, Guelph, ON, Canada), were dissolved and diluted in cold-filtered sterile triolein (Sigma, St. Louis, MO,

USA). Injection volume per egg was calculated based on 5.8 μL/58 g egg for chicken, 3.0 μL/30 g egg for pheasant and 2.0 μL/10 g egg for quail. The following species-specific dosing solutions of each compound were prepared: chicken (0.0494, 0.0963, 0.195, 0.416, 0.767, 1.57, 3.07 pmol/g egg for TCDD, 0.0438, 0.0867, 0.142, 0.335, 0.693, 1.38, 2.49 pmol/g egg for PeCDF, and 0.0742, 0.148, 0.245, 0.516, 1.05, 1.83, 4.02 pmol/g egg for TCDF); pheasant (0.0745, 0.0994, 0.224, 0.311, 0.820, 3.17, 6.68 pmol/g egg for TCDD, 0.141, 0.235, 0.388, 0.599, 1.07, 4.08, 6.76 pmol/g egg for PeCDF, and 0.131, 0.170, 0.288, 0.654, 1.12, 4.77, 14.2 pmol/g egg for TCDF), and Japanese quail (0.223, 0.497, 0.745, 1.24, 2.86 pmol/g egg for TCDD, 0.411, 0.911, 1.82, 2.61, 5.31, 11.16 pmol/g egg for PeCDF, and 0.418, 0.628, 1.59, 2.90, 4.81, 8.56 pmol/g egg for TCDF). Following dose preparation, injection vials were flooded with argon to preserve the triolein, capped and sterilized in an autoclave. Concentrations in triolein were confirmed by high resolution mass spectrometry (described below).

Egg injection was done in a laminar flow hood (NUAIRE, Plymouth, MN, USA). Eggs were candled to mark the centre of the air cell and the injection site was sterilized with 70% ethanol and then a single hole was drilled using a Dremel tool (Robert Bosch Tool Corporation, Racine, WI, USA). Triolein as a vehicle control or TCDD, PeCDF or TCDF stock solutions was injected into the air cell using a positive displacement pipettor (Gilson, Middleton, WI, USA) with sterile pipette tips changed after each injection. The injection site was then sealed with liquid paraffin wax (Royal Oak Sales Inc., Roswell, GA, USA) and a sterilized wooden applicator.

Eggs were incubated with the sealed injection site up at 37.5-37.7 ℃ with 50-60% humidity in a Petersime Rotary Incubator (Petersime Incubator Co., Gettysburg, OH,

USA) and rotated automatically every two hours. Eggs were transferred to the hatching trays of a Surepip hatcher (Agro Environmental Systems, Inc., Dallas, GA, USA) three days prior to the expected hatching date with only one treatment group per hatching tray. The hatcher was maintained at 37.5-37.5 ℃ with 70-75% humidity and each hatching tray was divided into individual compartments for each egg.

Hatching eggs were monitored one day prior to and two days after the expected hatching date. Once sufficiently dry, the hatchlings were moved into a Petersime Brood Unit (Petersime Incubator Co., Gettysberg, OH, USA) maintained at 30.0 ℃ and identified with a Swiftack (Heartland Animal Health Inc., Fair Play, MO, USA) identification tag bearing their unique egg number. Chicks were weighed and examined for abnormalities, and then raised for two weeks after the expected hatching date. Chicks were introduced to clean feed (Purina Game Bird Starter, Purina Mills, St. Louis, MO, USA) and water by dipping their beaks in both, which were then provided *ad libitum*. After the two-week grow-out period, 10 chicks were randomly selected from each treatment group, euthanized by cervical dislocation, and necropsied. The liver was removed, weighed, and divided into four portions; the first portion was placed in an I-Chem jar on ice for contaminant analysis, the second placed in a microtube containing RNAlater for mRNA analysis, the third portion was placed in a microtube that was frozen in liquid nitrogen for analysis of enzyme activity, and the fourth was placed in 10% formalin for assessment of histopathology. The liver tissue for mRNA and EROD analysis was then delivered to the Environmental Toxicology Laboratory, Toxicology Centre, University of Saskatchewan (Saskatoon, Saskatchewan, Canada).

2.2.3 Quantification of TCDD, PeCDF and TCDF Injection Solutions

Concentrations of injection solutions were confirmed by isotope dilution following EPA method 1613 (U.S.EPA., 1994) with ^{13}C surrogate standards (DF-CS-C100, Wellington Laboratories, Guelph, ON, Canada). Identification and quantification of TCDD, PeCDF and TCDF was performed using a Hewlett-Packard 5890 series high-resolution gas chromatograph interfaced with a Micromass® Autospec® high-resolution mass spectrometer (137 HRGC-HRMS) (Micromass®, Beverly, MD, USA). The mass spectrometer was operated in a Selected Ion-Monitoring (SIM) mode and the resolution for all reference gas peaks in all time windows was greater than 10,000. Concentrations of TCDD, PeCDF and TCDF were quantified by the internal standard isotope dilution method using mean relative response factors determined from standard calibration runs. Recoveries of ^{13}C-labeled PCDD/Fs internal standards and all other QA/QC criteria were within ranges specified by the EPA methods (U.S.EPA., 1994).

2.2.4 Total RNA Isolation and cDNA synthesis

Total RNA was extracted from approximately 30 mg of liver tissue with the RNeasy® Mini Kit (QIAGEN, Mississauga, ON, Canada) using a QIAshredder (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's protocol with one slight modification: a 50 % ethanol solution was used instead of a 70 % ethanol solution because it provided greater RNA yields (Head and Kennedy, 2007). Purified RNA was quantified using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Samples were checked for RNA integrity on a 1 % denaturing formaldehyde-agarose gel and visualized by staining the gel with ethidium bromide and

visualizing the bands under UV light using a VersaDoc 4000 MP imaging system (Bio-Rad, Hercules, CA, USA). The purified RNA samples were stored at -80 ℃ until analysis.

First-strand cDNA synthesis was performed using iScript™ cDNA Synthesis Kit (Bio-Rad)). A volume of 1 μg total RNA was combined with 4 μL of 5× iScript Reaction Mix, 1 μL of iScript Reverse Transcriptase, and RNase-free water to a final volume of 20 μL. Reaction mixes were incubated at 25 ℃ for 25 min, 42 ℃ for 20 min and, on completion, were inactivated at 85 ℃ for 5 min. The cDNA samples were stored at -80 ℃ until further analysis.

2.2.5 Cloning and sequencing of full-length Japanese quail CYP1A4 and CYP1A5 cDNA

A fragment of Japanese quail CYP1A4 and CYP1A5 was amplified using primers (Table 1) designed against conserved regions identified by aligning available homologous sequences from other avian species. PCR reactions were performed using a Bio-Rad MyCycler Thermal Cycler (Bio-Rad) in a volume of 20 μL, consisting of 10× Taq buffer with $(NH_4)_2SO_4$, 0.25 mM of each dNTP, 1.25 mM of $MgCl_2$, 0.125 μM of each primer, 1 μL of liver cDNA template, and 2.5 U Taq polymerase (Fermentas, Burlington, ON, Canada). The reaction mixture was initially denatured at 95 ℃ for 5 min followed by 40 cycles of amplification with the reaction profile of denaturing at 95 ℃ for 45 sec, annealing at 60 ℃ for 45 sec, and extension at 72 ℃ for 45 sec. Following amplification a final extension was performed at 72 ℃ for 10 min. A small volume of the amplified PCR fragments was visualized on a 1 % (w/v) agarose gel stained with ethidium bromide

and visualized under UV light on a VersaDoc 4000 MP imaging system (Bio-Rad). The remaining volume of PCR products was purified using a QIAQuick PCR purification system (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's protocol. Purified PCR products were cloned into the pGEM-T Easy Vector using a DNA ligation kit (Invitrogen, Carlsbad, CA, USA), and transformation into competent JM109 *E. coli* cells (Promega, Madison, WI, USA). Plasmids were isolated with a QIAGEN plasmid purification kit (QIAGEN) and the products were sequenced at the National Research Council of Canada's Plant Biotechnology Institute (University of Saskatchewan, Saskatoon, SK, Canada).

Table 2-1. PCR primers used for cloning and sequencing of Japanese quail CYP1A4 and CYP1A5.

Primer		Sequence (5' - 3')
Degenerate primers		
CYP1A4	Forward:	5'-ATGTACGCTGCCTTGTACCC-3'
	Reverse	5'-CCGTACTGAGGGGTGATGTC-3'
CYP1A5	Forward	5'-ACCTGGTCACCAAATTCCTG-3'
	Reverse	5'-CTCCAGGATGAAGGCTTCTG-3'
RACE primers		
CYP1A4	5' RACE	5'-CGTCCCGAATGTGCTCCTTATCAAAAG-3'
	3' RACE	5'-AATGTTCGCGTCCAACCTTCTGATA-3'
CYP1A5	5' RACE	5'-CGTCTCGGATGCTGTTCTTGTCATAGG-3'
	3' RACE	5'-AGTGGTGCCCTTCAGATCCCAAATG-3'

2.2.6 3′ and 5′ rapid amplification of cDNA ends (RACE)

Gene-specific primers (Table 2-1) were designed based on the partial cDNA sequences determined for Japanese quail CYP1A4 and CYP1A5. 3′ - RACE and 5′ - RACE PCR reactions were performed using SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol. A small volume of the amplified PCR fragments was visualized on a 1% (w/v) agarose gel stained with ethidium bromide and visualized under UV light on a VersaDoc 4000 MP imaging system (Bio-Rad). The remaining volume of PCR products was purified using a QIAQuick PCR purification system (QIAGEN, Mississauga, ON, Canada) according to the manufacturer's protocol. Purified PCR products were cloned into the pGEM-T Easy Vectors using a DNA ligation kit (Invitrogen, Carlsbad, CA, USA), followed by transformation into competent JM109 *E. coli* cells. Plasmids were isolated with a QIAGEN plasmid kit (QIAGEN, Mississauga, ON, Canada) and the products were then sequenced by the Plant Biotechnology Institute, National Research Council (NRC, Canada). Full-length cDNA sequences were assembled by aligning sequenced PCR products.

2.2.7 Real-time Polymerase Chain Reaction (Q-PCR)

Gene-specific primers against CYP1A4 and CYP1A5 from White-leghorn chicken were from Head and Kennedy (2007b) and from Common pheasant were from Hervé et al. (2009, submitted). Gene specific primers against Japanese quail CYP1A4 and CYP1A5 were designed based on the full-length sequences determined in this study.

To ensure amplification of desired transcripts the PCR products for each primer pair were sequenced as outlined above.

Real-time PCR was performed in 96-well PCR plates using an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). A separate 80 µl PCR reaction mixture consisting of gene-specific primers (Table 2-2), 40 µl of 2X SYBR Green master mix (Applied Biosystems, Foster City, CA, USA), 3 µL cDNA, and nuclease-free water to the final volume was prepared for each cDNA sample of interest and for each primer pair. The gene-specific Q-PCR primers for each species as well as the volumes and concentration of each component are shown (Table 2-2). A final reaction volume of 25 µL was transferred to each well and reactions were performed in triplicate. The PCR reaction mixture was denatured at 95 ℃ for 10 min before the first PCR cycle. The thermal cycle profile was denatured for 10 s at 95 ℃ and extension for 1 min at 60 ℃. A total of 40 PCR cycles were performed.

Table 2-2. PCR primers used for quantification of CYP1A4 and CYP1A5 transcript abundance in Japanese quail, Common pheasant and chicken.

Transcript		**Sequence (5' - 3')**	**Final Concentration (nM)**
β-Actin	Forward	AAATTGTGCGTGACATCAAGGA	(325)
	Reverse	GAGGCAGCTGTGGCCATCT	(325)
Japanese quail			
CYP1A4	Forward	ATGTACGCTGCCTTGTACCC	(325)
	Reverse	CTCCAGGATGAAGGCTTCTG	(325)
CYP1A5	Forward	TACAGGCAGCTGTGGATGAG	(325)
	Reverse	GATCTGAAGGGCACCACTG	(325)
Common pheasant			
CYP1A4	Forward	GAGCACATTCGGGATGTCA	(250)
	Reverse	CAGAGAGTTGGACACGGACA	(250)
CYP1A5	Forward	CATCCGAGATGTCACCGACT	(750)
	Reverse	TTGGGATCTGTGTGGCACTA	(750)
chicken			
CYP1A4	Forward	TAAGGACGTCAATGCTCGTTTC	(300)
	Reverse	CGTCCCGAATGTGCTCCTTAT	(300)
CYP1A5	Forward	ACAGCTGTGGAAGAGCACTACCA	(300)
	Reverse	TCTCCACGCACTGCTCGAT	(300)

To quantify Q-PCR results, the cycle at which the fluorescence signal was first significantly different from background (Ct) was determined for each reaction. The expression levels of CYP1A4 and CYP1A5 were normalized with reference to β-actin to derive the mean normalized expression (MNE) value as described by Simon (2003) (Eq. 1).

$$MNE = \frac{(E_{reference})^{Ct_{reference,mean}}}{(E_{target})^{Ct_{target,mean}}} \qquad (1)$$

Where: $E_{reference}$ and E_{target} represent the PCR efficiencies ($=10^{-1/slope}$) of the target gene (CYP1A4 and CYP1A5) and β-actin, respectively, as determined from the slope of a standard curve constructed using serial dilutions of a cDNA sample prepared by pooling random cDNA samples (Simon, 2003). Levels of expression relative to control were calculated (Equation 2).

$$N-\text{fold Change} = \frac{MNE_{experimental}}{MNE_{control}} \qquad (2)$$

2.2.8 Ethoxyresorufin-*O*-deethylase (EROD)

Microsome preparation and ethoxyresorufin-*O*-deethylase (EROD) assays were performed according to the methods of Kennedy and Jones (1994). Wherever possible all procedures were performed on ice using chilled equipment and reagents. Briefly, approximately 100 to 200 mg of liver tissue (frozen in liquid nitrogen) was rinsed in ice-cold phosphate buffer (0.08 M sodium phosphate, 0.02 M potassium phosphate, pH 7.4). Tissue was minced into small pieces with cold scissors and quantitatively transferred into

a 2 mL microcentrifuge tube. Tissue was homogenized with 10 strokes using a Fisher Scientific Powergen 125 (FTH-115) blade-type homogenizer. The mixture was kept on ice during the whole procedure. The homogenate was centrifuged at 9,000 g in a SORVALL® Legend RT+ Centrifuge (Thermo Fisher Scientific, Asheville, NC, USA) for 15 min at 4 ℃, and the supernatant from each sample, representing the S9 fraction, was transferred into separate ultracentrifuge tubes (SETON, Los Gatos, California, USA), and centrifuged at 100,000 g in a SORVALL® Ultraspeed Centrifuge (Thermo Fisher Scientific, Asheville, NC, USA) for 60 min at 4 ℃. The supernatant was discarded, the pellet was re-suspended in 0.6 mL of ice-cold phosphate buffer, and aliquots were stored at -80 ℃ until further use.

The EROD activities and total protein concentrations in each microsome preparation were assayed in 96-well plates. Dilutions of resorufin (Sigma) and bovine serum albumin (BSA, Sigma) were used to establish resorufin and protein standard curves according to Kennedy and Jones (1994). Each microsome sample was analyzed in triplicate together with a blank control. All wells contained 15 µL of microsomes, 50 µL of 7-ethoxyresorufin (7-ER, Sigma) working solution (final concentration 2 µM) and sodium phosphate buffer to a final volume of 235 µL for blank controls or 185 µL for wells containing microsomes. Following 5-min incubation at 37 ℃, the enzymatic reaction was initiated by adding 50 µL of NADPH to make a final concentration of 0.5 mM. Reactions were allowed to proceed for exactly 10 min at 37 ℃, after which time the reactions were terminated by adding 100 µL cold acetonitrile containing fluorescamine (2.16 mM, Sigma). Plates were immediately scanned using a fluorescence plate reader (POLARstar OPTIMA, BMG LABTECH, Offenburg, Germany) according

to Kennedy and Jones (1994) in order to quantify both resorufin formation and protein concentrations. Resorufin was quantified at 530 nm excitation and 590 nm emission wavelengths. Protein concentration was determined with a 400 nm excitation filter and a 460 nm emission filter.

2.2.9 Relative Potency and Relative Sensitivity Determination

Relative sensititity (ReS) and relative poteny (ReP) values were calculated in order to compare the potency of each compound within each species and to determine relative sensitivity of each species to each compound. The first step in the calculations was to determine the lowest observed effect concentration (LOEC) for each response. The threshold for effect (LOEC) was determined differently depending on whether the data met the assumptions of parametric statistics. The LOEC values for parametric data (EROD) were obtained by calculating the geometric mean of the least dose that stimulated a significant increase in EROD activity and the preceding dose. For data that were not normally distributed (mRNA expression), the LOEC was estimated as the point of intersection on the x-axis of the lower 95% confidence interval of the linear regression line. The ReS and ReP were calculated as:

$$\mathrm{ReS} = \frac{\mathrm{LOEC\ species\ A}}{\mathrm{LOEC\ chicken}} \qquad (3)$$

$$\mathrm{ReP} = \frac{\mathrm{LOEC\ TCDD\ specie\ s\ A}}{\mathrm{LOEC\ compound\ \ of\ interest\ \ in\ species\ \ A}} \qquad (4)$$

A calculation of relative responsiveness were not performed, however, a discussion of responsiveness is included here.

2.2.10 Statistical analyses

Values for all measurements were summarized as mean ± SEM. Statistical analyses were conducted using SPSS 16 (SPSS Inc., Chicago, IL, USA). The normality of each data set was assessed using the Kolomogrov–Smirnov one-sample test and homogeneity of variance was determined using the Levene's test, and both untransformed and Log transformed data were evaluated. CYP1A4 and CYP1A5 mRNA expression data were not normally distributed, so the Kruskal-Wallis (KW) test, with post-hoc Mann-Whitney U test was used for comparisons between treatment groups and the vehicle control. EROD data were analyzed by one-way ANOVA with a Dunnett's *post-hoc* test to make comparisons between treatment groups and the vehicle control. Differences were considered to be statistically different for CYP1A mRNA expression at $p \leq 0.1$ in order to minimize the probability of causing Type II error.

2.3 Results

2.3.1 Japanese quail CYP1A4 and CYP1A5

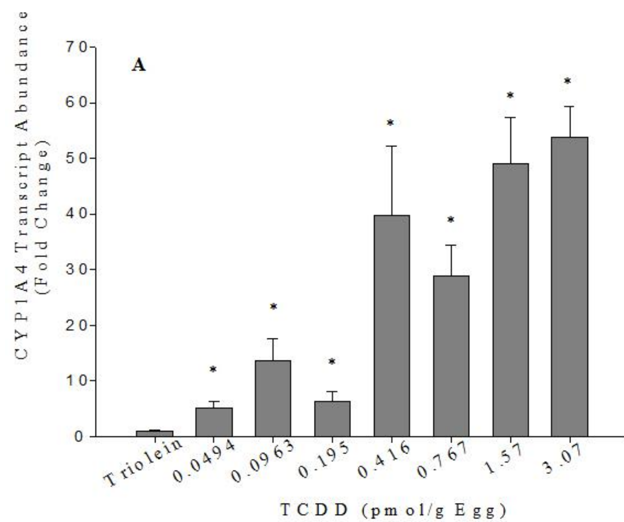
A partial cDNA sequence for an MFO enzyme designated as Japanese quail CYP1A1 is available in GenBank (Accession number: AB359052.1). However, to ensure accurate quantification of CYP1A4 and CYP1A5 mRNA expression by real-time PCR, as part of this study, these transcripts were cloned from Japanese quail hepatic tissue and sequenced. The nucleotide sequences for Japanese quail CYP1A4 and

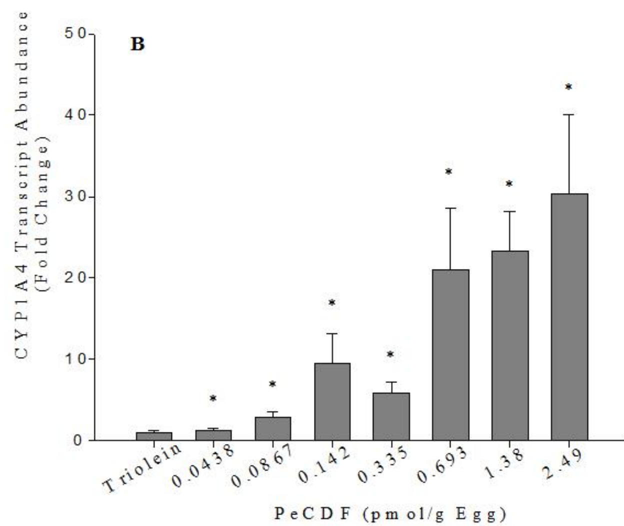
CYP1A5 have been submitted to GenBank under Accession numbers GQ906939 and GQ906938, respectively. The cloned full-length Japanese quail CYP1A4 cDNA consists of a 1593-bp open reading frame (ORF) encoding 530 amino acids. The Japanese quail CYP1A5 cDNA consists of a 1587-bp ORF encoding 528 amino acids.

The deduced amino acid sequence for Japanese quail CYP1A4 had a 90% overall amino acid identity with the chicken (*Gallus gallus*) (Accession No.: NP990478). The Japanese quail CYP1A5 protein sequence had 91% and 77% overall amino acid identities with the turkey (*Meleagris gallopavo*) (Accession No.: AY964644) and the Great cormorant (*Phalacrocorax carbo*) (Accession No.: AB239445), respectively. The Japanese quail CYP1A sequences also display great sequence homology with CYP1As of other avian species, including the Ring-necked pheasant (*Phasianus colchicus*) and Jungle crow (*Corvus macrorhynchos*).

2.3.2 Chemical-induced effects on hepatic CYP1A4 mRNA expression

White-leghorn chicken hepatic CYP1A4 mRNA expression was significantly greater than constitutive levels at all doses of each chemical. Maximum up-regulation of approximately 53-fold was observed at 3.07 pmol TCDD/g egg (Figure 2-1A), while a 30-fold up-regulation was observed at 2.49 pmol PeCDF/g egg (Figure 2-1B) and a 13-fold up-regulation was observed at 1.83 pmol TCDF/g egg (Figure 2-1C).


A
CYP1A4 Transcript Abundance (Fold Change)
0
10
20
30
40
50
60
70
Triolein
0.0494
0.0963
0.195
0.416
0.767
1.57
3.07
TCDD (pmol/g Egg)


B
CYP1A4 Transcript Abundance (Fold Change)
0
10
20
30
40
50
Triolein
0.0438
0.0867
0.142
0.335
0.693
1.38
2.49
PeCDF (pmol/g Egg)

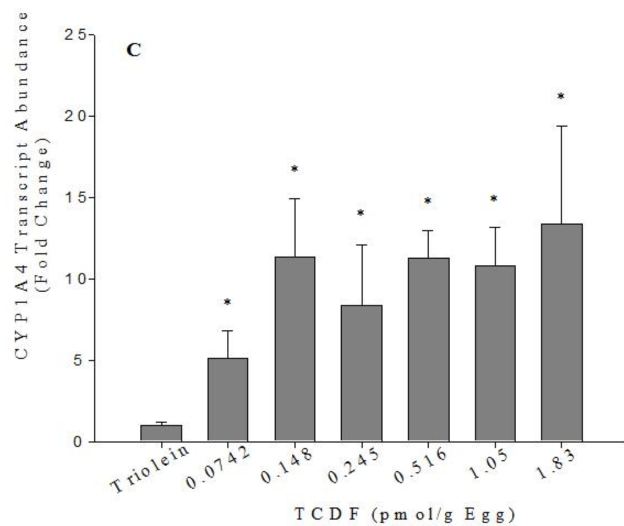
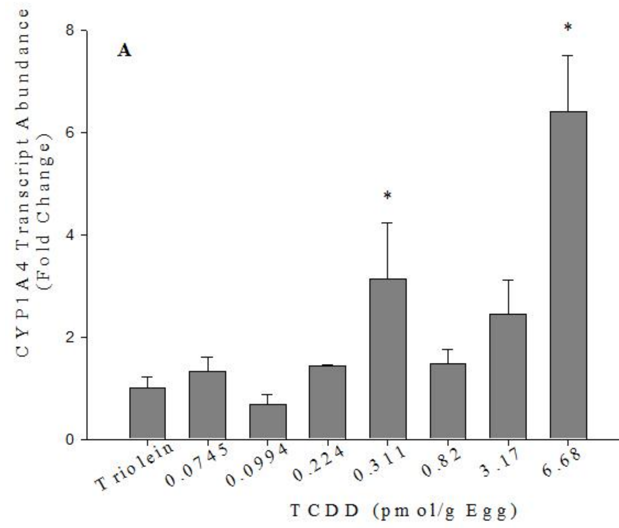
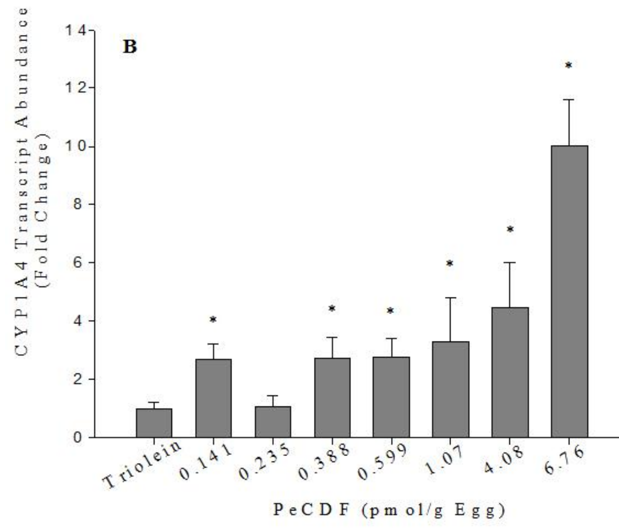

C
CYP1A4 Transcript Abundance (Fold Change)
0
5
10
15
20
25
Triolein
0.0742
0.148
0.245
0.516
1.05
1.83
TCDF (pmol/g Egg)

Figure 2-1. Effects of TCDD (A), PeCDF (B) and TCDF (C) on *CYP1A4* transcript abundance in the liver of the White-leghorn chicken. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of CYP1A4 mRNA expression in treatment groups relative to control group and bars represent standard error (N = 4-7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

Exposure to each of the three chemicals had much less of an effect on Common pheasant chicks. CYP1A4 mRNA expression was significantly up-regulated in chicks exposed to 0.311 and 6.68 pmol TCDD/g egg. The maximum response was approximately 6-fold at 6.68 pmol/g egg (Figure 2-2A). CYP1A4 mRNA expression was significantly up-regulated at 0.141, 0.388, 0.599, 1.07, 4.08 and 6.76 pmol PeCDF/g egg, to a maximum of approximately 10-fold at 6.76 pmol PeCDF/g egg (Figure 2-2B). CYP1A4 mRNA expression was significantly up-regulated at 0.654, 4.77 and 14.2 pmol TCDF/g egg, to a maximum of approximately 3-fold at 4.77 pmol TCDF/g egg (Figure 2-2C).


A
CYP1A4 Transcript Abundance (Fold Change)
Triolein
0.0745
0.0994
0.224
0.311
0.82
3.17
6.68
TCDD (pmol/g Egg)


B
CYP1A4 Transcript Abundance (Fold Change)
Triolein
0.141
0.235
0.388
0.599
1.07
4.08
6.76
PeCDF (pmol/g Egg)

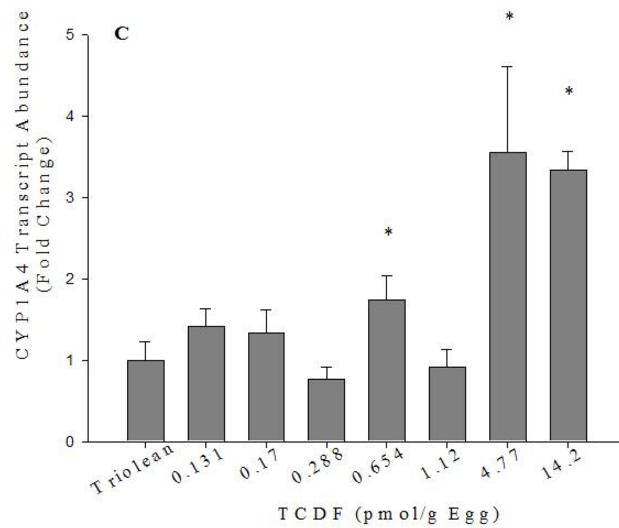
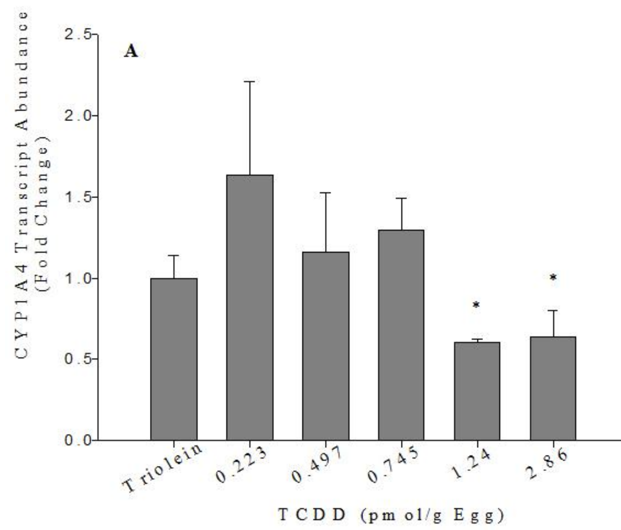
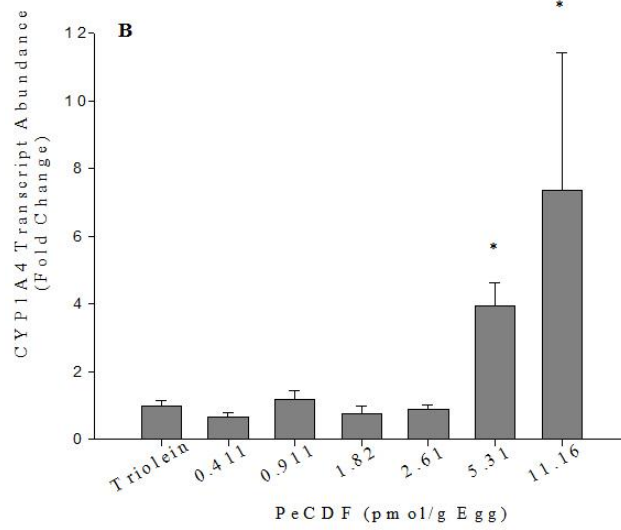

C
CYP1A4 Transcript Abundance (Fold Change)
Triolean
0.131
0.17
0.288
0.654
1.12
4.77
14.2
TCDF (pmol/g Egg)

Figure 2-2. Effects of TCDD (A), PeCDF (B) and TCDF (C) on *CYP1A4* transcript abundance in the liver of the Common pheasant. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of CYP1A4 mRNA expression in treatment groups relative to control group and bars represent standard error (N = 4-7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

Changes in Japanese quail hepatic CYP1A4 mRNA expression were observed. Both up-regulation and down-regulation were observed. CYP1A4 mRNA expression was not significantly up-regulated in response to TCDD. Significant down-regulation was observed at 1.24 and 2.86 pmol TCDD/g egg (Figure 2-3A). PeCDF exposure significantly up-regulated CYP1A4 mRNA expression at 5.31 and 11.16 pmol/g egg, to a maximum of approximately 7-fold at 11.16 pmol TCDD/g egg (Figure 2-3B). No significant up-regulation in CYP1A4 mRNA expression was observed in either of the TCDF exposure groups. However, a significant down-regulation of mRNA expression was observed at 1.59 and 2.90 pmol TCDF/g egg (Figure 2-3C).


A
CYP1A4 Transcript Abundance
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Triolein
0.223
0.497
0.745
1.24
2.86
TCDD (pmol/g Egg)


B
CYP1A4 Transcript Abundance
(Fold Change)
12
10
8
6
4
2
0
*
*
Triolein
0.411
0.911
1.82
2.61
5.31
11.16
PeCDF (pmol/g Egg)

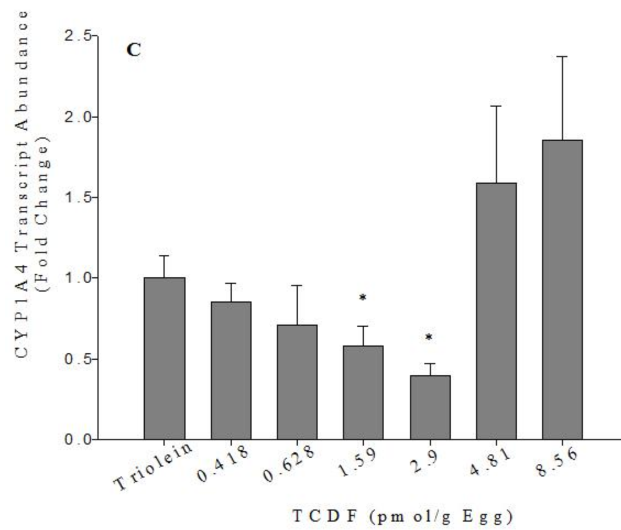
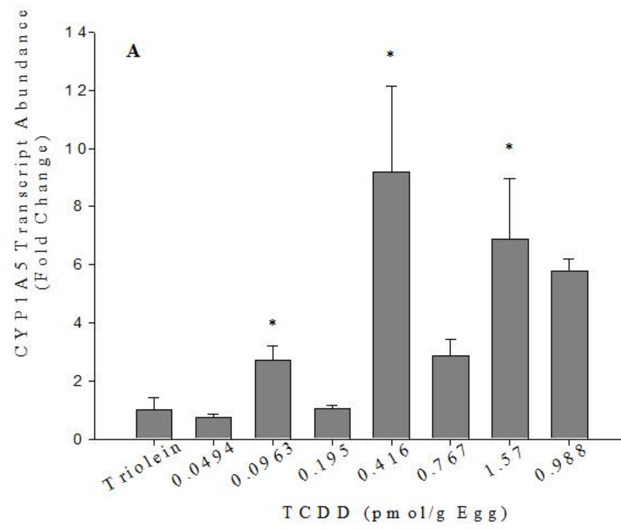
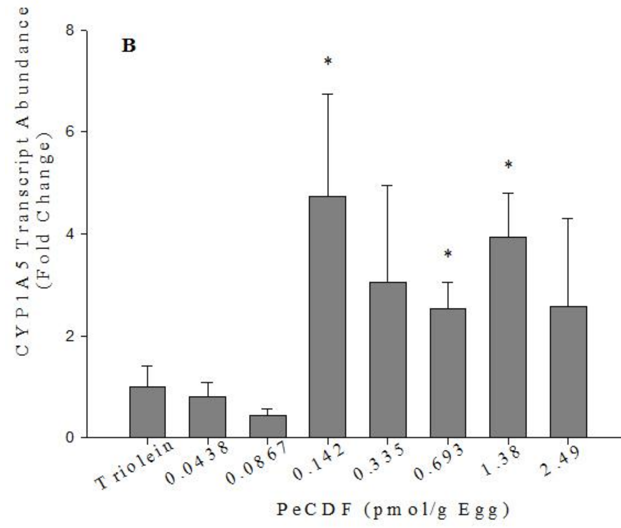

C
CYP1A4 Transcript Abundance
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Triolein
0.418
0.628
1.59
2.9
4.81
8.56
TCDF (pmol/g Egg)

Figure 2-3. Effects of TCDD (A), PeCDF (B) and TCDF (C) on *CYP1A4* transcript abundance in liver of Japanese quail. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of CYP1A4 mRNA expression in treatment groups relative to control group and bars represent standard error (N = 4-7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

2.3.3 Chemical-induced effects on hepatic CYP1A5 mRNA expression

White-leghorn chicken hepatic CYP1A5 mRNA expression was significantly up-regulated by 0.0963, 0.416, 0.767, 1.57 and 3.07 pmol TCDD/g egg, to a maximum of approximately 9-fold at 0.416 pmol TCDD/g egg (Figure 2-4A). CYP1A5 mRNA expression was significantly up-regulated at 0.142, 0.693 and 1.38 pmol PeCDF/g egg, to a maximum of approximately 4.7-fold at 0.142 pmol PeCDF/g ww egg (Figure 2-4B). CYP1A5 mRNA expression was significantly up-regulated at 0.148, 0.245, 0.516 and 1.83 pmol TCDF/g ww egg, to a maximum of approximately 8-fold at 0.245 pmol TCDF/g ww egg (Figure 2-4C).


A
CYP1A5 Transcript Abundance
(Fold Change)
0
2
4
6
8
10
12
14
*
*
*
Triolein
0.0494
0.0963
0.195
0.416
0.767
1.57
0.988
TCDD (pmol/g Egg)


B
CYP1A5 Transcript Abundance
(Fold Change)
0
2
4
6
8
*
*
*
Triolein
0.0438
0.0867
0.142
0.335
0.693
1.38
2.49
PeCDF (pmol/g Egg)

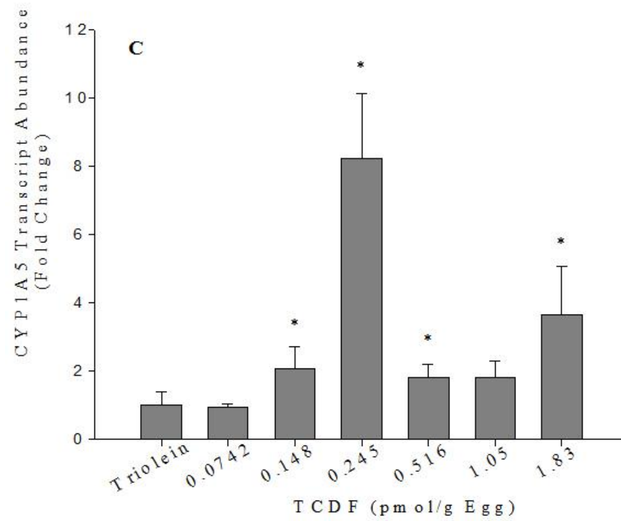
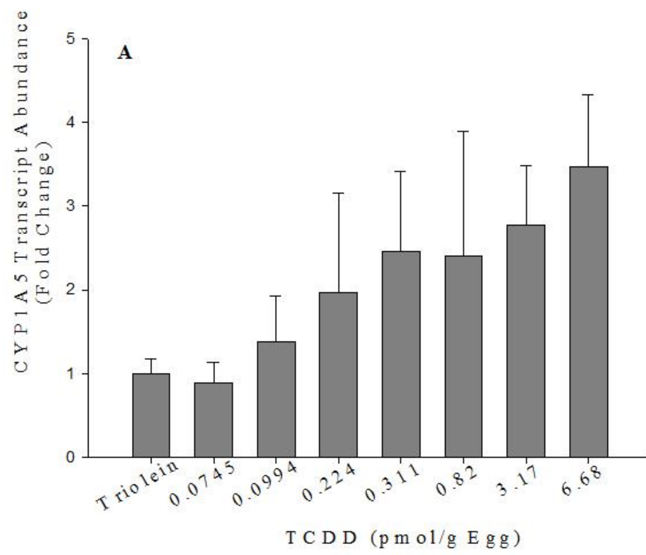
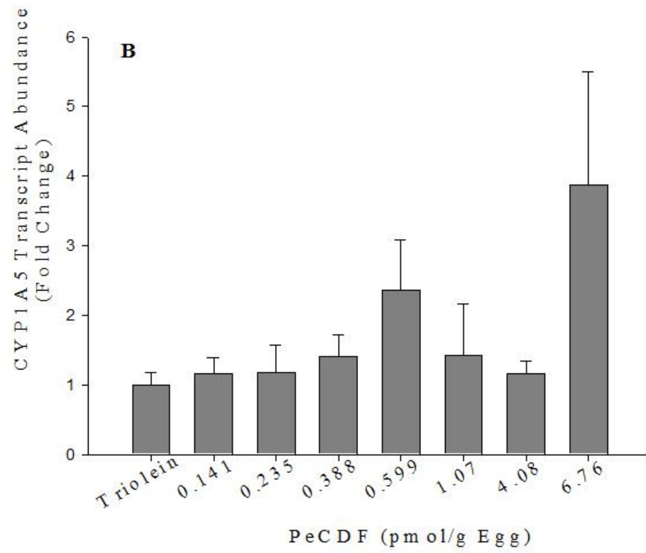

C
CYP1A5 Transcript Abundance
(Fold Change)
0
2
4
6
8
10
12
*
*
*
*
Triolein
0.0742
0.148
0.245
0.516
1.05
1.83
TCDF (pmol/g Egg)

Figure 2-4. Effects of TCDD (A), PeCDF (B) and TCDF (C) on CYP1A5 transcript abundance in the liver of the White-leghorn chicken. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of CYP1A5 mRNA expression in treatment groups relative to control group and bars represent standard error (N = 4-7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

The effects of TCDD, PeCDF and TCDF on CYP1A5 mRNA expression were less in Common pheasant and Japanese quail. No significant changes in Common pheasant hepatic CYP1A5 mRNA expression were observed in either the TCDD (Figure 2-5A) or PeCDF (Figure 2-5B) exposure groups. CYP1A5 mRNA expression was significantly downregulated at 0.170, 0.288 and 4.77 pmol TCDF/g egg (Figure 2-5C). No significant changes in Japanese quail hepatic CYP1A5 mRNA were detected in any of the TCDD (Figure 2-6A), PeCDF (Figure 2-6B) or TCDF (Figure 2-6C) exposure groups at any of the doses used.


A
CYP1A5 Transcript Abundance
(Fold Change)
0
1
2
3
4
5
Triolein
0.0745
0.0994
0.224
0.311
0.82
3.17
6.68
TCDD (pmol/g Egg)


B
CYP1A5 Transcript Abundance
(Fold Change)
0
1
2
3
4
5
6
Triolein
0.141
0.235
0.388
0.599
1.07
4.08
6.76
PeCDF (pmol/g Egg)

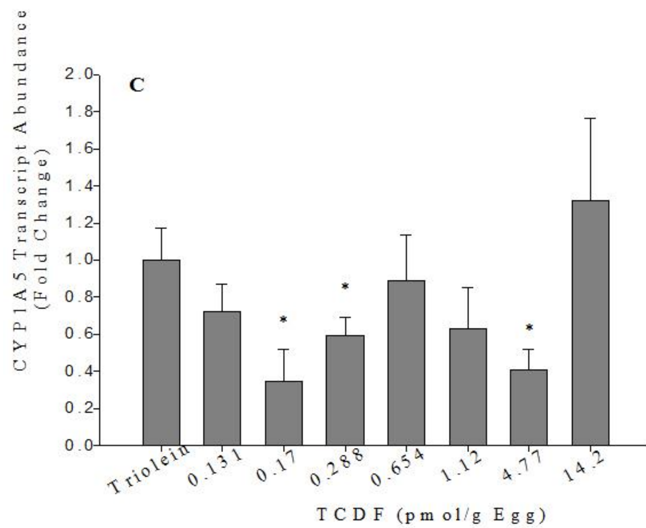
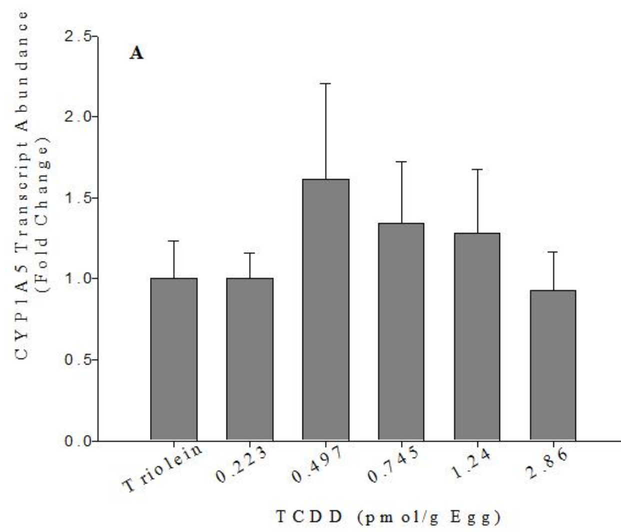
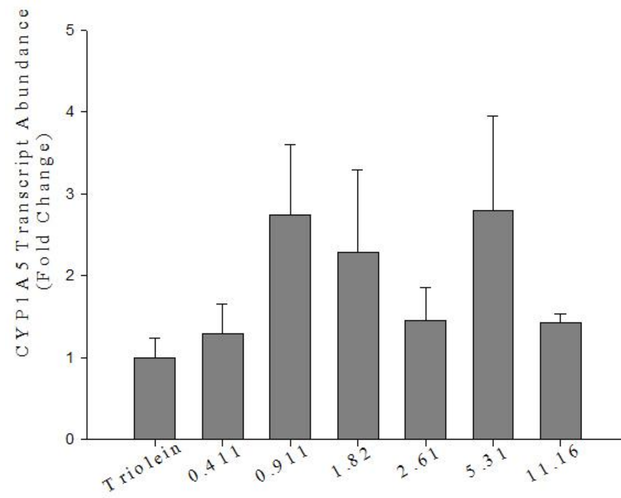

C
CYP1A5 Transcript Abundance
(Fold Change)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
*
*
*
Triolein
0.131
0.17
0.288
0.654
1.12
4.77
14.2
TCDF (pmol/g Egg)

Figure 2-5. Effects of TCDD (A), PeCDF (B) and TCDF (C) on CYP1A5 transcript abundance in the liver of the Common pheasant. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of CYP1A5 mRNA expression in treatment groups relative to control group and bars represent standard error (N = 4-7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).


A
CYP1A5 Transcript Abundance (Fold Change)
2.5
2.0
1.5
1.0
0.5
0.0
Triolein
0.223
0.497
0.745
1.24
2.86
TCDD (pmol/g Egg)


CYP1A5 Transcript Abundance (Fold Change)
5
4
3
2
1
0
Triolein
0.411
0.911
1.82
2.61
5.31
11.16

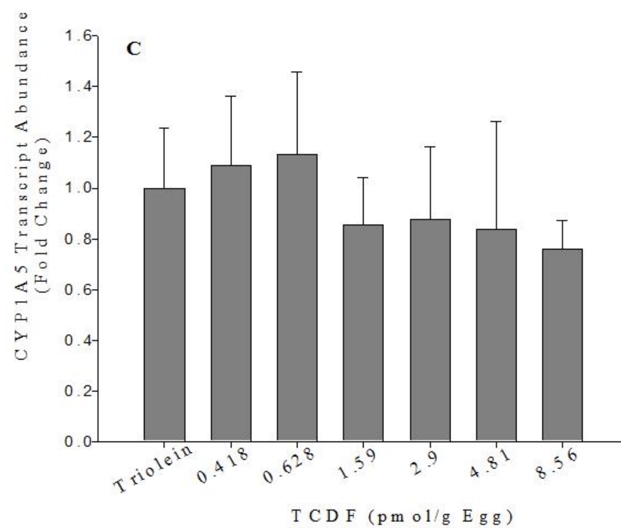
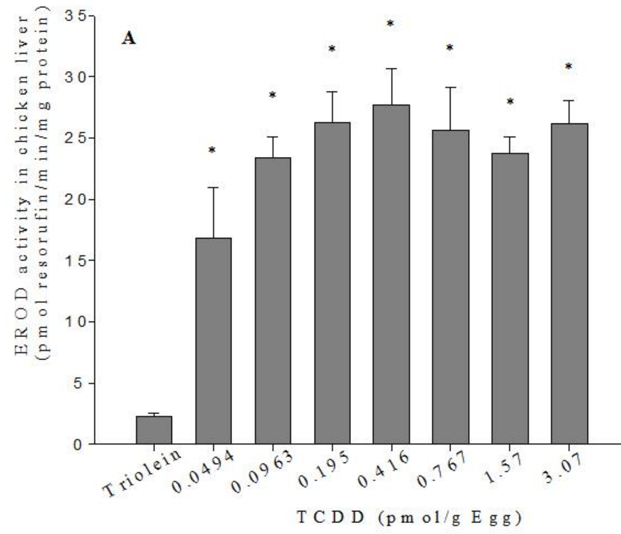
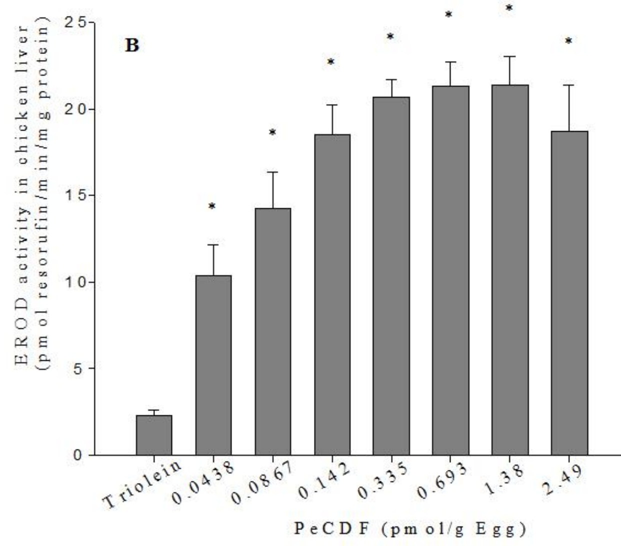

C
CYP1A5 Transcript Abundance (Fold Change)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Triolein
0.418
0.628
1.59
2.9
4.81
8.56
TCDF (pmol/g Egg)

Figure 2-6. Effects of TCDD (A), PeCDF (B) and TCDF (C) on CYP1A5 transcript abundance in the liver of the Japanese quail. Statistical analyses were performed using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U post-hoc test comparing each treatment group with the vehicle control group. Bars represent the percentage of CYP1A5 mRNA expression in treatment groups relative to control group and bars represent standard error (N = 4-7). Significant changes in gene expression are indicated by an asterisk ($p \leq 0.1$).

2.3.4 Chemical-induced effects on hepatic EROD activity

The EROD activity in White-leghorn chicken was significantly greater in each of the TCDD (Figure 2-7A), PeCDF (Figure 2-7B) and TCDF (Figure 2-7C) exposure groups, at all doses of each chemical. The EROD activity was induced to a maximum of approximately 12-fold at 0.416 pmol TCDD/g egg, 9-fold by 2.49 pmol PeCDF/g egg and 13-fold by 0.516 pmol TCDF/g egg.


A
EROD activity in chicken liver (pmol resorufin/min/mg protein)
35
30
25
20
15
10
5
0
Triolein
0.0494
0.0963
0.195
0.416
0.767
1.57
3.07
TCDD (pmol/g Egg)


B
EROD activity in chicken liver (pmol resorufin/min/mg protein)
25
20
15
10
5
0
Triolein
0.0438
0.0867
0.142
0.335
0.693
1.38
2.49
PeCDF (pmol/g Egg)

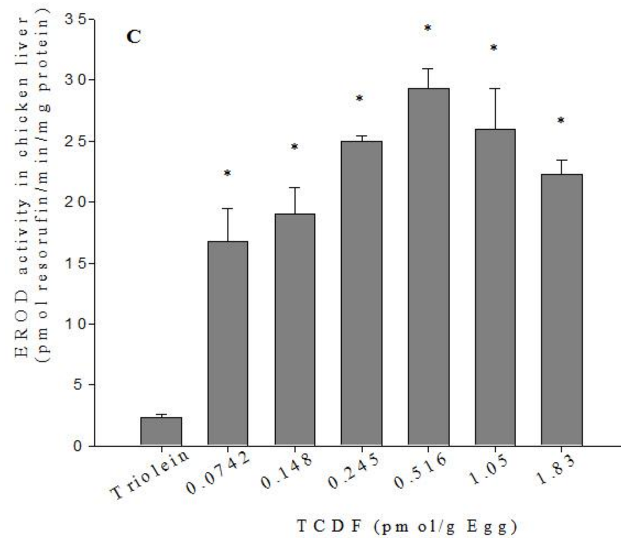
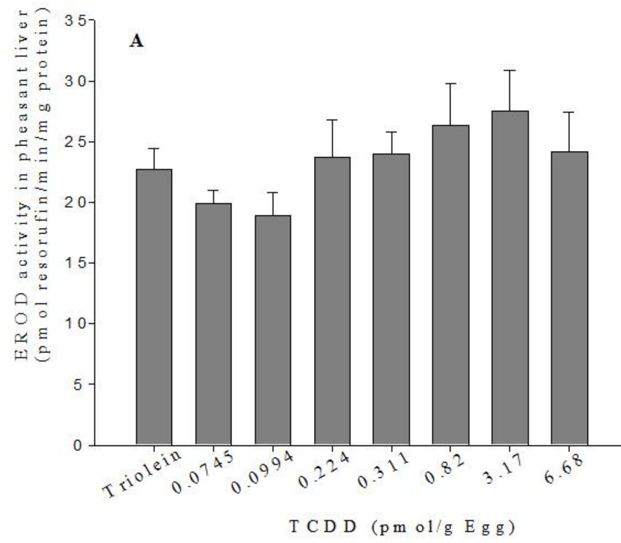
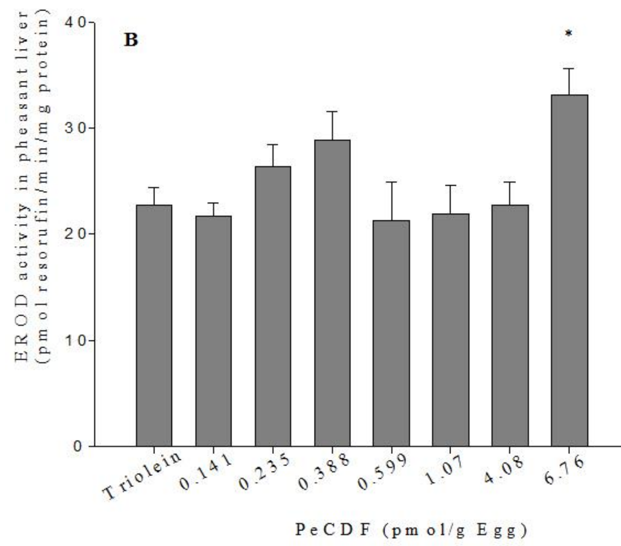

C
EROD activity in chicken liver (pmol resorufin/min/mg protein)
35
30
25
20
15
10
5
0
Triolein
0.0742
0.148
0.245
0.516
1.05
1.83
TCDF (pmol/g Egg)

Figure 2-7. Effects of TCDD (A), PeCDF (B) and TCDF (C) on EROD activity in the liver of the White-leghorn chicken. Statistical analyses were performed using one-way ANOVA followed by Dunnett's post-hoc test. Bars represent the percentage of EROD activity in treatment groups relative to the control group and bars represent standard error ($N = 4-7$). Significant changes in EROD activity are indicated by an asterisk ($p \leq 0.1$).

The EROD activity was not significantly greater in Common pheasant exposed to TCDD (Figure 2-8A). The EROD activity was significantly greater in the 6.76 pmol PeCDF /g egg exposure group, with a maximum response of approximately 1.5-fold (Figure 2-8B). EROD activity was significantly greater in the 0.654 pmol TCDF/g egg group, with the increase being approximately 1.4-fold (Figure 2-8C).


A
EROD activity in pheasant liver (pmol resorufin/min/mg protein)
Triolein
0.0745
0.0994
0.224
0.311
0.82
3.17
6.68
TCDD (pmol/g Egg)


B
EROD activity in pheasant liver (pmol resorufin/min/mg protein)
Triolein
0.141
0.235
0.388
0.599
1.07
4.08
6.76
PeCDF (pmol/g Egg)

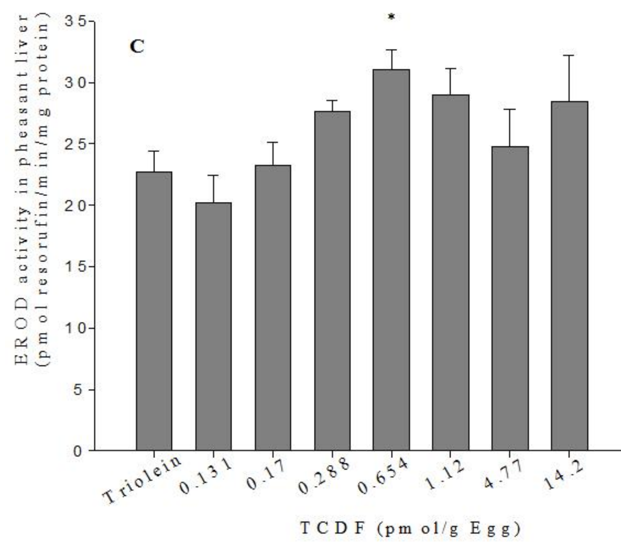
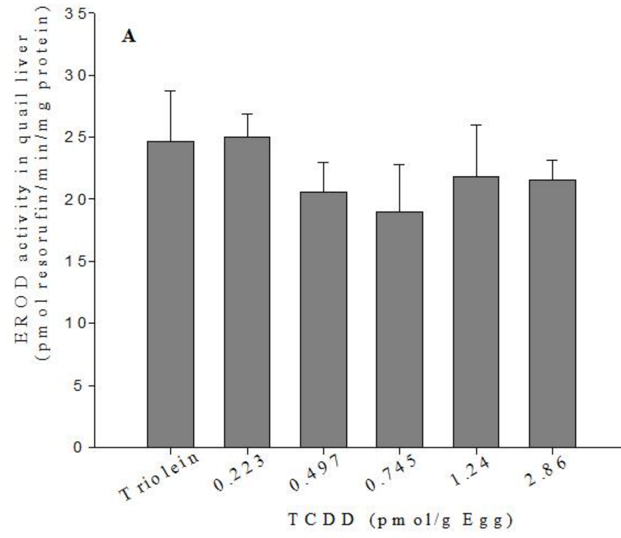
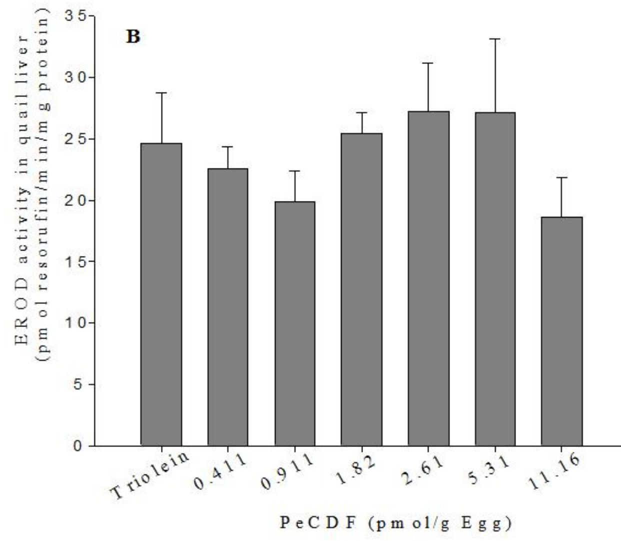

C
EROD activity in pheasant liver (pmol resorufin/min/mg protein)
Triolein
0.131
0.17
0.288
0.654
1.12
4.77
14.2
TCDF (pmol/g Egg)

Figure 2-8. Effects of TCDD (A), PeCDF (B) and TCDF (C) on EROD activity in the liver of the Common pheasant. Statistical analyses were performed using one-way ANOVA followed by Dunnett's post-hoc test. Bars represent the percentage of EROD activity in treatment groups relative to the control group and bars represent standard error (N = 4-7). Significant changes in EROD activity are indicated by an asterisk ($p \leq 0.1$).

Japanese quail EROD activity was not significantly greater in any of the exposure groups. The EROD activity was significantly decreased by 0.745 pmol TCDD/g egg (Figure 2-9A), while neither increases nor decreases were significant in PeCDF exposed groups (Figure 2-9B) or TCDF exposed groups (Figure 2-9C).


A
EROD activity in quail liver (pmol resorufin/min/mg protein)
35
30
25
20
15
10
5
0
Triolein
0.223
0.497
0.745
1.24
2.86
TCDD (pmol/g Egg)


B
EROD activity in quail liver (pmol resorufin/min/mg protein)
35
30
25
20
15
10
5
0
Triolein
0.411
0.911
1.82
2.61
5.31
11.16
PeCDF (pmol/g Egg)

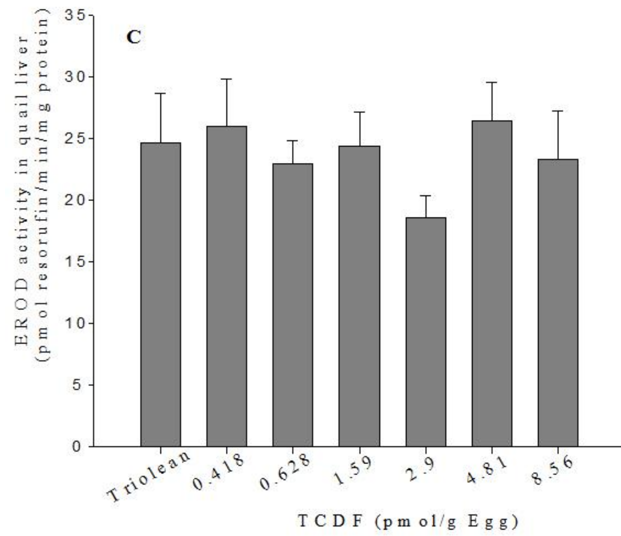

C
EROD activity in quail liver (pmol resorufin/min/mg protein)
35
30
25
20
15
10
5
0
Triolean
0.418
0.628
1.59
2.9
4.81
8.56
TCDF (pmol/g Egg)

Figure 2-9. Effects of TCDD (A), PeCDF (B) and TCDF (C) on EROD activity in the liver of the Japanese quail. Statistical analyses were performed using one-way ANOVA followed by Dunnett's post-hoc test. Bars represent the percentage of EROD activity in treatment groups relative to the control group and bars represent standard error (N = 4-7). Significant changes in EROD activity are indicated by an asterisk ($p \leq 0.1$).

2.3.5 Estimated LOEC values, Relative Sensitivity and Relative Potency

The LOEC values based on *CYP1A4* and *CYP1A5* mRNA abundance and EROD activity are shown (Table 2-3). Since either significantly greater magnitudes of responses of some endpoints were stimulated at the least dose injected, or no significant increase was observed, LOEC values could not be determined for all exposures. Therefore, the LOEC values for these situations are considered to be less than the least doses or greater than the greatest doses, respectively. The relative sensitivity values of each species (Table 2-4) and relative potencies values of each chemical (Table 2-5) were estimated based on the LOEC values. However, where LOEC values could not be established it was not possible to determine ReS or ReP values.

Table 2-3. Estimated LOEC values (pmol/g egg) for significant induction of biomarker responses in chicken, Common pheasant and Japanese quail exposed to TCDD, PeCDF or TCDF.

Biomarker	Chemical	White-leghorn chicken	Common pheasant	Japanese quail
CYP1A4	**TCDD**	≤ 0.0494*	0.466	> 2.86**
	PeCDF	≤ 0.0438*	0.0294	3.08
	TCDF	≤ 0.0742*	0.654	4.90
CYP1A5	**TCDD**	0.0621	> 6.68**	> 2.86**
	PeCDF	0.117	> 6.76**	> 11.16**
	TCDF	0.0980	> 14.2**	> 8.56**
EROD	**TCDD**	≤ 0.0494*	> 6.68**	> 2.86**
	PeCDF	≤ 0.0438*	5.26	> 11.16**
	TCDF	≤ 0.0742*	0.425	> 8.56**

[*] LOEC values were not determined as the lowest dose injected caused a significant increase in the biomarker response. The LOEC was assumed to be less than the lowest injected dose.

[**] LOEC values were not determined as no significant increase in the biomarker response was observed at any of the doses injected. The LOC was assumed to be greater than the highest injected dose.

Table 2-4. Rank-order of relative sensitivity (ReS) and ReS values (in bracket) of White-leghorn chicken (chicken), Common pheasant (pheasant) and Japanese quail (quail) to TCDD, PeCDF and TCDF based on CYP1A biomarker responses.

Response	Compound	Rank Order (ReS)
CYP1A4 mRNA	TCDD	Chicken (?) > Pheasant (?) > Quail (?)
	PeCDF	Pheasant (?) ≥ Chicken (?) > Quail (?) *
	TCDF	Chicken (?) > Pheasant (?) > Quail (?)
CYP1A5 mRNA	TCDD	Chicken (?) > Pheasant (?) ≥ Quail (?) *
	PeCDF	Chicken (?) > Pheasant (?) ≥ Quail (?) *
	TCDF	Chicken (?) > Pheasant (?) ≥ Quail (?) *
EROD	TCDD	Chicken (?) > Pheasant (?) ≥ Quail (?) *
	PeCDF	Chicken (?) > Pheasant (?) > Quail (?)
	TCDF	Chicken (?) > Pheasant (?) > Quail (?)

* Where LOEC values were not calculated because the lack of a significantly increase in the response (see table 3 for rationale) the ReS of one species was reported to be greater than or equal to that of another species. A (?) means no ReS value was calculated because no LOEC value was established.

Table 2-5. Rank-order of Relative potencies (ReP) and ReP values (in bracket) of TCDD, PeCDF and TCDF in White-leghorn chicken, Common pheasant and Japanese quail.

Response	Species	Rank Order (ReP)
CYP1A4 mRNA	White-leghorn chicken	Undetermined
	Common pheasant	PeCDF (15) > TCDD (1) > TCDF (0.7)
	Japanese quail	PeCDF (?) > TCDF (?) ≥ TCDD (?)
CYP1A5 mRNA	White-leghorn chicken	TCDD (1) > TCDF (0.6) > PeCDF (0.5)
	Common pheasant	Undetermined
	Japanese quail	Undetermined
EROD	White-leghorn chicken	Undetermined
	Common pheasant	TCDF (?) > PeCDF (?) > TCDD (?)
	Japanese quail	Undetermined

Values in brackets are the ReP values determined based on LOEC values reported in table 3. A (?) means no ReP value was calculated because no LOEC value was established.

2.4 Discussion

Differential sensitivities and responsiveness based on up-regulation of CYP1A4 and CYP1A5 mRNA expression and greater EROD activity were observed among in post-hatch White-leghorn chicken, Common pheasant and Japanese quail exposed to TCDD, PeCDF, or TCDF via injection into the air cell. The sensitivities of each of these members of the order Galliformes to the effects of dioxin like compounds have been classified based on the amino acid sequences of their AhR LBD. Specifically, chicken is classified in the most sensitive group (Type 1), Common pheasant is classified as moderately sensitive (Type 2), and Japanese quail is classified as insensitive (Type 3) (Head et al., 2008). TCDD, PeCDF and TCDF are full AhR agonists and studies have demonstrated CYP1A responsiveness to these chemicals in a variety of avian species (Kennedy et al., 1996; Lorenzen et al., 1997a; Gilday et al., 1998; Mahajan and Rifkind 1999; Head and Kennedy, 2007a; Hervé et al., 2009). Therefore, RePs based on responses of CYP1A enzymes were determined for the three DLCs within each species and the ReS of each species exposed to each compound.

2.4.1 Interspecies comparisons: Relative Sensitivities

The rank-orders of sensitivity to TCDD and TCDF stimulated induction of CYP1A4 and CYP1A5 based on mRNA expression and EROD activity of White-leghorn chicken > Common pheasant > Japanese quail are in agreement with the sensitivity ranking predicted by classification based on the amino acid sequence of the AhR LBD (Head et al. 2008). The rank-order of ReS to TCDD and TCDF reported here is identical to rank order based on LD_{50} values based on embryolethality (Cohen-Barnhouse et al.,

personal communication). In addition, Hervé et al. (2009) demonstrated the same rank-order of species sensitivity to TCDD and TCDF based on induction of CYP1A4 and CYP1A5 mRNA expression and EROD activity in primary cultures from each of the species studied here. Although actual ReP values could not be determined for CYP1A5 mRNA expression in Common pheasant and Japanese quail it is clear that based upon this endpoint the chicken is more sensitive to the effects of TCDF than either of the other two species.

Since the rank-order was different among the responses, sensitivities of the three species to PeCDF was less clear. For each compound the rank-order for ReS, based on EROD induction of chicken > Common pheasant > Japanese quail, is consistent with the predicted order of Head et al. (2008). The rank-order for ReS, based on CYP1A5 mRNA expression, of White-leghorn chicken > Common pheasant ≥ Japanese quail, suggests that Common pheasant and Japanese quail are approximately equally sensitive to the effects of PeCDF. In contrast, rank-order for ReS, based on induction of CYP1A4 mRNA expression, of Common pheasant ≥ chicken > Japanese quail, suggests that the Common pheasant may be at least as sensitive to the effects of PeCDF as is the chicken. Based on embryolethality LD_{50} values (Cohen-Barnhouse et al., personal communication) also observed greater sensitivity to PeCDF than to TCDD. The rank order based on embryolethality LD_{50} values (Cohen-Barnhouse et al., personal communication) was most similar to that based on CYP1A4 mRNA expression. Based on *in vitro* CYP1A4 and CYP1A5 mRNA expression and EROD induction in primary cell cultures, it has recently been demonstrated that chicken, Common pheasant and Japanese quail are equally sensitivity to PeCDF (Hervé et al., 2009).

2.4.2 Inter-compound Comparisons: Relative Potencies

Current World Health Organization (WHO) avian toxic equivalency factors (TEF) for TCDD, PeCDF, and TCDF are based on a limited number of studies that have been performed in chicken. Based on these studies, each compound has been assigned a TEF of 1 (Van den Berg et al., 1998). Although TCDD is generally accepted as being the most potent AhR agonist the biomarker inducing potency of PeCDF and TCDF relative to TCDD were determined in differentially sensitive avian species. Unfortunately, due to limitations in the volume of carrier that could be injected and the solubilities of the compounds of interest in triolein, it was not possible to accurately determine ReP values for all chemicals and all endpoints. However, the results of this study do suggest that PeCDF and TCDF may be as potent, if not more potent, than TCDD in some avian species. Specifically, in chicken it was observed that each compound is an equipotent inducer of CYP1A5 mRNA expression. Based on induction of EROD activity in liver of Common pheasant, both TCDF and PeCDF are more potent than TCDD. However, induction of EROD activity in TCDF and PeCDF exposed Common pheasant was weak, and was observed in only one exposure group. Based on CYP1A4 mRNA expression PeCDF may be as much as 15-fold more potent than TCDD and 20-fold more potent that TCDF in Common pheasant. Based on LD_{50} observations, the rank-order of ReP values observed for each of these species (chicken: TCDF > TCDD > PeCDF; Common pheasant and Japanese quail: PeCDF > TCDF > TCDD) (Cohen-Barnhouse et al., personal communication), which is consistent with the observation that TCDD may not be the most potent AhR agonist in these species. In addition, greater potency of PeCDF relative to TCDD in primary hepatocytes of Common pheasant has been reported (Hervé

et al. 2009). While those results were based upon induction of CYP1A4 and CYP1A5 mRNA expression *in vitro* as well as EROD activity, clearly there is merit for continued study of the effects of PeCDF on avian species. While it is unclear why the results from these two studies are not completely consistent, differences in the nature of the *in vitro* system used by Hervé et al. (2009) versus the egg injection protocol used here are potential explanations. The observation that PeCDF might be as potent as TCDD in chicken and Japanese quail and more potent than TCDD in Common pheasant is not without precedence. It has been reported that PeCDF was more potent as an inducer of EROD activity than TCDD in primary hepatocytes from double-crested cormorant (*Phalacrocorax auritus*) and Forster's tern (*Sterna forsteri*) (Sanderson et al., 1998). A similar observation has also been reported in green frog (*Rana esculenta*) hepatocytes (Rankouhi et al., 2005).

2.4.3 General Observations

Induction of EROD activity is a routine marker of exposure to dioxins and DLCs, including the chlorinated furans TCDF and PeCDF. Hervè et al. (2009) reported greater EROD activity in hepatocytes of White-leghorn chicken, Common pheasant, and Japanese quail exposed to TCDD, PeCDF, and TCDF than the constitutive expression in unexposed hepatocytes. In the current study, chicken was the only species in which EROD activity was induced by each chemical. It is possible that the lack of induction of EROD activity is related to low AhR activation as both CYP1A4 and CYP1A5 mRNA expression was low in Common pheasant and Japanese quail. Alternatively, basal hepatic EROD activity in Common pheasant and Japanese quail was 10-fold greater than

in chicken. A similar observation was made in wood duck (*Aix sponsa*) where basal hepatic EROD activity in 26-day-old embryos was 3-fold greater than in 19-day-old chicken embryos (Jin et al., 2001). Based on analysis of the AhR LBD wood duck would be classified as an insensitive (type 3) species (Head, 2006). Future studies should include greater doses of TCDD, PeCDF and TCDF in order to determine the ReP of these compounds *in ovo*. However, due to solubilities in triolien and the potential for greater background mortality due to increased volumes of carrier solvents, this will be difficult in the type of *in ovo* exposures conducted here.

Differences in the magnitude of the CYP1A4 and CYP1A5 mRNA expression in each exposure group were observed. Thus, it is important to consider the relative responsiveness as well as relative sensitivities of species. In each species and in response to each compound the maximum fold-change in mRNA expression was greater for CYP1A4 than CYP1A5. This is consistent with results from Hervè et al. (2009) who also demonstrated that CYP1A4 mRNA expression was greater in hepatocytes from chicken, Common pheasant, and Japanese quail exposed to TCDD, PeCDF and TCDF. It has also been reported that CYP1A4 mRNA expression was greater than CYP1A5 mRNA expression in chicken hepatocytes exposed to TCDD (Head and Kennedy 2007a; 2007b). Although transcription of both genes results from activation of the AhR it is possible that that transactivation of CYP1A4 is greater than transactivation of CYP1A5. Alternatively, differences in mRNA stability may account for the observed differences in transcript abundance *in vivo*, although no evidence for such an *in vitro* mechanism was reported by Head and Kennedy (2007a).

In addition to differences between CYP1A4 and CYP1A5 mRNA expression in the same species exposed to the same chemical, differences in the magnitude of mRNA expression were also observed among species exposed to the same compound. Specifically, CYP1A4 mRNA expression was greatest in chicken followed by Common pheasant and then Japanese quail. This observation is consistent with the predicted rank order of sensitivity proposed by Head et al. (2008). Magnitudes of CYP1A5 mRNA expression were also greatest in chicken compared to Common pheasant and Japanese quail, but no difference between maximal mRNA expression in Common pheasant and Japanese quail were observed.

Within each species the magnitude of CYP1A4 mRNA expression in response to each chemical also presents interesting findings. In chicken, CYP1A4 mRNA levels were greatest in the TCDD exposed organisms, followed by PeCDF and TCDF. However, in Common pheasant and Japanese quail, CYP1A4 mRNA levels were greatest in the PeCDF exposed birds. The observation that PeCDF stimulates greater expression of CYP1A4 mRNA than TCDD in Common pheasant is also consistent with the observation that PeCDF is at least as potent as an activator of CYP1A4 mRNA expression as TCDD. These results suggest that responsiveness, as it is related to the magnitude of CYP1A expression, may be related to the sensitivity of the species. If indeed changes in AhR ligand binding domain amino acid sequence of Common pheasant and Japanese quail (Karchner et al., 2006; Head et al., 2008) decrease binding affinity, as suggested by Hervè et al. (2009), then this may also explain decreased CYP1A responsiveness in less sensitive species, such as Common pheasant and Japanese quail.

Results demonstrating the utility of biomarkers of AhR activation in avian species predicted to be differentially sensitive to the effects of dioxins and DLCs were presented. Although each of the compounds studied is known to activate the AhR, each biomarker shows a unique response pattern. Where determination of ReS values was possible the general rank order of sensitivity in the TCDD and TCDF exposed groups was chicken > Common pheasant > Japanese quail. However, based on *CYP1A4* mRNA expression in the PeCDF exposed groups the rank order of sensitivity was Common pheasant $\geq$ chicken > Japanese quail. Of particular interest was the observation that PeCDF is more potent than TCDD in Common pheasant. Based on this observation it appears that further studies are required to address the current TEF values assigned to TCDD, PeCDF and TCDF in avian species.

Finally, the results suggest that for most of the avian species, Q-PCR analysis of CYP1A expression; in particular CYP1A4 mRNA expression may be a more sensitive biomarker of exposure than analysis of EROD induction. Analysis of CYP1A4 mRNA may be particularly beneficial for the analysis of exposure in less sensitive (i.e. Type 2 and Type 3) species. In addition to the increased sensitivity of the Q-PCR approach, Q-PCR detection of mRNA is not inhibited by DLCs. Analysis of EROD activity may underestimate exposure as competition between the substrate (ethoxyresorufin) and the inducer (the DLC) may cause a decrease in EROD activity (Petrulis and Bunce, 1999).

CHAPTER 3
LITERATURE CITED

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APPENDIX
cDNA sequence and deduced amino acid sequences of Japanese quail CYP1A4 (Accession No.: GQ906939) and CYP1A5 (Accession No.: GQ906938)

Figure I. cDNA sequence and deduced amino acid sequences of Japanese quail CYP1A4 (Accession No.: GQ906939) and CYP1A5 (Accession No.: GQ906938). Numbers in both sides correspond to nucleotide position. The start codon (ATG) and stop codon (TAG or TGA) for translation are boxed. The polyadenylation signal in the 3'-untranslated region (UTG) is shown in bold letters and underlined.

CYP1A4

```
                                                              1  ac   2
3       gcggggacctggtgacaggatcgggccctcgtgggacagcagcagcagcagccagaggtt  62
                 M  A  A  G  P  Q  A  V  M  A  Q  V  S  S  S  G  L
63      cacttccagatggcagcggggccgcaggcagtgatggcacaggtgagcagctcaggtctc  122
        I  S  S  T  E  V  L  V  A  A  A  T  F  C  L  L  L  L  L  T
123     atctcatccaccgaggtgctggtggcagctgccactttctgcctgctcctgctgctgacc  182
        Q  T  R  R  Q  N  V  P  K  G  L  R  S  P  P  G  P  R  G  L
183     cagacccgccggcagaatgtacccaaggggctgcgcagcccccccaggaccccgtgggctc  242
        P  L  L  G  N  V  L  E  L  R  K  D  P  H  L  V  L  T  E  M
243     ccactgctgggtaatgtgctggagctgaggaaagacccacacctggtgctcactgagatg  302
        S  R  K  Y  G  D  V  M  E  V  T  I  G  S  R  P  V  V  V  L
303     agccgcaaatacggggatgtgatggaggtgaccatcggctcccggcccgtggtggtgctc  362
        S  G  L  D  T  V  R  Q  A  L  V  R  Q  A  E  D  F  M  G  R
363     agcgggctggacaccgtcaggcaagccttggtgaggcaagcagaagacttcatgggacgc  422
        P  D  L  P  S  F  K  Y  V  S  N  G  H  S  L  A  F  S  Y  E
423     cccgacctgcccagctttaagtatgtctccaatggccacagcctggcattcagctacgaa  482
        C  G  D  A  W  K  A  R  R  K  L  A  Q  N  A  L  K  T  F  S
483     tgcggggatgcgtggaaagcccgcaggaaactggcacagaacgccttgaagaccttctcc  542
        I  A  A  S  P  T  A  S  S  S  C  L  L  E  E  H  V  S  T  E
543     attgccgccagccccactgcctcctccagctgcctcctggaggagcatgtctccactgag  602
        A  S  Y  L  V  T  K  F  L  Q  L  M  E  E  K  Q  T  F  N  P
603     gccagctacctggtcaccaaattcctgcagctgatggaggagaagcaaaccttcaacccc  662
        N  N  Y  L  V  V  S  V  A  N  V  I  C  A  I  C  F  G  K  R
663     aacaactacctggtggtgtcggtggccaatgtcatctgcgccatttgctttggcaagcgc  722
        Y  D  H  D  D  Q  E  L  L  N  V  V  N  M  N  T  E  F  G  D
723     tatgaccatgacgaccaggagctgctcaacgtggtgaacatgaacactgagtttggggat  782
        V  A  A  A  G  N  P  S  D  F  I  P  L  L  R  Y  L  P  N  R
783     gtggctgctgctggcaacccctctgacttcatcccgctgctccggtacctccccaaccgt  842
        A  M  A  T  F  K  D  V  N  T  R  F  D  A  F  I  E  K  I  V
843     gctatggctacttttaaggatgtcaatacccgtttcgatgccttcatagagaaaattgtc  902
        Q  N  H  Y  T  T  F  D  K  E  H  I  R  D  V  T  D  S  L  I
903     cagaaccattacaccacttttgataaggagcacattcgggacgtcacagactcattgatt  962
        G  Q  C  Q  E  K  K  T  G  G  N  V  R  V  Q  P  S  D  K  S
```

```
963   gggcagtgccaggagaagaagacagggggaatgttcgcgtccaaccttctgataagagc 1022
      I  I  S  I  V  N  D  L  F  G  A  G  F  D  T  V  T  T  A  L
1023  atcatctccatcgtcaacgacctctttggggcaggctttgacaccgtgacaactgccctg 1082
      S  W  C  V  M  Y  A  A  L  Y  P  H  I  Q  K  K  I  Q  A  E
1083  tcttggtgcgtgatgtatgctgccttgtacccccacatccagaagaagattcaggcagag 1142
      L  D  Q  I  I  G  R  E  R  R  P  R  L  S  D  R  S  M  L  P
1143  ctggatcagatcattggccgggagaggagaccacgactgtctgaccgaagcatgctgccc 1202
      Y  T  E  A  F  I  L  E  V  F  R  H  S  S  L  L  P  F  T  I
1203  tacacagaagcctttatcctggaggtgttccggcactcttcccttctgcccttcaccatc 1262
      P  H  S  T  T  K  D  T  V  L  N  G  Y  F  I  P  K  N  T  C
1263  ccacatagtacaacaaaagacactgtactgaatggctacttcatccccaagaacacctgc 1322
      V  F  I  N  Q  W  Q  V  N  H  D  E  K  I  W  K  D  P  S  S
1323  gtgttcatcaaccagtggcaagtgaaccacgatgagaagatctggaaggacccctcctcc 1382
      F  N  P  E  R  F  L  N  A  A  G  T  E  I  N  R  T  E  G  D
1383  ttcaatcccgagcgcttcctcaatgcagcaggcaccgaaatcaacaggacagagggtgac 1442
      K  V  V  I  F  G  L  G  K  R  R  C  I  G  E  S  I  G  R  W
1443  aaagtggtgatctttggcctggggaagaggcgttgcatcggggagtccatcgggcgctgg 1502
      E  V  F  L  F  L  T  T  I  L  Q  Q  L  E  I  N  L  A  P  G
1503  gaggtcttcctcttcctgaccaccatcctgcagcagctggagatcaacctggcccctggg 1562
      Q  Q  V  D  I  T  P  Q  Y  G  L  T  M  K  Y  K  Q  C  E  C
1563  cagcaggtggacatcacccctcagtacgggctgaccatgaagtacaagcagtgtgagtgc 1622
      F  Q  M  K  K  R  F  P  T  K  S  S  A  *
1623  ttccagatgaagaaacgcttccccaccaagagctctgcgtgaggagggaggagagagccg 1682
1683  agccttgtggggttgaggactcgcacctcttgcatagagttgctcccaatgcttcctgca 1742
1743  ggcatccacatccctctgccatgctcaggatgagggctctgaaatgactccacacagga 1802
1803  atccctcagcatctacatgctggtgacagcctgactactagaaatatatacttatataag 1862
1923  atgaatgcaaagaggaaccaagcatagagacacagtaaacactctcggcttcaaaaaaa 1982
1983  aaaaaaaaaaa  1993
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CYP1A5

```
1       acgcgggagcagaagagatcctgcttggttcaggggctggatgccctctgcctctctctg  60
                                            M  G  P  E  E  V  M  V
61      ctgatcttcaaggacctgcagttctttcaggtggcaatggggccagaagaagtgatggtg  120
        Q  V  G  S  P  G  L  I  S  S  T  E  V  L  V  A  A  A  T  F
121     caggtgggcagcccaggtctcatctcatccaccgaggtgctggtggcagctgccactttc  180
        C  L  L  L  L  L  T  Q  T  R  R  Q  N  V  P  K  G  L  R  S
181     tgtctgctcctgctgctgacccagacccgccggcagaatgtacccaaggggctgcgcagc  240
        P  P  G  P  H  G  L  P  V  L  G  S  A  L  E  L  R  K  D  T
241     cccccaggaccccatgggctcccagtgctgggcagtgcattggagctgaggaaggacacg  300
        H  L  V  L  T  E  M  S  R  K  Y  G  D  V  M  E  V  T  I  G
301     cacctggtgctcactgagatgagccgcaaatatggggatgtgatggaggtgaccatcggc  360
        S  R  P  V  V  V  L  S  G  L  E  T  I  K  Q  A  L  V  R  Q
361     tcccggcctgtcgtggtgctcagcgggctggaaaccatcaagcaagccttggtgaggcaa  420
        A  E  D  F  M  G  R  P  D  L  Y  S  F  R  H  V  T  D  G  Q
421     gcagaagacttcatgggacgccccgacctctacagcttccgacacgttacggatggacag  480
        S  L  T  F  S  T  D  T  G  E  M  W  K  A  R  R  K  L  A  Q
481     agcctgaccttcagcaccgacacgggggaaatgtggaaagcccgcaggaaactggcacag  540
        N  A  L  K  N  F  S  I  A  A  S  P  T  A  S  S  S  C  L  L
541     aatgccctgaagaacttctccatcgctgccagccccacggcctcctccagctgcctcctg  600
        E  E  H  V  T  N  E  A  S  Y  L  V  T  K  F  L  Q  L  M  E
601     gaggagcacgtcaccaacgaggccagctacctggtcaccaaattcctgcagctgatggag  660
        E  K  Q  S  F  D  P  Y  R  Y  T  V  V  S  V  A  N  V  I  C
661     gagaagcagagctttgacccctatcgctacacggtggtgtcagtggccaacgttatctgt  720
        A  I  C  F  G  K  R  Y  D  H  E  D  Q  E  L  L  N  V  V  N
721     gccatttgctttggcaagcgctacgaccatgaagaccaggagctgctcaacgtggtgaac  780
        V  V  D  E  F  V  N  V  T  A  V  G  N  L  A  D  F  I  P  L
781     gtggtggatgagtttgtgaatgtgactgctgttggcaacctggctgacttcatcccgctg  840
        L  Q  Y  L  P  S  R  N  M  D  L  F  L  D  F  N  K  R  L  M
841     ctccagtacctccccagccgcaacatggatttgtttctggatttcaacaagcggttaatg  900
        K  L  L  Q  A  A  V  D  E  H  Y  K  T  Y  D  K  N  S  I  R
901     aaactgctacaggcagctgtggatgagcactacaagacctatgacaagaacagcatccga  960
        D  V  T  D  S  L  I  E  Q  C  M  E  K  K  A  E  G  S  G  A
961     gacgtcaccgactccctcattgagcagtgcatggagaaaaaagccgaaggcagtggtgcc 1020
        L  Q  I  P  N  E  K  I  I  N  L  V  N  D  I  F  G  A  G  F
1021    cttcagatcccaaatgagaagatcatcaacctggtgaatgacatctttggggcaggcttt 1080
        D  T  V  T  T  A  L  S  W  S  L  M  Y  L  V  T  Y  P  H  I
1081    gacactgtgacaaccgccctgtcctggagcctcatgtacctcgtgacgtacccccacatc 1140
        Q  K  K  I  Q  A  E  L  D  Q  T  I  G  R  E  R  R  P  R  L
1141    cagaagaagattcaggcagagctggatcagaccattggccgggagaggagaccacggctg 1200
        S  D  R  S  M  L  P  Y  T  E  A  F  I  L  E  M  F  R  H  S
1201    tctgaccgaagcatgctgccctacacagaagccttcatcctggagatgttccggcactcc 1260
        S  F  I  P  F  T  I  P  H  S  T  T  R  D  T  V  L  N  G  Y
1261    tccttcatacccttcaccatcccacacagcacaaccagggacacagtgctgaatggctac 1320
        Y  I  P  K  D  R  C  V  F  I  N  Q  W  Q  V  N  H  D  E  K
1321    tatatcccaaaggaccgctgcgtgtttatcaaccagtggcaagtgaaccacgatgagaaa 1380
        L  W  K  D  P  Q  T  F  N  P  E  R  F  L  S  A  E  G  T  E
1381    ctttggaaggatccacagactttcaacccagagcggttcctcagtgctgaagggactgaa 1440
        L  N  K  V  D  A  E  K  V  M  T  F  G  L  G  K  R  R  C  I
1441    ttgaacaaagtggatgcagagaaagtgatgacttttggcttagggaaaaggaggtgcatt 1500
        G  E  N  I  A  R  W  E  V  F  L  F  L  S  T  L  L  Q  Q  L
1501    ggggaaaacatagccaggtgggaggtgttccttttcctgtccacattgctccagcaactg 1560
        E  F  S  I  R  D  G  E  K  A  D  M  T  P  I  Y  G  L  T  M
1561    gagttcagcatccgcgatggtgagaaggcggacatgacgcctatatatggactgaccatg 1620
        K  H  K  R  C  E  H  F  Q  V  K  K  R  F  S  T  K  S  S  N
```

1621 aagcacaagagatgtgagcactttcaagtcaagaaacgcttctccacaaagagctcaaac 1680
      *
1681 taagctattcatccatagcacagacactgccacttgggtgccctgaggtgccctgggtct 1740
1741 ggcataacctcgcagaaccgtgc**aataaa**caaaagctattgaaggtcaaaaaaaaaaaaa 1800
1801 aaaaaaaaaaaaaaaaaa 1818