Prediction of the Sensitivity of Avian Species to the Embryotoxic Effects of Dioxin-Like compounds

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ABSTRACT

The main goal of this thesis was to develop new methods and knowledge that will explain and predict species differences in sensitivity to dioxin-like compounds (DLCs) in birds. The important achievements and results obtained from the four experimental chapters of this thesis are summarized as follow: (1) an efficient luciferase reporter gene (LRG) assay was developed for use with 96-well cell culture plates; (2) the results obtained from LRG assay were shown to be highly correlated to available *in ovo* toxicity data; (3) amino acids at positions 324 and 380 within the aryl hydrocarbon receptor 1 ligand binding domain (AHR1 LBD) were shown to be responsible for reduced Japanese quail *(Coturnix japonica)* AHR1 activity to induce a dioxin-responsive reporter gene in comparison to chicken (*Gallus gallus domesticus*), and ring-necked pheasant *(Phasianus colchicus)* AHR1 in response to different DLCs; (4) AHR1 LBD sequences of 86 avian species were studied and differences at amino acid sites 256, 257, 297, 324, 337 and 380 were identified. It was discovered that only positions 324 and 380 play a role in AHR1 activity to induce a dioxin-responsive gene; (5) in COS-7 cells expressing chicken AHR1, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) are equipotent inducers of the reporter gene and bind with similar affinity to chicken AHR1, however, in the cells expressing pheasant, Japanese quail and common tern (*Sterna hirundo*) AHR1, PeCDF is a stronger inducer than TCDD. PeCDF also binds with higher affinity to pheasant and quail AHR1 than TCDD.

The results of this thesis show that embryo lethal effect of DLCs in avian species can be predicted by use of two new non-lethal methods: (1) the LRG assay and (2) determination of the identity of the amino acids at positions

324 and 380. The findings and methods described in this thesis will be of use for environmental risk assessments of DLCs.

RESUMÉ

Le principal objectif de la présente thèse consistait à élaborer de nouvelles méthodes et connaissances qui expliqueraient et prédiraient les différences entre les espèces d'oiseaux relativement à la vulnérabilité aux composés semblables aux dioxines. Les réalisations et résultats importants obtenus à partir des quatre chapitres expérimentaux de la thèse sont résumés comme suit : 1) un essai efficace sur un gène rapporteur de la luciférase a été conçu pour être utilisé avec des plaques de culture cellulaire à 96 cupules; 2) les résultats de cet essai ont démontré une très forte corrélation aux données sur la toxicité *in ovo* disponibles; 3) il a été démontré que les acides aminés en position 324 et 380 sont responsables de la diminution de l'activité du récepteur 1 d'hydrocarbure d'aryle pour produire un gène rapporteur sensible aux dioxines chez la Caille du Japon (*Coturnix japonica*) par rapport au récepteur 1 d'hydrocarbure d'aryle chez le poulet (*Gallus gallus domesticus*) et le Faisan de Colchide *(Phasianus colchicus)* en réponse à différents composés semblables aux dioxines; 4) les séquences du domaine de liaison aux ligands du récepteur 1 d'hydrocarbure d'aryle de 86 espèces aviaires ont été étudiées et les sites d'acides aminés 256, 257, 297, 324, 337 et 380 ont été recensés. Il a été découvert que seules les positions 324 et 380 jouent un rôle dans l'activité du récepteur 1 d'hydrocarbure d'aryle pour produire un gène rapporteur sensible aux dioxines; 5) dans des cellules COS-7 exprimant le récepteur 1 d'hydrocarbure d'aryle du poulet, la 2,3,7,8-tétrachlorodibenzo-*p*-dioxine et le 2,3,4,7,8-pentachlorodibenzofurane sont des inducteurs équivalents du gène rapporteur et se lient avec une affinité semblable au récepteur 1 d'hydrocarbure d'aryle du poulet; cependant, dans les cellules exprimant le récepteur 1 d'hydrocarbure d'aryle du Faisan, de la Caille du

Japon et de la Sterne pierregarin (*Sterna hirundo*), le 2,3,4,7,8-pentachlorodibenzofurane est un inducteur plus puissant que la 2,3,7,8-tétrachlorodibenzo-*p*-dioxine. Le 2,3,4,7,8-pentachlorodibenzofurane se lie également avec une plus grand affinité au récepteur 1 d'hydrocarbure d'aryle du Faisant et de la Caille que la 2,3,7,8-tétrachlorodibenzo-*p*-dioxine.

Les résultats de la présente thèse démontrent que les effets embryolétaux des composés semblables aux dioxines dans les espèces aviaires peuvent être prédits à l'aide de deux nouvelles méthodes non létales : 1) l'essai sur un gène rapporteur de la luciférase et 2) la détermination de l'identité des acides aminés aux positions 324 et 380. Les résultats et les méthodes décrits dans la présente thèse seront utilisés pour les évaluations des risques environnementaux des composés semblables aux dioxines.

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I would like to dedicate this thesis to the memories of my mother, Marzieh, and my sister, Minoo. I believe that they are always around to support me during the hard times of my life. I dedicate this thesis to my wife, Shirin Tajik and to my sons, Arman and Ryan.

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[^{3}H]TCDD	2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-p-dioxin
Å	Angstrom
aa	amino acid
AHR	aryl hydrocarbon receptor
AHRE	aryl hydrocarbon response element
AIP	AHR-interacting protein
Ala or A	Alanine
ANOVA	analysis of variance
AP	adaptor primer
ARNT	aryl hydrocarbon receptor nuclear translocator
bHLH/PAS	basic helix-loop-helix/PER-ARNT-SIM
B_{max}	maximal binding capacity
BP	base pair
C	Chicken
CALUX	chemically activated luciferase expression
CASTp	computed atlas of surface topography of proteins server
CC	common cormorant
CCPM	corrected counts per minute
cDNA	complementary DNA
CMV	Cytomegalovirus
CYP1A	cytochrome P4501A
CYP1A4	cytochrome P4501A4
CYP1A5	cytochrome P4501A5
DBD	DNA binding domain
DLC	dioxin-like compound
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DSSP	define secondary structure of proteins
EC_{50}	median effective concentration
$EC_{threshold}$ or EC_{thr}	threshold effect concentration
EROD	ethoxyresorufin *O*-deethylase
FBS	fetal bovine serum
FICZ	6-formylindolo [3,2-b] carbazole
Gg	gallus gallus
H	Hours
HAP	hydroxyapatite
Hsp90	heat shock protein 90
IC_{50}	50% inhibitory concentration
ID	inhibitory domain

Ile or I	Isoleucine
IVTT	*in vitro* transcription and translation
JQ or Q	Japanese quail
K_d	dissociation constant
kDa	kilo Dalton
K_{OW}	octanol-water partition coefficient
LBD	ligand binding domain
LD_{50}	lethal dose for 50% of the population
LRG	luciferase reporter gene assay
MIN	Minute
MultiProt	multiple alignment of protein structures
NMR	nuclear magnetic resonance
NOEL	no observable effect level
no-RT	without the reverse transcriptase enzyme
NSB	non-specific binding
NT	non-transfected
NWRC	National Wildlife Research Centre
OHRI	Ottawa Hospital Research Institute
OLDERADO	on-line database ensemble representatives and domains
OLIGO-DT	short sequence of deoxy-thymine nucleotides
PBS	phosphate-buffered saline
PCB	polychlorinated biphenyl
PCB 126	3,3’,4,4’,5-pentachlorobiphenyl
PCDDs	polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
PCR	polymerase chain reaction
PDB	protein data bank
PeCDF	2,3,4,7,8-pentachlorodibenzofuran
PER	period circadian protein
PI	protease inhibitor cocktail
ProSA	protein structure analysis
P-S-T-rich	proline-serine-threonine rich
QPCR	quantitative reverse transcription-polymerase chain reaction
Q-Rich	glutamine-rich
RACE	rapid amplification of cDNA ends
ReP	relative potency
ReS	relative sensitivity
RMSD	root-mean-square deviation
RNP or P	ring-necked pheasant
SB	specific binding

SD	standard
SE	standard error
Ser or S	Serine
SIM	single-minded protein
ss gene	spineless gene
TAD	transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzo-furan
TEF	toxic equivalency factor
TEQ	toxic equivalent or TCDD equivalent
UPL	unprogrammed lysate
Val or V	Valine
WHO	World Health Organization

STATEMENT OF CONTRIBUTIONS

Chapter 2

Overall data interpretation and manuscript writing	Reza Farmahin
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AHR1 expression in COS-7 cells and IVTT	Reza Farmahin Dongmei Wu
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Luciferase reporter gene assays	Reza Farmahin

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Real-Time PCR	Reza Farmahin

CHAPTER 1 : INTRODUCTION

1.1. GENERAL INTRODUCTION AND RATIONALE

Contamination of the environment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and dioxin-like compounds (DLCs) has raised concerns to the public, scientists and regulatory agencies for the past half-century. DLCs accumulate in the food chain, mainly in the fatty tissues of animals, and many DLCs are highly toxic. Although global concentrations of DLCs in biota have declined due to regulations taken by national and international organizations and governments, some areas of the planet are still contaminated with these chemicals. In some DLC-contaminated areas wild species of animals are exposed to relatively high levels of DLCs and risk assessments are required. Such assessments usually involve difficult and contentious arguments among stakeholders (the public, governments and industry) due to differences in perceived risk and damage to the environment.

Risk assessments for wild birds are particularly difficult because (1) the sensitivity of avian species to DLC toxicity ranges over several orders of magnitude, and (2) limited or no toxicity data regarding DLCs are available for most avian species. Toxicity data, obtained from feeding studies or egg injection experiments, require a large number of birds or eggs, and experiments with wild birds are often not possible. Moreover, it is not logical (or ethical) to remove fertile eggs from populations at risk or in decline. To address these problems, the studies described in this thesis were directed toward the establishment of new methods to predict *in ovo* sensitivity to the toxic effects of DLCs for *all* species of birds, from diverse taxa and feeding guilds. The work was built upon important characteristics of the avian aryl hydrocarbon receptor (AHR) discovered by Karchner and colleagues

(Karchner *et al.*, 2006) as well as hundreds of studies on dioxins and the AHR conducted by other scientists.

1.2. DIOXIN-LIKE COMPOUNDS

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are a group of 75 structurally related compounds (congeners) which include TCDD. Based on toxicological effects similar to that of TCDD, a wider group of halogenated aromatic compounds have been recognized as DLCs. These include all PCDDs, all polychlorinated dibenzofurans (PCDFs) and some polychlorinated biphenyl (PCB) congeners (Figure 1.1) (Van den Berg *et al.*, 1998).

1.2.1. History and sources of DLCs

Humans have been producing dioxins and DLCs for about 200 years. The first evidence of unintentional production of dioxins was found in a chemical plant in Lampertheim, Germany, where they were manufacturing washing soda using the LeBlanc process as early as 1827 (Safe, 1998). Generally, dioxins are unwanted by-products created during the manufacture of other chemicals, such as herbicides, disinfectants and wood preservatives. For example, between 1897 and 1915 the Dow Chemical Company produced chlorine in long shallow troughs constructed of tarred lumber. They also used carbon sticks with both the cathode and anode impregnated with paraffin. The use of petroleum products, which include tar and paraffin, provided a source of naturally occurring furans, which could have been chlorinated during the manufacturing process, creating a high volume, high concentration chlorinated furan waste stream (Ritter and Allen, 2008).

The other main source of DLCs in the environment is combustion, such as the incineration of municipal and industrial waste, and the burning of wood

and gasoline. Natural processes like volcanic eruptions and forest fires are also sources of DLCs emission (Freeman and de Tejada, 2002). Dioxin contamination of the environment is largely through airborne dioxins, as they adhere to small particles that can travel long distances in the atmosphere. This means that the Canadian environment is exposed to dioxins and furans created in other countries. Dioxins work their way up the food chain by accumulating in and being stored by body fat (Health Canada, 2008).

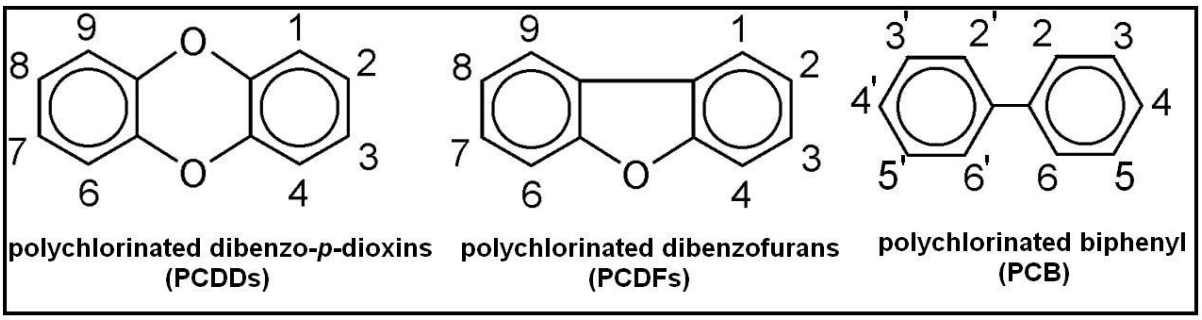

Figure 1.1. Structures of dioxin-like compounds.

1.2.2. Toxic equivalency factors

The potencies and toxic effects of various DLCs are considerably different among avian species; the relative potencies of DLCs compared to the potency of TCDD range from 0.00001 to 1(Van den Berg *et al.*, 1998) and therefore, avian toxic equivalency factors (TEFs) were assigned by the World Health Organization (WHO). The TEF concept was developed to facilitate risk assessments for PCDD, PCDF, and polychlorinated biphenyl (PCB) mixtures by relating the toxicity of these DLCs to the toxicity of TCDD. TEFs were determined for selected congeners of DLCs compared to the reference congener, TCDD, which by definition has a TEF of 1 (Safe, 1990). The

following four criteria are required for the inclusion of a DLC into the TEF concept: (1) a compound must show a structural relationship to PCDD and PCDF; (2) a compound must bind to the AHR; (3) a compound must elicit AHR-mediated biochemical and toxic responses; and, (4) a compound must be persistent and accumulate in the food chain (Van den Berg *et al.*, 1998). There are 75 PCDD, 135 PCDF and 209 PCB congeners in total, of which only 29 (7 PCDDs, 10 PCDFs, 12 PCBs) are currently included in the TEF concept (Van den Berg *et al.*, 1998; Van den Berg *et al.*, 2006).

Using TEF values, the potency of the DLC mixtures can be predicted by calculating the sum of the potency-adjusted doses (to TCDD) of the individual compounds (the TCDD or toxic equivalents, or the TEQ value). The TEQ is calculated by multiplying the concentration of each DLC congener by the corresponding TEF. The concept of TEQ was developed to improve risk assessment and regulatory control. The TEQ concentrations for samples are calculated using the following equation:

$$TEQ = \Sigma_{n1} [PCDD_i \times TEF_i] + \Sigma_{n2} [PCDF_i \times TEF_i] + \Sigma_{n3} [PCB_i \times TEF_i]$$

1.2.2.1. Criticisms of the TEF Concept

The TEF/TEQ approach, which considers the cumulative toxic effects of DLCs in a mixture to be additive, was supported by some experimental evidence (Viluksela *et al.*, 1998; Walker *et al.*, 2005). However, based on receptor theory, compounds which compete for the same receptor site may cause antagonistic effects if mixtures contain partial agonists. Some studies have found an antagonistic effect on AHR activation, which suggests that in some instances the TEQ method overestimates the toxic potency of a mixture

(Haag-Gronlund *et al.*, 1998; Hestermann *et al.*, 2000; Safe, 1998; Schroijen *et al.*, 2004; Van Birgelen *et al.*, 1994). On the other hand, there is evidence that the TEQ method might underestimate toxic potency.

Another major criticism of the TEF approach is the difference in species responsiveness. Studies of the biological and toxicological effects of DLCs have shown that there are extensive and important species differences in the functional responses elicited by these compounds (Lin and Lu, 2001; Peterson *et al.*, 1993; Smart and Daly, 2000).

1.2.3. Environmental levels of DLCs

Although environmental levels of DLCs have declined in many global regions, some areas are still contaminated with high levels of these chemicals in the soil and biota. For example, along the Tittabawassee River in Michigan, USA, relatively high concentrations of 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) were reported in sediments and flood-plain soil (Hilscherova *et al.*, 2003). In the same area, relatively high concentrations of TCDF and PeCDF (84 and 95 ng $TEQ_{WHO\text{-}Avian}$/kg) were reported in belted kingfisher (*Ceryle alcyon*) eggs and nestlings (Seston *et al.*, 2012).

1.3. THE ARYL HYDROCARBON RECEPTOR

About 30 years ago, the AHR was discovered to be a specific binding receptor for TCDD (Poland *et al.*, 1976). Today it is known that the AHR belongs to the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS)[1] super

[1] One of the first members of this super family (cloned one year before the AHR) is the aryl hydrocarbon receptor nuclear translocator (ARNT), which acts as a dimerization partner of the AHR (Hoffman *et al.*, 1991; Poland *et al.*, 1976). Other members are PER (a circadian

family. This is a group of structurally related proteins that are involved in the detection of and adaptation to environmental change (Gu *et al.*, 2000). The AHR is expressed in virtually all the cells in vertebrates, which highlights its importance and diverse functions (Hahn, 2002). Most, if not all, biological responses to DLCs act through a common mechanism that begins with the binding of DLCs to the AHR.

1.3.1. AHR structure

The AHR gene has been duplicated and diversified in vertebrate animals (Hahn, 2002). In contrast to humans and other mammals, which possess a single AHR, some species have multiple isoforms of the AHR (Hahn *et al.*, 2006). In birds, for example, there are at least two AHR isoforms: AHR1 and AHR2 (Hahn, 2002; Yasui *et al.*, 2004). Yasui and colleagues identified AHR2 in the great cormorant and black-footed albatross (Yasui *et al.*, 2004; Yasui *et al.*, 2007).

The N-terminus of the AHR protein contains a basic domain (a stretch of basic residues), followed by helix-loop-helix (HLH) domains. The basic domain is critical for specific AHR–aryl hydrocarbon response element (AHRE)[2] interaction (Schmidt and Bradfield, 1996), and the HLH domain mediates heterodimerization between AHR and ARNT (Fukunaga *et al.*, 1995; Huang *et al.*, 1993). Following the basic-HLH, there are two PAS domains,

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transcription factor) and SIM (the fruit fly *(Drosophila)* "single-minded" protein involved in neuronal development), which shares a highly conserved PAS domain, named after these first three members (PER, ARNT, SIM) (Gu *et al.*, 2000; Kewley *et al.*, 2004).

[2] The target sequence for AHR and ARNT heterodimer in DNA is 5'-**TNGC**GTG-3'. The AHR binds one half-site (indicated by bold) while the ARNT binds the other half-site (indicated by underline) (Bacsi *et al.*, 1995; Swanson *et al.*, 1995). This core consensus is called the aryl hydrocarbon response element (AHRE) (Saatcioglu *et al.*, 1990), the dioxin responsive element (DRE), or the xenobiotic responsive element (XRE) (Whitlock, Jr., 1999) in the literature.

PAS A and PAS B, which were also found in PER, ARNT and SIM, hence the name PAS. The PAS domains contribute to the binding of AHR to Hsp90 and AIP (AHR-interacting protein; an immunophilin-like chaperone) in the cytoplasm and ligand binding and heterodimerization between AHR and ARNT in the nucleus (Hao *et al.*, 2011; Ma and Whitlock, Jr., 1997; Soshilov and Denison, 2008). The ligand binding domain (LBD) of AHR and the PAS region overlap, and the LBD lies within the conserved N-terminal domain (Burbach *et al.*, 1992). The C-terminal region of the receptor comprises the transactivation domain (TAD), which is divided into acidic, glutamine-rich (Q-Rich) and proline-serine-threonine rich (P-S-T-rich) subdomains (Ma *et al.*, 1995; Ramadoss and Perdew, 2005). Between the PAS-B domain and the TAD, there is an inhibitory domain responsible for the suppression of the TA activity of AHR in the absence of an agonist; this region was named the inhibitory domain (ID) (Ma *et al.*, 1995).

1.3.2. Mechanism of action

The AHR binds to heat shock protein 90 (hsp90) and additional chaperone proteins in the cytoplasm. Hsp90 seems to ease ligand binding by maintaining the unliganded receptor in an appropriate conformation (Pongratz *et al.*, 1992). However, AHR ligand binding activity does not absolutely require the presence of receptor-bound hsp90 (Phelan *et al.*, 1998). Activation of AHR by ligand binding causes a series of sequential signaling events, including dissociation of AHR from chaperone proteins, translocation into the nucleus, and dimerization with the aryl hydrocarbon nuclear translocator (ARNT) protein. Heterodimerization of liganded AHR with ARNT in the nucleus enables the dimer to bind to AHRE located in the upstream region of several

dioxin-responsive genes, during which considerable alteration of the chromatin structure at the enhancer occurs (Figure 1.2) (Whitlock *et al.*, 1996). The binding to AHRE results in DNA bending (Kinoshita *et al.*, 2004), and triggers the recruitment of coactivators and chromatin-modifying complexes to open the chromatin for promoter accessibility and recruitment of the general transcription machinery to the gene (Beedanagari *et al.*, 2010). Liganded AHR may also suppress (down-regulate) transcription of some genes (Tijet *et al.*, 2006) through mechanisms that are as yet unclear (Riddick *et al.*, 2004).

1.3.3. AHR role in physiology and toxicology

From the earliest days of AHR research, scientists have searched for answers about the normal function of this receptor, apart from binding to xenobiotics. Our current understanding is that the AHR plays a key regulatory role in various physiological functions including cellular proliferation and migration, angiogenesis, immune regulation, neuronal effects, adaptive metabolism, development, and detoxification in a variety of organs and cell systems. The spineless (ss) gene (the fruit fly homolog of the mammalian AHR) is necessary for development of the distal segments of the antenna and leg (Duncan *et al.*, 1998; Emmons *et al.*, 1999). No specific [^{3}H]TCDD binding has been detected in any invertebrate species (Denison *et al.*, 1985; Hahn *et al.*, 1994). It is proposed that during the evolutionary history of AHR, it has changed from a protein that does not bind ligand (invertebrates), to a protein that is a ligand-activated transcription factor (Hahn *et al.*, 2006).

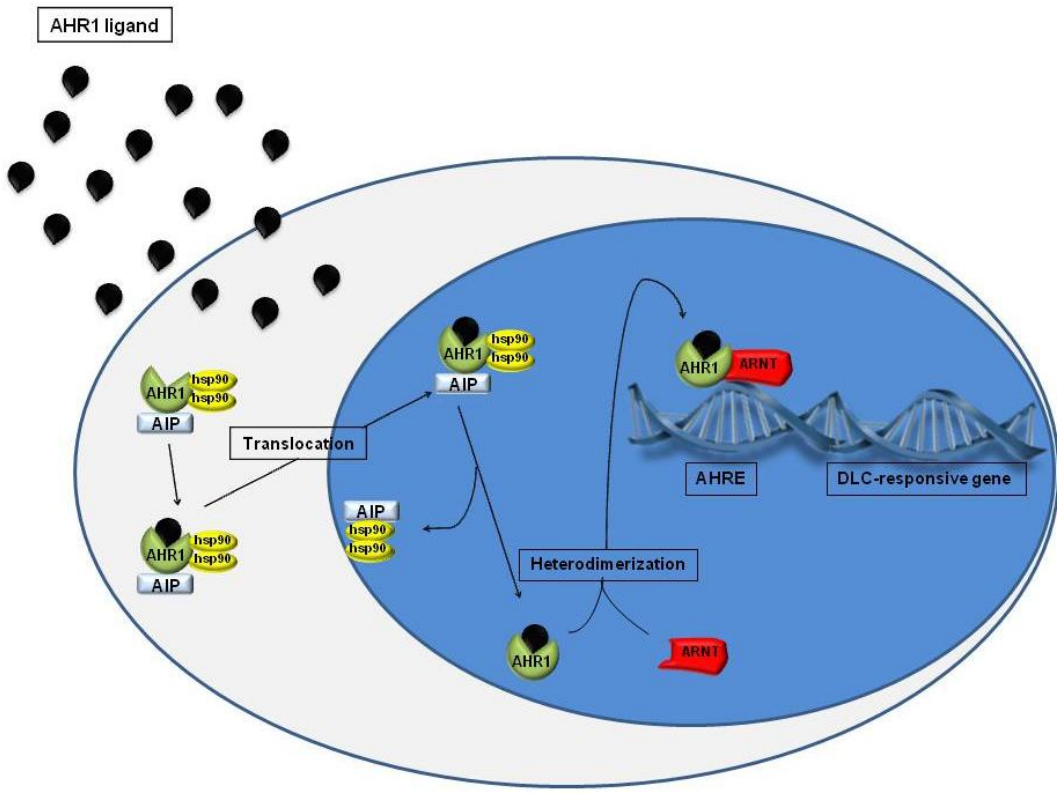


Figure 1.2. The molecular mechanism of activation of gene expression by the AHR.

1.3.3.1. Role of AHR in physiological system

Studies on the response to DLCs in AHR knockdown animals and newly discovered endogenous ligands increase our understanding of key endogenous regulatory roles for the AHR in normal physiology and development.

Several normal processes in tissues and cells are reported to be disrupted, directly or indirectly, by exposure to DLCs. If DLC toxicity is a reflection of dysregulated physiological functions, then the AHR likely has a normal role in these processes. Important processes that are disrupted by DLC exposure include cardiovascular development and function (Aragon *et*

al., 2008; Thackaberry *et al.*, 2005; Walker *et al.*, 1997; Walker and Catron, 2000; Wang *et al.*, 2010), cell differentiation (Vorderstrasse *et al.*, 2004), cell proliferation, death and aging regulation (Ge and Elferink, 1998; Huang and Elferink, 2005; Ray and Swanson, 2009), circadian rhythms (Garrett and Gasiewicz, 2006; Shimba and Watabe, 2009), inflammatory responses (Sibilano *et al.*, 2012; Stevens *et al.*, 2009; Vondracek *et al.*, 2011), palate development (Imura *et al.*, 2010; Takagi *et al.*, 2000), prostatic function (Arima *et al.*, 2010; Fritz *et al.*, 2008; Vezina *et al.*, 2008; Vezina *et al.*, 2010; Vezina *et al.*, 2009), and invasive tumor growth (Wang *et al.*, 2011; Zhang *et al.*, 2012). In several of these processes, some of the most consistent effects of TCDD include dysregulation of cell cycle, cellular proliferation and differentiation, and cell–cell communication. This suggests that the most sensitive tissues to TCDD would be those undergoing differentiation and proliferation, such as in the fetus, or the immune system.

The development of mice lacking the AHR (Lahvis and Bradfield, 1998), constitutively active AHR (McGuire *et al.*, 2001), and conditionally expressed AHR (Walisser *et al.*, 2005) increased our understanding of its physiological functions. Use of these models suggests that the AHR has physiological involvement in the development and function of angiogenesis (Ichihara *et al.*, 2007; Lahvis *et al.*, 2000), cardiovasculature systems (Zhang *et al.*, 2010), hematopoiesis (Gasiewicz *et al.*, 2010), immune system (Kimura *et al.*, 2008; Thurmond *et al.*, 2000; Veldhoen *et al.*, 2008), melanogenesis (Jux *et al.*, 2011), development of female reproductive tissues (Robles *et al.*, 2000), mammary gland development (Hushka *et al.*, 1998), prostate development (Jana *et al.*, 1999), maintenance of pregnancy (Abbott *et al.*, 1999), wound

healing (Carvajal-Gonzalez *et al.*, 2009), tumorigenicity (Mulero-Navarro *et al.*, 2005), and aging (Hirabayashi and Inoue, 2009).

Many studies provide evidence of endogenous ligands for the AHR. For example, several photo-oxidation products of tryptophan, such as 6-formylindolo [3,2-b] carbazole (FICZ), bind to the AHR with very high affinity (Rannug *et al.*, 1987). Several sulfoconjugates of FICZ have been found in human urine (Rannug *et al.*, 1987) and could indicate that the endogenous tryptophan derivatives are AHR ligands. A study has also provided evidence of the potential ability for tryptophan photoproducts to modulate light-dependent regulation of the circadian rhythm through the triggering of AHR signaling (Mukai and Tischkau, 2007). Indoleamine-2,3-dioxygenase (a major pathway of tryptophan metabolism, especially in inflammation and in dendritic cells) is activated by TCDD, and its products, kynurenine and kynurenic acid, can activate AHR (DiNatale *et al.*, 2010; Mezrich *et al.*, 2010; Moroni *et al.*, 2012). Arachidonic acid derivatives (Aboutabl *et al.*, 2009; Seidel *et al.*, 2001) are another class of endogenous substances that bind the AHR. Tissue concentrations of these compounds can be as high as the mM range, and many of them are metabolized by cytochrome P450[3] isozymes regulated by the AHR, and are involved in inflammatory responses (Chiaro *et al.*, 2008; Nebert and Karp, 2008). Based on many studies on the AHR that have provided convincing evidence of the existence of endogenous ligands, it is likely that one or more of these compounds will prove to be physiological ligands for the AHR.

[3] Cytochrome P450 enzymes are mixed function monooxygenases located on the smooth endoplasmic reticulum membrane (Murray and Reidy, 1990) that are capable of transforming lipophilic compounds into more soluble derivatives. There are many different members of the CYP family, and they are responsible for oxidative metabolism (Phase I) of a wide number of compounds. Among the various families of cytochrome P450s, CYP1, CYP2, and CYP3 are involved in the majority of xenobiotic metabolism.

1.3.3.2. Role of AHR in DLC toxicity

AHR-deficient mice are highly resistant to TCDD toxicity, and it has been suggested that most of the pathological changes induced by TCDD are mediated entirely by the AHR (Fernandez-Salguero *et al.*, 1996). The AHR is involved in AHR-mediated DLC toxicity in two different ways: (1) induction of metabolizing enzymes, which results in the production of toxic metabolites, and (2) aberrant changes in global gene transcription beyond those observed in the AHR gene battery.

The AHR mediates induction of a large and varied group of important Phase I and Phase II enzymes. CYP1A genes are probably the most well-known AHR-induced genes. The CYP1 enzymes are responsible for both metabolically activating and detoxifying numerous DLCs (Nebert *et al.*, 2004). For some congeners, the metabolism results in detoxification; CYP1A oxidizes the contaminant to a more polar derivative that is eliminated from the body. Other congeners, particularly highly-chlorinated dioxins, are extremely resistant to metabolism (Hu and Bunce, 1999). In such cases, the metabolites are not eliminated and can continue to regulate transcription of dioxin-responsive genes. CYP1A induction can be associated with the generation of reactive oxygen species, carcinogens and toxic metabolites (Nebert *et al.*, 2004; Schlezinger *et al.*, 2000). In avian species, CYP1A4 catalyses EROD and aryl hydrocarbon hydroxylase activity (Gilday *et al.*, 1996; Rifkind *et al.*, 1994) and CYP1A5 preferentially catalyzes the oxidation of uroporphyrinogen to uroporphyrin. Uroporphyrinogen oxidation has an important role in the uroporphyria caused by DLCs (Gilday *et al.*, 1996; Sinclair *et al.*, 1997), and uroporphyrin concentration and EROD activity are used as biomarkers of DLC

exposure in avian tissues (Head and Kennedy, 2010; Herve *et al.*, 2010b; Herve *et al.*, 2010c; Kennedy *et al.*, 1993; Kennedy *et al.*, 2003). A positive correlation was reported between the expression level of hepatic CYP1A enzyme and DLC concentrations in yolk sacs of bald eagles *(Haliaeetus leucocephalus)* (Elliott *et al.*, 1996a). Similar findings were reported for great blue herons (*Ardea herodias*) (Bellward *et al.*, 1990), double-crested cormorants (*Phalacrocorax auritus*) (Sanderson *et al.*, 1994), black-crowned night herons (*Nycticorax nycticorax*) (Rattner *et al.*, 1994) and ospreys (*Pandion haliaetus*) (Elliott *et al.*, 2001).

More recently, global changes in gene expression studies have been conducted, and have led to the identification of novel adverse effects of TCDD exposure in cellular processes and functions. Several gene expression studies showed that hundreds of genes are up- or down-regulated by TCDD exposure, which confirmed the complicated actions of this compound (Frueh *et al.*, 2001; Kurachi *et al.*, 2002; Puga *et al.*, 2000). The diversity of transcriptional responses makes it difficult to identify specific genes responsible for lethality and other major forms of dioxin toxicity. Results of a study of rats exposed to TCDD suggest that hepatic toxicity is likely not caused by dysregulation of a single gene. However, hepatic toxicity is due to the alteration of multiple genes, possibly under coordinate control of the AHR, which disrupt pathways, such as energy or lipid metabolism (Moffat *et al.*, 2010).

1.3.4. AHR and differential sensitivity to DLCs

The AHR structure contributes to the differential sensitivity to DLCs in several animal models. In 1976, a 10-fold difference was reported between two strains of mice (non-responsive DBA/2 mouse, and responsive C57BL/6

mouse) in CYP1A induction, lethality and teratogenicity following TCDD exposure (Poland *et al.*, 1976). This difference in dioxin sensitivity was later attributed to a single nucleotide polymorphism at position 375 (the equivalent position of amino acid residue 380 in chicken) in the AHR LBD (Poland and Knutson, 1982; Poland *et al.*, 1994; Ema *et al.*, 1994). Several other studies reported the importance of this amino acid in vertebrates such as the common tern (*Sterna hirundo*), mice, rat and human (Backlund and Ingelman-Sundberg, 2004; Ema *et al.*, 1994; Karchner *et al.*, 2006; Murray *et al.*, 2005; Pandini *et al.*, 2007; Pandini *et al.*, 2009; Poland *et al.*, 1994; Ramadoss and Perdew, 2004). It is also shown that the amino acid at position 319 (equivalent to 324 in chicken) plays an important role in the transactivation ability of AHR and the ability to bind to a ligand, due to its involvement in LBD cavity volume and its steric effect (Pandini *et al.*, 2009). Mutation at position 319 in mouse eliminated AHR DNA binding (Pandini *et al.*, 2009). The importance of amino acid at position 324 (equivalent to 319 in mice) was also shown in the common tern (Karchner *et al.*, 2006).

1.3.4.1. AHR1 structure and avian sensitivity to DLC

Birds express at least two AHR paralogs, designated AHR1 and AHR2 (Hahn, 2002). AHR1 is more transcriptionally active than AHR2 (Yasui *et al.*, 2007) and the basal level of expression of AHR1 is several-fold greater than that of AHR2 in the liver of birds (Mol *et al.*, 2012; Yasui *et al.*, 2007).

Available data suggests that chickens are more susceptible to the toxic and biochemical effects of DLCs than other avian species. Egg injection studies showed some species to be between 10- and 1000-fold less sensitive to the embryotoxic effects of DLCs than chickens. For example, while the

0.15-0.3 µg/kg TEQ concentration caused 50% mortality (LD50) in chicken embryos (Powell *et al.*, 1996), herring gulls (average egg concentration of DLCs is 0.07-1 µg/kg TEQ; Jermyn-Gee *et al*., 2005) were shown to have high reproductive success in the Great Lakes region. The first study that attempted to elucidate the role of avian AHR1 domains and key amino acids within avian AHR1 in avian differential sensitivity was performed in 2006. Karchner and colleagues (2006), using chimeric AHR1 constructs and combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive to DLC toxicity) and common tern (low sensitive to DLC toxicity), showed that the LBD is responsible for differences in TCDD sensitivity between the chicken and common tern. More specifically, they showed that the identities of amino acids at sites 324 and 380 within the LBD contributed to the differential sensitivity of chicken (Ile324_Ser380) and tern (Val324_Ala380) AHR1 exposed to TCDD (Karchner *et al.*, 2006). This finding was the basis of the hypothesis that birds can be classified into three main groups for risk assessment purposes, based on the identities of these two amino acids: high sensitivity (type 1; Ile324_Ser380), moderate sensitivity (type 2; Ile324_Val380) and low sensitivity (type 3; Val324_Ala380) (Head *et al.*, 2008; Karchner *et al.*, 2006).

1.4. TOXICOKINETICS OF DLCS

1.4.1. Absorption

The three routes of dioxin exposure are ingestion, skin contact and inhalation. The uptake of PCDDs from dermal and pulmonary absorption is far more limited than orally (Diliberto *et al.*, 1993; Nessel *et al.*, 1992).

Absorption of TCDD when orally administered to rats was reported to be 88% (Diliberto *et al.*, 1996). Similarly, the absorption of orally administered TCDF and PeCDF in oily vehicles in rat, hamster and guinea-pig was 70-90% (Birnbaum *et al.*, 1980; Brewster *et al.*, 1987; Koga *et al.*, 1989). In human, the absorption of digested TCDD in adults was 87% (Poiger and Schlatter, 1986) and absorption of PCDD/F congeners in breast-fed infants was 90% (McLachlan, 1993). A study examined DLC bioavailability using chickens exposed through their diet to soil contaminated with all 2,3,7,8-substituted PCDD/PCDFs and showed that bioavailability was chlorination-dependent ranging from 80% for tetrachlorinated to less than 10% for octachlorinated congeners (Stephens *et al.*, 1995). The bioavailability of orally ingested [^{3}H]TCDD in adult female ring-necked pheasant varied based on the matrix of the diet (Nosek *et al.*, 1992). The greatest and lowest bioavailability of [^{3}H]TCDD occurred from a suspension of crickets (58%) and earthworms (30%). This difference suggests a longer retention time of insects in the pheasant gastrointestinal tract leading to greater bioavailability. Similarly, [^{3}H]TCDD bioavailability for European starlings *(Sturnus vulgaris)* orally exposed to suspensions of earthworms (14%) and hard-bodied invertebrates (44%) was reported (Martin *et al.*, 1989).

Dermal permeation of TCDD is strongly dose-dependent in rats (Banks *et al.*, 1990), but the bioavailability after dermal exposure is likely less than 1% (Van den Berg *et al.*, 1994). Dermal uptake of different DLCs is generally slow, though it depends on the vehicle (Jackson *et al.*, 1993); dermal uptake has a good inverse correlation with the octanol-water partition coefficient (KOW) of DLCs (Banks and Birnbaum, 1991; Brewster *et al.*, 1989).

1.4.2. Distribution

The distribution of a compound after absorption is initially regulated by its binding to components in blood, and its ability to diffuse through blood vessels and tissue membranes (Lakshmanan *et al.*, 1986). Highly lipohilic dioxins are expected to distribute in adipose tissue or other fat rich tissues. It is shown that the pattern of TCDD tissue distribution is dose-dependent. For example, a study found that the liver to fat concentration ratio was 0.21 in the lowest dose group, and 3.4 in the highest dose group (Diliberto *et al.*, 2001). This result led to the hypothesis that TCDD was sequestered by an inducible hepatic protein, which later proved to be CYP1A2. This hypothesis was supported by the results of a study of CYP1A2 knockout mice, which showed that even in the high dose treatment, the liver/fat ratio was approximately 0.2 (Diliberto *et al.*, 1997). Overall, lipid solubility played a key role in the disposition of TCDD at low doses, but was strongly affected by hepatic binding to CYP1A2.

1.4.3. Metabolism

Metabolism plays a major role in regulating the elimination of DLCs. It has been shown that CYP1A1 is responsible for TCDD metabolism (Olson *et al.*, 1994), and not the CYP1A2 protein (Tai *et al.*, 1993). The human metabolic rate and liver clearance of TCDD are both extremely low (Wendling *et al.*, 1990). It is reported that two TCDD metabolites, 2,3,7-trichloro-8-hydroxydibenzo-*p*-dioxin and 1,3,7,8-tetrachloro-2-hydroxydibenzo-*p*-dioxin, accounted for approximately 40% of the total TCDD eliminated in a man exposed to high levels of TCDD (Sorg *et al.*, 2009). Oxidized metabolites were identified, and oxidation of PCDDs and PCDFs preferentially occurs at position

2, 3, 7 or 8 (Poiger *et al.*, 1982). Sulfur-containing metabolites have also been identified as minor metabolites, with the S substitution preferentially at the 4 position (Kuroki *et al.*, 1989). In a study where adult pheasants were administrated [^{3}H]TCDD, only parent compound and no metabolites were found after 128 days (Nosek *et al.*, 1992).

1.4.4. Elimination

Dioxins are eliminated very slowly, and the rate of PCDD and PCDF elimination is highly dependent on the position of the chlorine atoms. The slowest elimination rates in all the laboratory species studied was seen in congeners with a 2,3,7,8-chlorine substitution pattern (Poiger *et al.*, 1982). Excretion in almost all the studied species takes place through bile and feces, in the form of hydroxylated or conjugated metabolites (Van den Berg *et al.*, 1994). Feces were found to be the major excretion route in mice exposed to TCDD (Gasiewicz *et al.*, 1983). Guinea-pigs, unlike other rodents, eliminate TCDD more than its polar metabolites. Unchanged TCDD in feces is passively diffused into the intestinal lumen from blood (Olson, 1986). Biliary excretion rates of PCDDs, PCDFs, and PCBs in bald eagle were estimated as 0.015-0.02% per day (Kumar *et al.*, 2002). The half-life of TCDD in rat is reportedly in the range of 17 to 31 days, depending on the strain and experimental conditions. Most studies with rodents have found similar elimination rates for TCDD from the liver and adipose tissue (Abraham *et al.*, 1988; Allen *et al.*, 1975; Fries and Marrow, 1975; Piper *et al.*, 1973; Rose *et al.*, 1976). Overall, the half-life of TCDD in human is estimated to be 7 to 11 years. However, it seems that the TCDD half-life varies, depending on its tissue concentration. For example, the half-life calculated from one study with a human volunteer

ingesting a single dose of [^{3}H]TCDD was reported to be six years (Poiger and Schlatter, 1986), while the half-life of TCDD in Victor Yushchenko (Ukrainian president who was poisoned with a high dose of dioxin) was reported to be 15.4 months (Sorg *et al.*, 2009). The half-life for TCDD elimination in different bird species is variable (Martin *et al.*, 1989; Mineau *et al.*, 1984). The half-lives of [^{3}H]TCDD in ring-necked pheasant hatchling and adult hen (that were not producing eggs) were 13 and 378 days (Nosek *et al.*, 1992). A half-life of 100 days for whole body elimination was estimated for TCDD in herring gulls (Braune and Norstrom, 1989). European starling elimination was shown to be faster than herring gull (half-life=7.2 days) (Martin *et al.*, 1989).

1.5. DLC TOXICITY IN BIRDS

One of the most important signs of bird toxicity to DLCs is edema that occurs prior to death. This toxic syndrome has been referred to as 'chick edema disease', due to the diagnostic importance of the bloated appearance it causes in chicks. Chick edema manifests clinically by drooping, dyspnea and death. At necropsy, there are large accumulations of fluid in body cavities, and swollen liver and kidneys. An outbreak of chick edema disease, caused by a toxic fat with high levels of PCDD that was added to the birds' food, was a serious economic problem for the American poultry industry in the mid-1950s (Cantrell *et al.*, 1969).

Other signs of DLC toxicity were described for the Great Lakes Embryo Mortality, Edema, and Deformities Syndrome (GLEMEDS). This occurred in fish-eating birds and followed the onset of high levels of DLCs in the Great Lakes in the 1960s. GLEMEDS was characterized by an incidence of embryonic and chick mortality, growth retardation, and developmental

abnormalities, such as bill deformities, club feet, missing eyes and defective feathering. At necropsy, there was subcutaneous, percardial and peritoneal edema, liver enlargement, liver necrosis and porphyria (Giesy *et al.*, 1994b; Gilbertson *et al.*, 1991).

Various avian species were administered DLCs and the common toxic effects of DLCs were determined to be: mortality, weight loss, edema, hemorrhage, hatchability and tissue necrosis (Bruggeman *et al.*, 2003; Hoffman *et al.*, 1996; Hoffman *et al.*, 1998; Tumasonis *et al.*, 1973). Reproductive effects of dietary exposure to DLCs are also common. For example, the offspring of chickens exposed to Aroclor 1254 in drinking water exhibited poor hatching success, deformities and liver abnormalities (Tumasonis *et al.*, 1973). Other studies reported decreased sperm numbers (Bird *et al.*, 1983) and the laying of smaller clutches with reduced hatching success (Fernie *et al.*, 2001) in American kestrels treated with PCBs. There is also evidence showing that the brains of great blue heron hatchlings, collected from colonies highly contaminated with PCDD and PCDF, exhibited a high frequency of intercerebral asymmetry. The asymmetry was significantly correlated with the level of TCDD and TCDD-TEQs in eggs taken from the same nest (Henshel *et al.*, 1997).

1.6. THESIS OVERVIEW

1.6.1. Rationale

High levels of DLCs in the Great Lakes of North America contributed to the population decline of some species of wild birds in the 1960s (Giesy *et al.*, 1994a; Gilbertson *et al.*, 1991), and adverse health effects of DLCs on birds have been reported in many studies (Bruggeman *et al.*, 2003; Hoffman *et al.*,

1996; Hoffman *et al.*, 1998; Tumasonis *et al.*, 1973). Regulation of DLCs has resulted in decreases in contamination in many areas in comparison to the past several decades (U.S.EPA, 2006). However, these chemicals continue to have harmful effects on birds at some DLC-contaminated sites (Clark *et al.*, 2009; Franceschini *et al.*, 2008; Fredricks *et al.*, 2011b). Available toxicity data for DLCs in birds are largely derived from studies conducted with the domestic chicken, which may not be an appropriate surrogate for most avian species because chickens are far more sensitive to DLC toxicity than most other avian species. Insufficient toxicity data for various species of birds causes difficulty for risk assessments (U.S.EPA, 2001). Available *in ovo* and *in vitro* methods to generate toxicity data typically require a large number of fertilized eggs. However, to obtain and use fertilized eggs of wild (or rare) species for toxicity studies is neither possible nor ethical. Recently, it was shown that the LBD (not DBD or TAD), and more specifically the amino acid identities at position 324 and 380 in the AHR1 LBD, were associated with differences between chicken and tern sensitivity to TCDD (Karchner *et al.*, 2006). This formed the basis of the hypothesis that birds can be classified into different groups of sensitivity to DLCs for risk assessment purposes, based on the identities of these two amino acids within the AHR1 LBD (Head *et al.*, 2008; Karchner *et al.*, 2006).

The main goal of this thesis was to develop methods and knowledge that allow us to predict the sensitivity of any bird to DLCs by using a luciferase reporter gene (LRG) assay and/or the amino acid sequence of the AHR1 LBD. This research expanded our understanding of the molecular structure of avian AHR1 and the mechanisms underlying differential avian sensitivity to DLC. The research will provide a link between molecular characteristics of

AHRs in birds and existing *in vivo* and *in vitro* data on DLC toxicity. The data generated through this thesis will be of use to risk assessors because it will facilitate the determination of avian sensitivity to DLCs in an ecosystem.

1.6.2. Specific research hypotheses and objectives

The main goal of my PhD research was to develop new methods and knowledge that will explain and predict species differences in sensitivity to DLCs in birds. The specific hypotheses and objectives for the four experimental chapters are as follows.

Hypothesis: The LRG assay can be used as an alternative to the Ethoxyresorufin-O-deethylase (EROD) assay to predict avian *in ovo* (egg injection) sensitivity to DLCs.

Objective: Determine EC_{50} values for selected DLCs in COS-7 cells expressing wild-type avian AHR1 using the LRG assay and validate the predictability of the results obtained from this assay by comparing them to available LD_{50} data from egg injection studies.

Hypothesis: The major determinant of the sensitivity of avian AHR1 to DLCs resides within the LBD and not the DBD or TAD.

Objective: Compare the linear regression equations and correlation coefficients obtained between (1) *in ovo* LD_{50} and LRG EC_{50} data for COS-7 cells expressing wild-type avian AHR1; and (2) *in ovo* LD_{50} and LRG EC_{50} data for COS-7 cells expressing chicken mutant AHR1 (chicken DBD- one of twelve different types of avian LBD-chicken TAD).

Hypothesis: The sensitivity of birds to DLCs is determined by the amino acids at sites 324 and 380 in the AHR1 LBD.

Objective: (1) alteration of amino acids at sites 324 and 380 in Japanese quail AHR1 and chicken AHR1 and determine the ability of mutant constructs to induce a CYP1A reporter gene in the LRG assay to quantify the impact of the exchanged amino acid on AHR1 activity; (2) perform homology modeling to study whether the side chains of these residues contribute to the binding cavity surface.

Hypothesis: The amino acid residues at sites 256, 257, 297 and 337 in avian AHR1 do not play a role in the determination of species sensitivity to DLCs.

Objective: (1) alteration of amino acids at site 256, 257, 297 and 337 in chicken AHR1 and determine the ability of mutant constructs to induce a CYP1A reporter gene in the LRG assay to elucidate the impact of the exchanged amino acid on AHR1 activity; (2) to perform homology modeling to study whether the side chains of these residues contribute to the binding cavity surface.

Hypothesis: TCDD and PeCDF are equipotent inducers of the reporter gene in cells expressing chicken, pheasant, Japanese quail and common tern AHR1.

Objective: Obtain concentration response curves for TCDD and PeCDF and compare the relative potency of these chemical to induce a CYP1A5 reporter gene in COS-7 cell expressing chicken, pheasant, Japanese quail and common tern AHR1.

Hypothesis: The differences in PeCDF relative potency among avian species is due to differences in its binding affinity to AHR1 in each species.

Objective: Measure the binding affinity of PeCDF-AHR1 and TCDD-AHR1 using the modified cell-based binding assay and the hydroxyapatite (HAP) binding assay.

Hypothesis: Two amino acids at positions 324 and 380 contribute to the differences in PeCDF potency among species.

Objective: Alteration of amino acids at position 324 and 380 in chicken and Japanese quail AHR1 and determine the impact of exchanged amino acids on relative potency of PeCDF to induce a CYP1A5 reporter gene in COS-7 cells expressing wild and mutant AHR1 constructs in the LRG assay.

Hypothesis: The European Starling and chicken are equally sensitive to DLCs.

Objective: Measure induction of CYP1A4/5 mRNA in primary hepatocyte cultures of both species exposed to TCDD, PeCDF and TCDF.

CHAPTER 2 : SEQUENCE AND *IN VITRO* FUNCTION OF CHICKEN, RING-NECKED PHEASANT AND JAPANESE QUAIL AHR1 PREDICT *IN VIVO* SENSITIVITY TO DIOXINS

(Modified from Farmahin, *et al*., 2012. *Environ. Sci. Technol.* **46**(5), 2967-2975)

2.1. ABSTRACT

There are large differences in sensitivity to the toxic and biochemical effects of dioxins and dioxin-like compounds (DLCs) among vertebrates. Karchner *et al.* (2006) showed that the difference in sensitivity between domestic chicken (*Gallus gallus domesticus)* and common tern (*Sterna hirundo)* to aryl hydrocarbon receptor 1 (AHR1)-dependent changes in gene expression following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is based upon the identities of the amino acids at two sites within the ligand binding domain of AHR1 (chicken – highly sensitive; Ile324_Ser380 vs. common tern – 250-fold less sensitive than chicken; Val325_Ala381). Here, I tested the hypotheses that (*i*) the sensitivity of other avian species to TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) is also determined by the amino acids at sites that are equivalent to sites 324 and 380 in chicken, and (*ii*) Ile324_Ala380 and Val324_Ser380 genotypes confer intermediate sensitivity to DLCs in birds. I compared ligand-induced transactivation function of full-length AHR1s from chicken, common tern, ring-necked pheasant (*Phasianus colchicus*; Ile324_Ala380) and Japanese quail (*Coturnix japonica*; Val324_Ala380) and three Japanese quail AHR1 mutants. The results support the hypothesis that avian species can be grouped into three general classes of sensitivity to DLCs. Both

AHR1 genotype and *in vitro* transactivation assays predict *in vivo* sensitivity. Contrary to the assumption that TCDD is the most potent DLC, PeCDF was more potent than TCDD at activating Japanese quail (13- to 26-fold) and common tern (23- to 30-fold) AHR1. The results support and expand previous *in vitro* and *in vivo* work that demonstrated ligand-dependent species differences in AHR1 affinity. The findings and methods will be of use for DLC risk assessments.

2.2. INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs) and coplanar polychlorinated biphenyls (PCBs) are chemicals collectively referred to as dioxins or dioxin-like compounds (DLCs) due to their structural similarities and common mechanisms of biochemical and toxic action. There are large difference in sensitivity to DLCs among species and strains of animals. For approximately 35 years there has been interest in discovering the fundamental mechanisms that account for differences in sensitivity among species. In my opinion, an understanding of the reasons for differential sensitivity to DLC toxicity among species will lead to development of better methods for environmental and human health risk assessments of this class of environmental contaminants.

There is considerable evidence that most, if not all, toxic effects of DLCs are initiated by activation of the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor, and member of the basic-helix-loop-helix (bHLH)-PAS family of genes. AHR-deficient mice are highly resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxicity (Fernandez-Salguero *et al.*, 1996) and earlier studies with mouse strains (Poland *et al.*, 1994), mice (Goryo *et al.*,

2007), rats (Boutros *et al.*, 2011) and birds (Head *et al.*, 2008; Karchner *et al.*, 2006) indicate that the molecular properties of the AHR itself and AHR-mediated gene expression provide insight into mechanisms underlying differential sensitivity to DLCs (Okey, 2007). Some of the largest differences in sensitivity to DLCs occur among species of birds, with the domestic chicken (*Gallus gallus domesticus*) being considerably more sensitive than other avian species to several AHR-mediated effects, including induction of cytochrome P4501A (CYP1A), hepatotoxicity, edema and embryolethality (Head *et al.*, 2008).

There are at least two isoforms of the AHR (AHR1 and AHR2) in birds. Expression of AHR1 was reported to be several-fold greater than that of AHR2 in avian liver (Mol *et al.*, 2012; Yasui *et al.*, 2007) and AHR1 appears to be more transcriptionally active than AHR2 (Yasui *et al.*, 2007). Karchner and colleagues discovered that the identities of two amino acids within the ligand-binding domain (LBD) of AHR1 (Ile324 and Ser380 in chicken; Val325 and Ala381 in common tern [*Sterna hirundo*]) confer differences in sensitivity to TCDD between the more sensitive chicken and the relatively insensitive common tern (Karchner *et al.*, 2006). Using site-directed mutagenesis, the results of that study showed that replacing Val and Ala in the common tern AHR1 with the corresponding chicken amino acids imparted high-affinity binding of TCDD to the tern AHR1. In addition, a reporter gene assay that used cells transfected with wild-type or mutant AHR1 constructs confirmed the importance of the identities of amino acids at sites 324/325 and 380/381 for AHR1 function. Subsequently, the AHR1 LBDs of several avian species were sequenced (Head *et al.*, 2008). Based on these sequences, and data from *in vitro* and egg injection studies, the authors

hypothesized that birds could be grouped into three general classes of DLC sensitivity: type 1 (chicken-like; Ile324_Ser380), type 2 (ring-necked pheasant [*Phasianus colchicus*]-like; Ile324_Ala380) or type 3 (Japanese quail [*Coturnix japonica*]-like; Val324_Ala380).

Here I report the results of *in vitro* studies that were designed to examine, in greater detail, the role of amino acids at LBD sites 324/325 and 380/381. An efficient reporter gene assay was developed for measuring AHR1-dependent gene expression in monkey kidney cells (the COS-7 line, which has low endogenous AHR1 expression) that were transiently transfected with seven different avian AHR1 constructs and exposed to TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) or 2,3,7,8-tetrachlordibenzofuran (TCDF). The AHR1 constructs were (a) full-length chicken, ring-necked pheasant, Japanese quail or common tern AHR1 or (b) three mutants of Japanese quail AHR1 that contained amino acid mutations at sites 324 and/or 380. Comparison of the results obtained with the reporter gene assay with results from a recently published *in ovo* study of toxicity support the hypothesis that the genotype of the AHR1 LBD is predictive of sensitivity to DLCs in birds. The present work provides further evidence that the classification of birds into groups based on sensitivity to DLCs would be of considerable use for environmental risk assessments.

2.3. MATERIALS AND METHODS

2.3.1. Cloning and sequence analysis of AHR1 cDNA

The methods for cloning and sequence analysis of ring-necked pheasant and Japanese quail AHR1 cDNA are indicated below; the methods for chicken

and common tern AHR1 cloning and sequencing are described elsewhere (Karchner *et al.*, 2006).

2.3.1.1. Total RNA and mRNA isolation

Liver of ring-necked pheasant and Japanese quail were obtained from birds bred and housed at Couvoir Simetin (Mirabel, QC, Canada). Immediately after the birds were euthanized, livers were collected, frozen in liquid nitrogen, and stored at -80˚C. Total RNA was extracted using RNeasy kits (Qiagen, Mississauga, ON, Canada) and mRNA was isolated by use of Oligotex kits (Qiagen) according to procedures provided by the manufacturer. Approximately 0.5 mg total RNA was used to obtain the concentrated mRNA solutions used subsequently.

2.3.1.2. cDNA synthesis

Single-stranded cDNA was synthesized from mRNA (1 µg) by use of a modified lock-docking oligo (dT) primer that contained two degenerate nucleotides at the 3' end. These nucleotides position the primer at the beginning of the poly A+ tail, thus eliminating the 3' heterogeneity inherent with conventional oligo (dT) priming. Double-stranded cDNA for the AHR1 of ring-necked pheasant and Japanese quail was synthesized by use of Marathon cDNA amplification kits (Clontech, Foster City, CA, USA). Following double-stranded cDNA synthesis, blunt ends were created and Marathon adaptors were ligated to both ends of the double-stranded cDNA.

2.3.1.3. 3' and 5' rapid amplification of cDNA ends (RACE)

3' and 5' RACE procedures were performed using oligonucleotide primers (Table 2.1) designed to recognize pheasant and Japanese quail sequences coupled with adaptor primers (AP1 or AP2). PCR products were run on 1.2% agarose gels, followed by band excision and purification with Nucleotrap gel extraction kits (Clontech).

2.3.1.4. Ligation, transformation, sequencing and phylogenetic analysis

All DNA fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into JM109 competent cells. Plasmid DNA was purified with QIAprep spin miniprep kits (Qiagen) and sequenced on an Applied Biosystems 3730 DNA Analyzer at the Ontario Hospital Research Institute (Ottawa, ON, Canada). Reported sequences indicate consensus between at least three different colonies at the nucleotide level. Sequences were analyzed using Sequencher 4.8 (Gene Code Corporation, Ann Arbor, MI, USA) and aligned using ClustalW. The tree was constructed using the minimum evolution criterion in PAUP*4.0 with the neighbour-joining tree as the starting tree. Only the N-terminal half of the protein (~400 amino acids) was used because the alignment is less certain in the C-terminal half.

2.3.2. Expression constructs

Full-length chicken, ring-necked pheasant, and Japanese quail AHR1 cDNAs were amplified with Pfu Ultra High-Fidelity DNA polymerase (Stratagene, La Jolla, California, USA) using CS-forward and CS-reverse primers (Table 2.1).

For chicken AHR1 amplification, an AHR1 construct (pcDNA-GgAHR)(Karchner *et al.*, 2006) was used as the template. At the 5'-end of the forward primer, a CACC 5'-overhang and the Kozak consensus sequence (CACCATGA) were added to ensure directional ligation into the pENTR/D-TOPO vector (Invitrogen, Burlington, ON, Canada) and efficient initiation of translation, respectively. At the 3'-end of the reverse primer, the TGA stop codon was removed to allow downstream V5 epitope expression. PCR products were run on 1.2% agarose gels and bands were excised. Bands were purified with the Nucleotrap gel extraction kit, ligated into pENTR/D-TOPO vectors and subcloned into pcDNA 3.2/V5-DEST vectors (Invitrogen). Chicken, ring-necked pheasant and Japanese quail AHR1 constructs were sequenced to ensure accuracy. Other constructs used were: common tern AHR1 (pcDNA-ShAHR; Karchner *et al.* 2006), *Renilla* luciferase vector (phRL-CMV; Promega), CYP1A5 reporter vector (GL4-ccCYP1A5) and cormorant ARNT1 (pcDNA-ccArnt1). GL4-ccCYP1A5 and pcDNA-ccArnt1 were kindly provided by Dr. Hisato Iwata, Ehime University, Japan (Lee *et al.*, 2009; Yasui *et al.*, 2007).

2.3.3. Site-directed mutagenesis of Japanese quail AHR1

Three mutant Japanese quail AHR1 constructs were prepared using site-directed mutagenesis to change the amino acid residues at sites 324 and 380 (schematic shown in Figure 2.1); the mutant forms were V324I_A380S (chicken-like mutant), A380S (unknown mutant; to my knowledge, no avian species sequenced to date have this genotype) and V324I (pheasant-like mutant). Primer sequences are indicated in Table 2.1; all mutations were constructed using Pfu

Ultra High-Fidelity DNA polymerase. PCR products were treated with *DpnI* endonuclease (New England Biolabs, Beverly, MA, USA) and then transformed to MAX Efficiency DH5α Competent Cells (Invitrogen). Full-length sequencing of each of the three mutant constructs was conducted to ensure that each contained the desired sequence.

2.3.4. AHR1 expression in COS-7 cells

2.3.4.1. Preparation of TCDD, PeCDF and TCDF solutions

A detailed description of the preparation of TCDD, PeCDF and TCDF solutions is provided elsewhere (Herve *et al.*, 2010b). In brief, serial dilutions of TCDD, PeCDF and TCDF were prepared from stock solutions in dimethyl sulfoxide (DMSO) and concentrations were determined by isotope dilution following EPA method 1613 (U.S.EPA, 1994) by high-resolution gas chromatography high-resolution mass spectrometry.

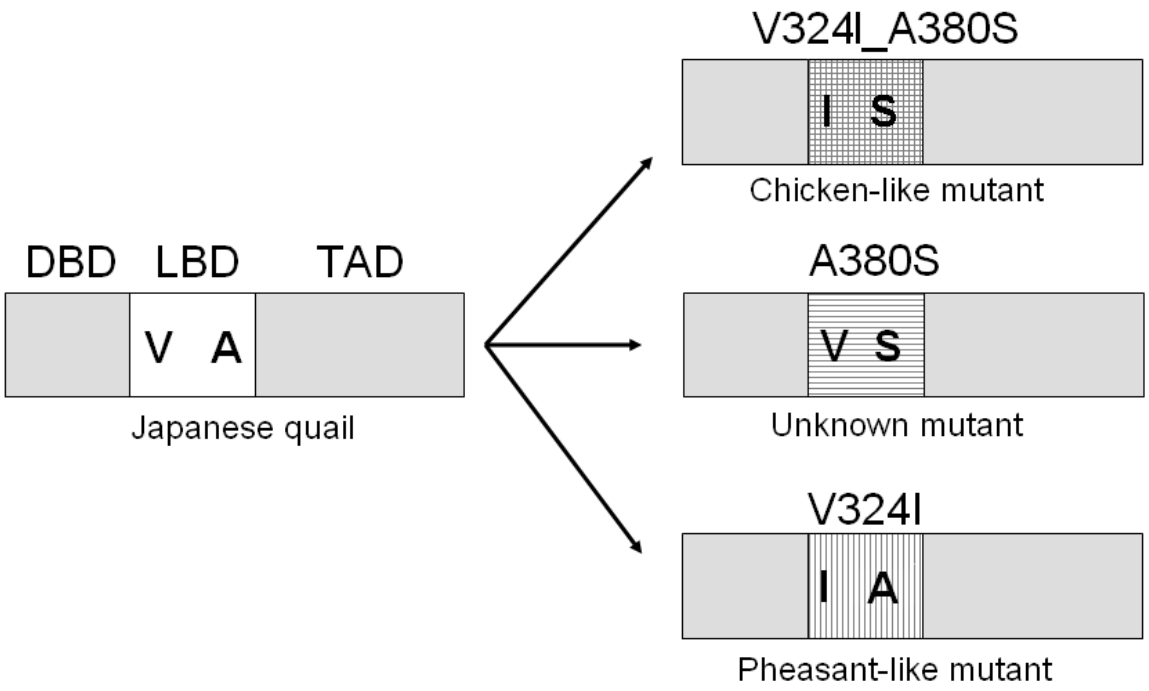


Figure 2.1. Schematic illustration of the three mutant AHR1 constructs that were prepared by site-directed mutagenesis to change V324 and/or A380 within the ligand binding domain (LBD) of Japanese quail AHR1 to produce the chicken-like mutant (V324I_A380S), the unknown mutant (A380S) or the pheasant-like mutant (V324I). The three mutants contained both the Japanese quail DNA-binding domain (DBD) and transactivation domain (TAD).

Table 2.1. PCR conditions for 3' and 5' RACE, amplification of full-length avian AHR1, and site-directed mutagenesis were: a) 94° C (30 s), 5 cycles of 94° C (5 s) and 74°C (4 min), 5 cycles of 94° C (5 s) and 72°C (4 min), and 25 cycles of 94° C (5 s) and 70°C (4 min); b) 94°C (45 s), 35 cycles of 94°C (45 s), 60°C (45 s) and 72°C (3 min), and a final elongation at 72°C (30 min); c) 95˚C (1 min), 21 cycles of 95˚C (30sec), 55˚C (1 min), and 68˚C (18 min). *AP1 and *AP2 are commercially available primers from the Marathon cDNA amplification kit (Clontech, Foster City, CA, USA). Bold fonts in the site-directed mutagenesis primer sequences indicate the nucleotides that cause mutations in the Japanese quail AHR1.

3' and 5' RACE	Pheasant-forward	CTACGGAAGCGTAACATGAAATTGC
	Pheasant-reverse	GACAAGGCGTGCATTTGCCTGTACC
	Quail-forward	CAACCACCTTCCATCCTTGAGATACGG
	Quail-reverse	GCTTCCGCAGATGTTCTGCTCCTTCTT
	AP1*	CCATCCTAATACGACTCACTATAGGGC
	AP2*	ACTCACTATAGGGCTCGAGCGGC
Amplification of full length avian AHR1	CS-forward	CACCATGAACCCCAATGTCACCTAC
	CS-reverse	CATAAATCCACTAGATGCCAAATCTGG
Site-directed mutagenesis	V324I-forward	AACAGGATACCAGTTC**A**TTCATGCAGCTGATATG
	V324I-reverse	CATATCAGCTGCATGAA**T**GAACTGGTATCCTGTTC
	A380S-forward	GACCGGATTACATCATT**T**CCACGCAAAGACCTCTTAC
	380S-reverse	GTAAGAGGTCTTTGCGTGG**A**AATGATGTAATCCGGTC

2.3.4.2. Cell culture, transfection and luciferase assay

COS-7 cells were provided by Dr. R. Haché (University of Ottawa, Ottawa, ON, Canada) and were maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS; Wisent, St. Bruno, QC, Canada), 1% MEM non-essential amino acids (Invitrogen) and 1% penicillin-streptomycin (Invitrogen; 10,000 unit/ml penicillin, 10,000 µg/ml streptomycin). Cells were plated at a concentration of 10,000 cells/well in 96-well plates in dextran-coated and charcoal-treated DMEM (Invitrogen) supplemented with 10% charcoal stripped FBS and 1x penicillin-streptomycin (Invitrogen). Transfection was carried out 18h after plating. DNA and Fugene 6 transfection reagent (Roche, Laval, QC, Canada) were diluted in Opti-MEM (Invitrogen). A total of approximately 50 ng of DNA was complexed with 0.15 µl of Fugene 6 transfection reagent and the mixture (5 µl) was then added to each well. A *Renilla* luciferase vector *(*phRL-CMV*)*, carrying the cytomegalovirus *(*CMV*)* promoter, was used as a transfection control. The ratio of firefly luciferase luminescence units to *Renilla* luciferase luminescence units is defined throughout this study as the *luciferase ratio*. The amounts of transfected expression vectors were 8 ng of chicken, ring-necked pheasant, Japanese quail, common tern or mutated avian AHR1, 1.55 ng of cormorant ARNT1, 5 ng of CYP1A5 reporter construct and 0.75 ng of *Renilla* luciferase vector. The total amount of transfected DNA was kept constant at 50 ng by adding salmon sperm DNA (Invitrogen). For each 96-well plate, COS-7 cells were transfected with (a) one of the four wild-type constructs (chicken, ring-necked pheasant, Japanese quail or common tern) or one of the three mutant Japanese quail constructs, (b) the ARNT construct, (c)

the CYP1A5 reporter construct and (d) *Renilla* luciferase vector. Cells were dosed 5 h after transfection with DMSO or DMSO solutions of TCDD, PeCDF or TCDF at 0.05% final DMSO concentration. Cells were removed from the incubator 18–20 h after dosing to measure luciferase activity. Luminescence was measured using Dual-Glo luciferase assay kits (Promega) in a LuminoSkan Ascent luminometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3.4.3. AHR1 protein expression

COS-7 cells were plated in 6-well plates and transfected with wild-type or mutant avian AHR1 using Fugene 6 (Roche). Forty-eight hours after transfection, cells were lysed in IPH buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EDTA, 2 mM dithiothreitol and protease inhibitor cocktail; Roche). Protein concentration was determined using Bradford reagent (Bio-Rad, Mississauga, ON, Canada). Cell lysates (30 µg aliquots) were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Invitrogen). Anti-V5-HRP antibody (Invitrogen) was used at 1:5000 dilution for detecting V5-AHR1. Anti-β-actin-peroxidase (Sigma-Aldrich, Oakville, ON, Canada) was used at 1:25000 as a loading control. The blots were then visualized by enhanced chemiluminescence.

2.3.5. Concentration-response curves

Duplicate concentration-response curves were obtained for each DLC and each AHR1 construct, and the duplicate curves are referred to as Study 1 and Study 2. For each 96-well plate, the luciferase ratios obtained from four replicate

DMSO-treated wells and four wells treated with each concentration of TCDD, PeCDF or TCDF were determined. The mean luciferase ratio obtained for DMSO-treated wells was subtracted from the luciferase ratio values obtained for DLC-treated wells. GraphPad (GraphPad Prism 5.0 software; San Diego, CA, USA) was used for curve fitting and data were fit to a four parameter logistic model (Head and Kennedy, 2007b).

2.3.6. Relative sensitivity (inter-species comparisons)

The slopes and maximum responses (efficacies) of the concentration-response curves (luciferase ratio vs. log DLC concentration) were not usually identical among the AHR1 constructs, and relative sensitivity (ReS) values were determined at three points (EC_{20}, EC_{50} and EC_{80}) on the concentration-response curves. Because the chicken is more sensitive than most, if not all, avian species to the toxic and biochemical effects of potent DLCs, ReS values for wild-type and mutant AHR1 constructs were expressed relative to the sensitivity observed in cells containing the chicken AHR1 construct. For example, the ReS for the 50% response (ReS_{50}) of Japanese quail AHR1 was: chicken TCDD EC_{50} (0.21nM) ÷ quail TCDD EC_{50} (21nM) = 0.01. The general types of concentration-response curves obtained and the locations of EC_{20}, EC_{50} and EC_{80} values are shown in Figure 2.2.

2.3.7. Relative potency (inter-compound comparisons)

The systematic framework proposed by Villeneuve *et al.* (2000) was used to obtain ReP values. For each construct, the luciferase ratio data were first

converted to the percent maximum luciferase ratio elicited by TCDD (% $TCDD_{max}$). The normalized luciferase ratio data were then plotted against log DLC concentration and curves were fitted using a four parameter logistic equation(Head and Kennedy, 2007b). Three points ($Y_{20, 50, 80}$), which corresponded to $EC_{20, 50 \text{ and } 80}$ values that were observed for TCDD, were used to calculate ReP values for PeCDF and TCDF. For example, the PeCDF ReP_{50} value for the Japanese quail construct was: quail TCDD EC_{50} ÷ quail PeCDF Y_{50}. The general types of concentration-response curves obtained and the locations of EC_{20}, $EC_{50,}$ EC_{80}, Y_{20}, Y_{50} and Y_{80} values are shown in Figure 2.3.

2.3.8. *In vitro* transcription and translation (IVTT) of avian AHR1

FluoroTect $Green_{Lys}$ tRNA-labeled AHR1 proteins for chicken, ring-necked pheasant, Japanese quail and mutant (V324I, A380S and V324I_A380S) constructs were synthesized by use of TnT Quick-Coupled Reticulocyte Lysate Systems kits (Promega) following the manufacturer's instructions. SDS-PAGE was performed using 20 µl of fluorescently-labelled TnT reaction products and 10% SDS polyacrylamide gels. A Typhoon 9210 Imager (Molecular Dynamics Inc, Sunnyvale, CA, USA) was used to detect the fluorescent bands (excitation: 532 nm; emission: 580 nm).

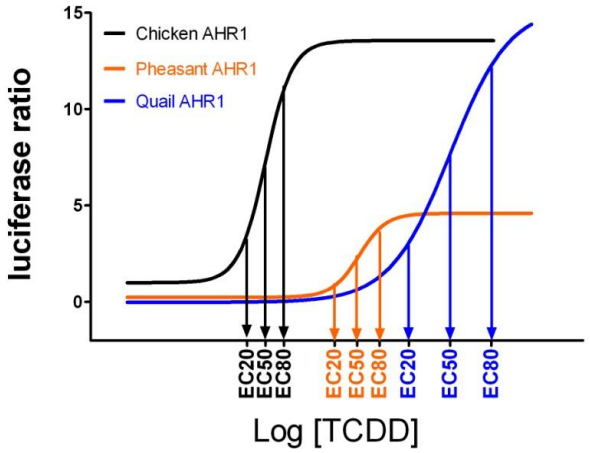


Figure 2.2. Conceptual diagram that illustrates how relative sensitivity (ReS) values were determined for COS-7 cells transfected with pheasant or quail AHR1 constructs treated with TCDD. $EC_{20, 50, \text{ and } 80}$ values were first determined for the AHR1s of chicken, pheasant and quail. $ReS_{20, 50, \text{ or } 80}$ values of pheasant = chicken $EC_{20, 50, \text{ or } 80}$ ÷ pheasant $EC_{20, 50, \text{ or } 80}$. Similarly, $ReS_{20, 50, \text{ or } 80}$ values of quail = chicken $EC_{20, 50, \text{ or } 80}$ ÷ quail $EC_{20, 50, \text{ or } 80}$.

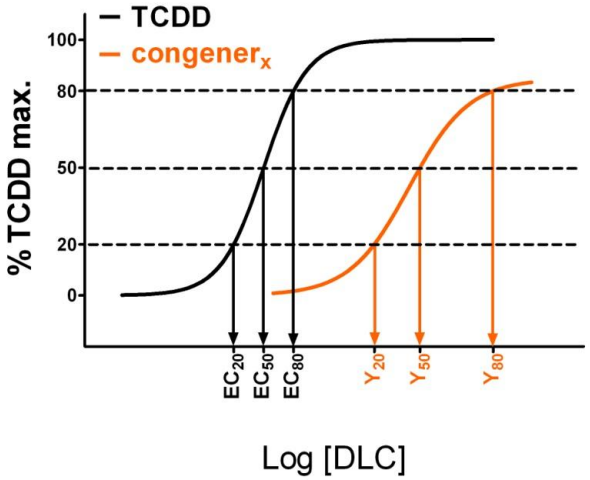


Figure 2.3. Conceptual diagram that illustrates how relative potency (ReP) values were determined for DLC congener$_x$ (i.e., PeCDF or TCDF) in COS-7 cells transfected with another AHR1 construct (e.g., chicken construct). $EC_{20, 50, \text{ and } 80}$ values were first determined for TCDD. Three points ($Y_{20, 50, \text{ and } 80}$) were used to calculate ReP values. The $ReP_{20, 50, \text{ or } 80}$ = TCDD $EC_{20, 50, \text{ or } 80}$ ÷ congener$_x$ $EC_{20, 50, \text{ or } 80}$.

2.4. RESULTS

2.4.1. AHR1 characterization

The full-length sequences for ring-necked pheasant AHR1 (RNP-AHR1; accession number: HM053553) and Japanese quail AHR1 were determined (Figure 2.4). Two allelic variants were found in Japanese quail AHR1 (JQ-AHR1*1; accession number: HM053555 and JQ-AHR1*2; accession number: HM053554). The proteins encoded by RNP-AHR1, JQ-AHR1*1 and JQ-AHR1*2 contained 860 aa (96.6 kDa), 857 aa (96.1 kDa) and 859 aa (96.3 kDa), respectively, with JQ-AHR1*2 containing insertions of two glutamine residues in the transactivation domain (positions 640 and 641) that were not present in JQ-AHR1*1. The amino acid sequences of chicken, ring-necked pheasant and Japanese quail AHR1 have 96% or greater identity overall (Table 2.2). The full-length AHR1 sequences reported here are consistent with the partial sequences reported previously (Head *et al.*, 2008; Karchner *et al.*, 2006). The amino acid residues at two key positions within the LBD were: chicken: Ile324_Ser380; ring-necked pheasant: Ile324_Ala380; Japanese quail: Val324_Ala380; common tern: Val325_Ala381. IVTT-synthesized full-length chicken, ring-necked pheasant, Japanese quail AHR1 proteins and mutant Japanese quail AHR1 proteins migrated to similar positions on SDS polyacrylamide gels and were expressed to approximately the same degree (Figure 2.5). AHR1 protein expression levels in COS-7 cells transfected with wild-type or mutant AHR1 constructs were similar (Figure 2.6). Finally, phylogenetic analysis of the avian AHR1 sequences with AHRs of selected vertebrate species indicates that they form a monophyletic group with other AHR1 forms (Figure 2.7).

2.4.2. AHR1 expression in COS-7 cells

2.4.2.1. Relative sensitivity (inter-species comparisons)

The concentration-dependent effects of TCDD, PeCDF and TCDF on AHR1-associated reporter gene expression in COS-7 cells that were transfected with (a) constructs encoding full-length chicken, ring-necked pheasant, Japanese quail, common tern or (b) mutant AHR1s are shown in Figure 2.8. The EC_x and ReS_x values for each DLC and each AHR1 construct are indicated in Table 2.3; three general sensitivity classes of avian species (chicken-like, pheasant-like or quail-like) are highlighted in light, moderate or dark grey, respectively. The range of ReS values (ReS_{20-80}) is of particular value for comparing the AHR1 constructs because it accounts for differences in individual EC values (i.e., EC_{20}, EC_{50} and EC_{80}) that occurred because the concentration-response curves did not always have identical slopes and maximum responses. Among the wild-type AHR1s, the rank-order of ReS in TCDD-exposed cells was chicken > pheasant > quail ≈ common tern (the $ReS_{average}$ values were: chicken = 1.0, pheasant = 0.13, quail = 0.011, tern = 0.014). Based on these $ReS_{average}$ values, pheasant AHR1 was 8-fold less sensitive, quail AHR1 was 90-fold less sensitive, and tern AHR1 was 70-fold less sensitive than AHR1 of chicken exposed to TCDD (Figure 2.9). The difference in sensitivity to PeCDF among the constructs was not as large as that observed for TCDD. For example, the quail AHR1 was only 5-fold less sensitive than chicken AHR1 to PeCDF (the ReS value for chicken was 1.0; the $ReS_{average}$ value for quail was 0.19). The range in sensitivity to TCDF among the four wild-type AHR1 constructs was more similar to the range in sensitivity observed for

TCDD (e.g. quail and tern were 33- and 34-fold less sensitive than chicken to TCDF, respectively).

The $ReS_{average}$ values for the chicken-like mutant (V324I_A380S) for each DLC (0.7 for TCDD; 1.1 for PeCDF; 1.0 for TCDF) were similar to the ReS in chicken (1.0). Similarly, the $ReS_{average}$ values for the pheasant-like mutant (V324I) of 0.13 for TCDD, 0.42 for PeCDF and 0.1 for TCDF were similar to the $ReS_{average}$ values in pheasant (0.13 for TCDD; 0.49 for PeCDF; 0.09 for TCDF). The $ReS_{average}$ values for the unknown mutant (A380S) appeared to be slightly higher than the $ReS_{average}$ values for the pheasant-like mutant (V324I), but further studies would be needed to determine if there are indeed differences in ReS values between the A380S mutant and the V324I mutant.

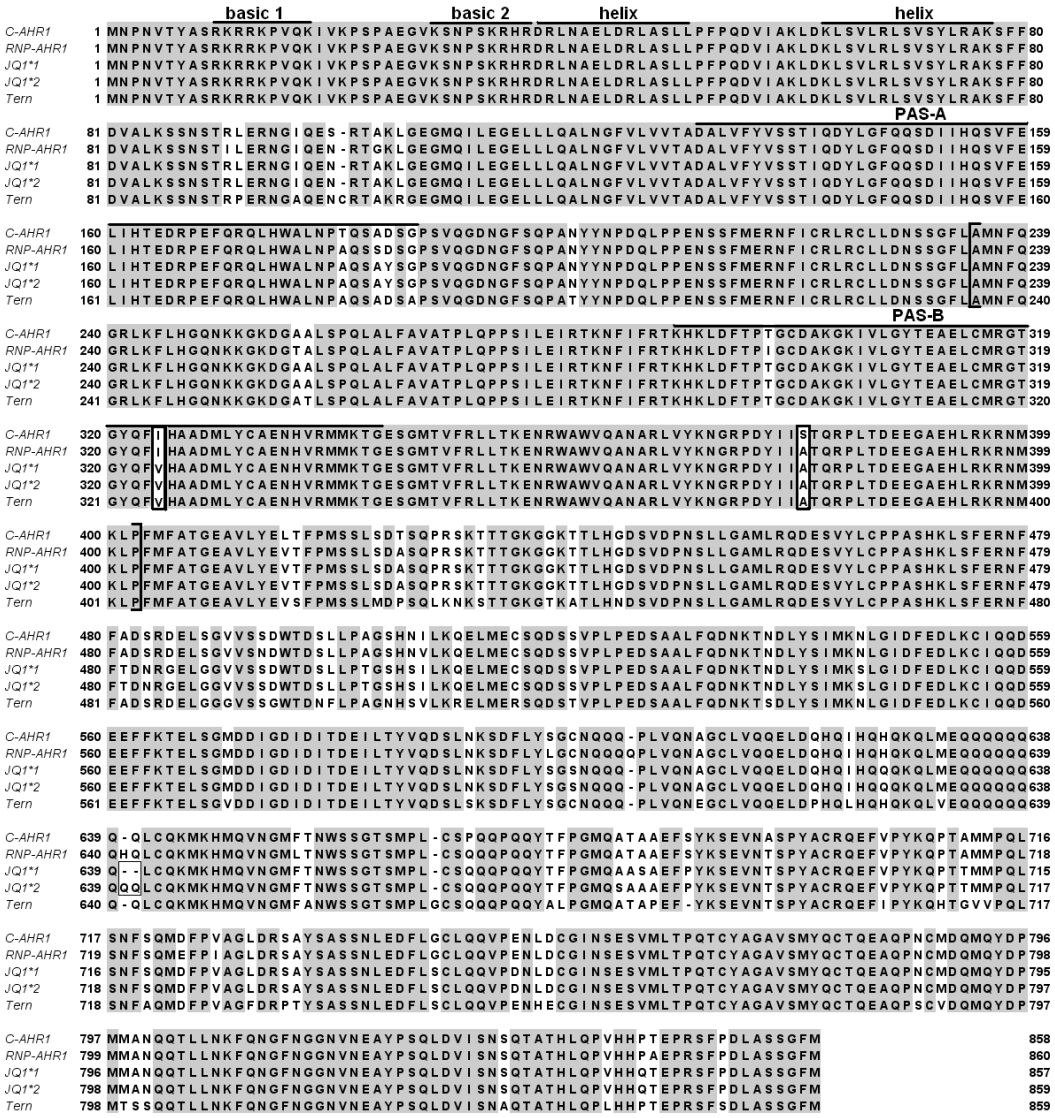


Figure 2.4. Alignment of aryl hydrocarbon receptor 1 (AHR1) amino acid sequences. The amino acid sequences of chicken (C-AHR1), ring-necked pheasant (RNP-AHR1), two allelic forms of Japanese quail (JQ1*1 and JQ1*2) and common tern were aligned using CLUSTALW2. Identical amino acids are shaded. The ligand binding domain (LBD) is indicated within brackets; the basic-helix-loop-helix (bHLH) and PAS domains are shown by lines above the amino acids. Boxed areas indicate key amino acids (sites 324 and 380) within the LBD AHR1 and insertion/deletion (indel sites) in two allelic variants of Japanese quail AHR1 (sites 640 and 641). Accession numbers are: chicken AHR1 (AF260832), ring-necked pheasant AHR1 (HM053553), Japanese quail AHR1*1 (HM053555), Japanese quail AHR1*2 (HM053554).

Table 2.2. AHR1 amino acid sequence for chicken (accession number: AF260832) was aligned with the AHR1 sequences of ring-necked pheasant (accession number: HM053553), two Japanese quail AHR1 variants (accession numbers: HM053555 and HM053554) and common tern AHR1 (accession number: AF192503). The percent identity among the sequences is indicated.

	RNP_AHR1	JQ_AHR1*1	JQ_AHR1*2	Tern_AHR1	protein length (aa)
Chicken_AHR1	97	97	97	91	858
RNP_AHR1		96	96	90	860
JQ_AHR1*1			99	91	857
JQ_AHR1*2				91	859
Tern_AHR1					859

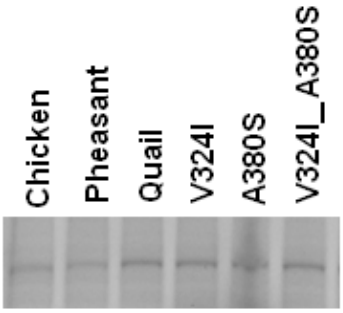


Figure 2.5. Representative example of electrophoretic analysis of fluorescently-labelled chicken, ring-necked pheasant, Japanese quail and mutated Japanese quail AHR1s (V324I, A380S and V324I_A380S) that were expressed by *in vitro* transcription and translation (IVTT). AHR1 proteins were labelled with FluoroTect Green$_{Lys}$ and loaded on a 10% SDS polyacrylaminde gel. A Typhoon 9210 Imager was used to detect the bands. It is noted that the analysis of several bands with all constructs did not indicate differences in the intensity of AHR1s among the constructs.

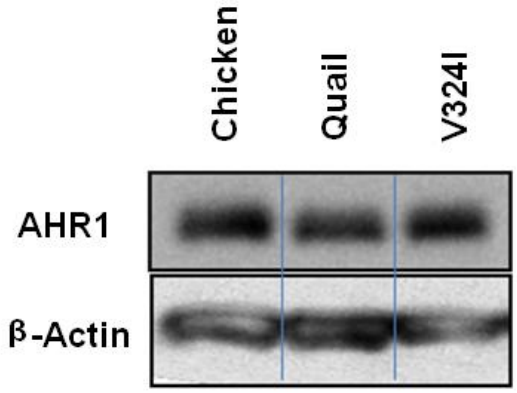


Figure 2.6. Representative example of a western blot showing the expression of wild-type (chicken and quail) or mutant (V324I) avian AHR1 protein in COS 7 cells (upper panel). β-actin (lower panel) was included as an internal control. Forty-eight hours after transfection, cell lysates were separated using SDS-PAGE, transferred to nitrocellulose membranes and probed with anti-V5-HRP antibody for detection of AHR1 expression. The same blot was stripped and re-probed for β-actin using anti-β-actin-peroxidase antibody.

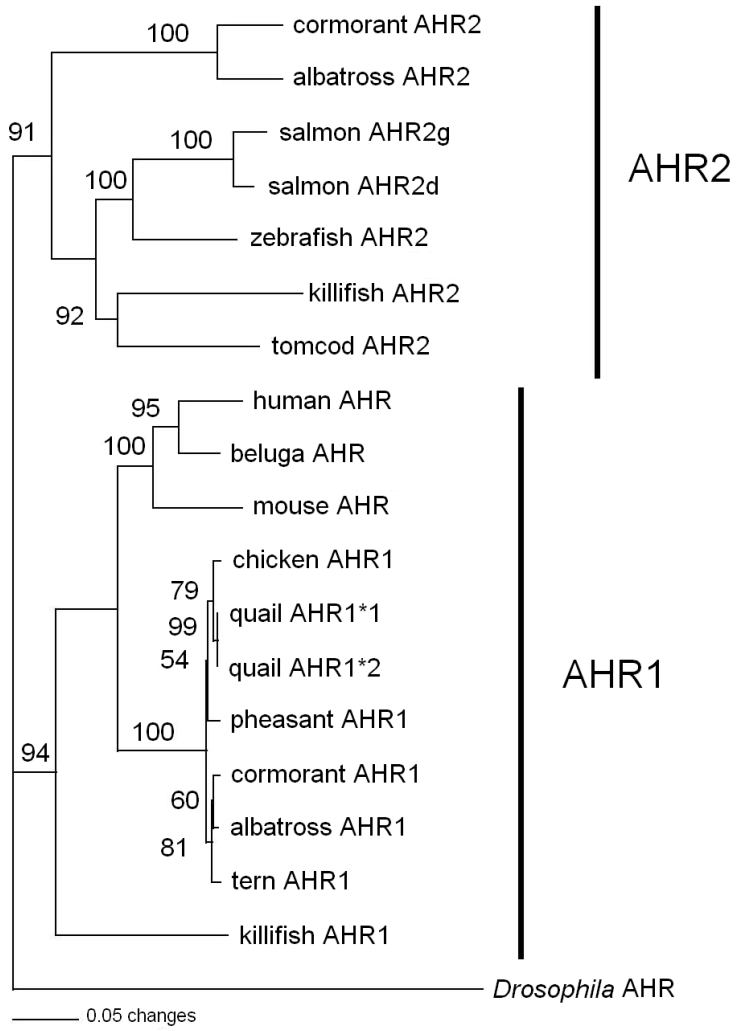


Figure 2.7. Phylogenetic analysis of selected vertebrate AHR amino acid sequences. Sequences were aligned using ClustalW®. The tree was constructed by use of the minimum evolution criterion in PAUP*4.0® with the neighbour-joining tree as the starting tree. Only the N-terminal half of the protein (~400 amino acids) was used because the alignment is less certain in the C-terminal half. Numbers at the nodes represent bootstrap values based on 100 re-samplings of the data. The AHR1 and AHR2 clades are indicated. Accession numbers for AHR2 are: great cormorant AHR2 (AB287294), Hawaiian black-footed albatross AHR2 (AB106110), Atlantic salmon AHR2g (AY052499), Atlantic salmon AHR2d (AF495590), zebrafish AHR2 (AF063446), killifish AHR2 (U29679), Atlantic tomcod AHR2 (AF050489); accession numbers for AHR1 include: human AHR (L19872), beluga whale AHR (AF332999), mouse AHR (M94623), chicken AHR1 (AF260832), Japanese quail AHR1*1 (HM053555), Japanese quail AHR1*2 (HM053554), ring-necked pheasant AHR1 (HM053553), great cormorant AHR1 (AB109545), Hawaiian black-footed albatross AHR1 (AB106109), common tern AHR (AF192503), killifish AHR1 (AF024591), *Drosophila melanogaster* AHR (AF050630).

2.4.2.2. Relative potency (inter-compound comparisons)

ReP values were obtained from data that were normalized to the maximum luciferase ratios elicited by TCDD for each AHR1 construct (Figures 2.3 and 2.10) and ReP values are indicated in Table 2.4 and Figure 2.11. Perhaps the most interesting results were with PeCDF because it was considerably more potent than TCDD in cells transfected with Japanese quail AHR1 ($ReP_{average}$ = 20) or common tern AHR1 (ReP = 26). PeCDF was slightly more potent than TCDD in the pheasant ($ReP_{average}$ = 3.7), pheasant-like mutant ($ReP_{average}$ = 3.6) and unknown mutant ($ReP_{average}$ = 3.6). The $ReP_{average}$ values for the DLCs in COS-7 cells transfected with the chicken AHR1 (1.0 for TCDD; 0.84 for PeCDF; 0.45 for TCDF) were generally similar to the chicken like-mutant AHR1 $ReP_{average}$ values (1.0 for TCDD; 1.1 for PeCDF; 0.91 for TCDF).

2.4.2.3. In vitro - In vivo comparisons

An important goal of my research on avian AHR1 is to develop in vitro methods that can be used to predict the in vivo potencies of DLCs among avian species. The in vitro results obtained in the present study were compared to the LD20, LD50 and LD80 values of TCDD, PeCDF and TCDF determined in a companion study (Cohen-Barnhouse *et al.*, 2011) where these compounds were injected into chicken, pheasant or Japanese quail eggs. As shown in Figure 2.12 (panel A and B), there was a significant correlation between in ovo LD20, LD50 and LD80 values and in vitro EC20, EC50 and EC80 values obtained with the luciferase reporter gene assay. It is also determined the correlations between in ovo LD50 values and ethoxyresorufin O-deethylase (EROD) EC50 values in

chicken, pheasant and Japanese quail hepatocyte cultures (Figure 2.12, panel B). The coefficient of correlation between in ovo LD20, LD50, and LD80 values and in vitro EC20, EC50, and EC80 values was higher for the LRG assay (r^2=0.71, p<0.005; r^2=0.77, p<0.005; r^2=0.70, p<0.005, respectively) than for the EROD assay (r^2=0.51, p<0.05). The relationship between the LRG and EROD assays was significant (r^2=0.72, p<0.005; Figure 2.12, panel C). The ReP and ReS values derived from egg injection studies, primary hepatocyte studies and the present study with transfected COS-7 cells are summarized in Table 2.5.

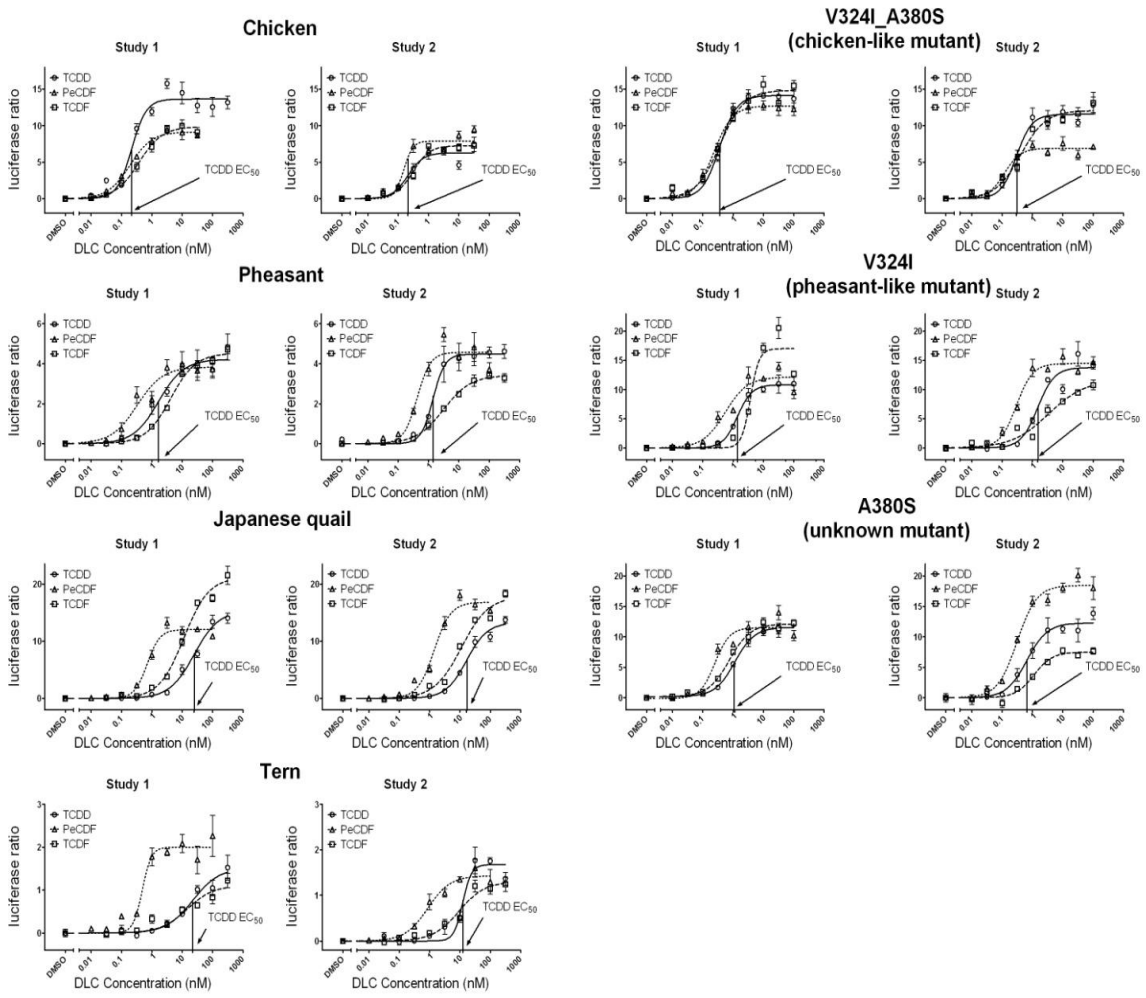


Figure 2.8. Concentration-dependent effects of TCDD, PeCDF and TCDF on AHR1 response element (AHRE)-driven luciferase reporter gene activity in COS-7 cells that were transfected with either (a) full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail, common tern, or (b) mutant constructs of Japanese quail AHR1. COS-7 cells were also transfected with cormorant ARNT1, CYP1A5 reporter vector and *Renilla* luciferase vector. Cells were exposed to DMSO or graded concentrations (0–300 nM) of TCDD, PeCDF or TCDF for 18-20h and luciferase ratios (the ratio of firefly luciferase luminescence units to *Renilla* luciferase luminescence units) were determined. Two studies were done for each DLC and each construct. Data points indicate the mean luciferase ratio determined in four wells; bars represent SE of the mean values.

Table 2.3. COS-7 cells transfected with chicken, ring-necked pheasant, Japanese quail, common tern or three Japanese quail mutant AHR1s were exposed to graded concentrations of TCDD, PeCDF or TCDF. Luciferase ratio values were determined and data were fit to a four-parameter logistic model using GraphPad Prism 5®. Concentration-response curves were plotted for each curve and the 20%, 50%, and 80% effective concentrations ($EC_{20,\ 50,\ and\ 80}$) were calculated. Two separate experiments, each of which used a 96-well plate to generate a concentration-response curve, were conducted for each DLC; four replicates of each concentration of each DLC were used. Relative sensitivity (ReS) is defined as $EC_{20,\ 50\ or\ 80\ (compound\ A)}$ in chicken ÷ $EC_{20,\ 50\ or\ 80\ (compound\ A)}$ in the species of interest. Three general classes of avian species (chicken-like, pheasant-like or quail-like) are highlighted in light, moderate or dark grey, respectively. The A380S genotype (unknown mutant) is not highlighted.

chemical	AHR1 construct	EC20(nM)		EC50(nM)		EC80(nM)	
		Study 1	Study 2	Study 1	Study 2	Study 1	Study 2
TCDD	Chicken	0.10	0.09	0.22	0.21	0.47	0.47
	V324I_A380S	0.14	0.13	0.32	0.29	0.72	0.66
	A380S	0.38	0.22	1.1	0.65	3.2	1.9
	Pheasant	0.44	0.74	1.7	1.4	6.2	2.6
	V324I	0.65	0.62	1.4	1.5	6.7	2.7
	Tern	5.5	3.0	22	12	88	48
	Quail	6.8	5.5	25	17	90	50
PeCDF	Chicken	0.06	0.04	0.19	0.17	0.61	0.74
	V324I_A380S	0.09	0.05	0.23	0.12	0.62	0.27
	A380S	0.12	0.11	0.25	0.31	0.53	0.88
	Pheasant	0.08	0.21	0.33	0.42	1.3	0.85
	V324I	0.17	0.12	0.54	0.29	2.2	0.60
	Tern	0.30	0.26	0.48	0.87	0.78	2.9
	Quail	0.36	0.59	0.70	1.4	1.4	3.4
TCDF	Chicken	0.11	0.12	0.37	0.30	1.3	0.73
	V324I_A380S	0.11	0.11	0.37	0.33	1.3	0.95
	A380S	0.23	0.43	0.72	1.1	2.3	2.9
	Pheasant	1.1	0.75	4.2	3.1	16	13
	V324I	2.3	0.5	3.7	3.5	14	4.3
	Tern	3.6	2.3	14	9.4	57	38
	Quail	3.1	2.7	11	11	42	47

		ReS_{20}	ReS_{50}	ReS_{80}	$ReS_{average}$	ReS_{20-80}
TCDD	Chicken	1	1	1	1	1
	V324I_A380S	0.7	0.7	0.7	0.69	0.7
	A380S	0.3	0.3	0.2	0.28	0.2-0.3
	Pheasant	0.2	0.1	0.1	0.13	0.1-0.2
	V324I	0.2	0.1	0.1	0.13	0.1-0.2
	Tern	0.02	0.01	0.01	0.014	0.01-0.02
	Quail	0.02	0.01	0.01	0.011	0.01-0.02
PeCDF	Chicken	1	1	1	1	1
	V324I_A380S	0.7	1	1.5	1.1	0.7-1.5
	A380S	0.4	0.6	1.0	0.68	0.4-1
	Pheasant	0.3	0.5	0.6	0.49	0.3-0.6
	V324I	0.3	0.4	0.5	0.42	0.3-0.5
	Tern	0.2	0.3	0.4	0.27	0.2-0.4
	Quail	0.1	0.2	0.3	0.19	0.1-0.3
TCDF	Chicken	1	1	1	1	1
	V324I_A380S	1.0	1.0	0.9	0.96	0.9-1
	A380S	0.3	0.4	0.4	0.37	0.3-0.4
	Pheasant	0.1	0.1	0.1	0.09	0.1
	V324I	0.1	0.1	0.1	0.1	0.1
	Tern	0.04	0.03	0.02	0.029	0.02-0.04
	Quail	0.04	0.03	0.02	0.030	0.02-0.04

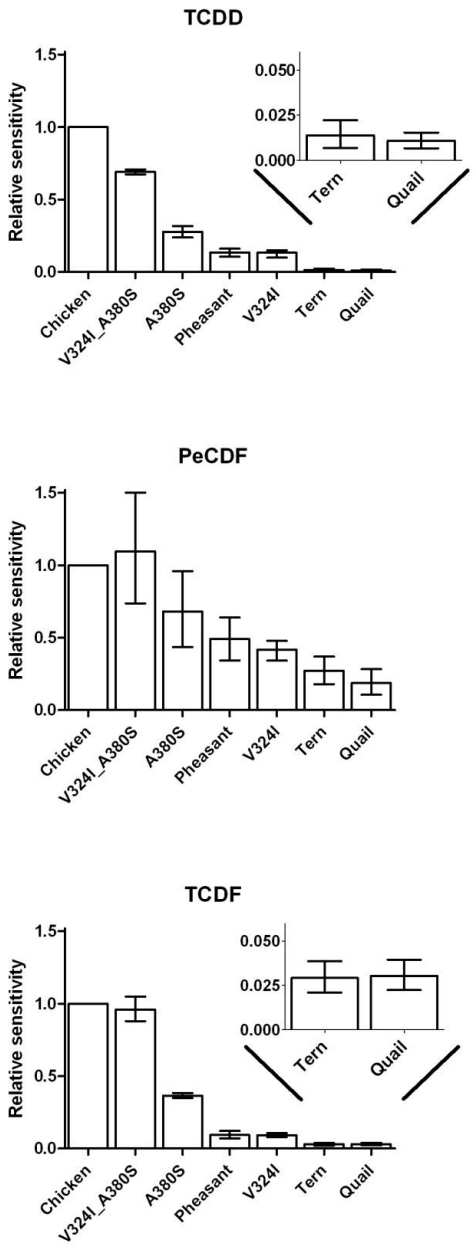


Figure 2.9. Relative sensitivity (ReS) values of full-length and mutant AHR1 constructs transiently transfected into COS-7 cells when exposed to TCDD, PeCDF or TCDF. Large bars represent mean ReS values and error bars indicate the maximum and minimum ReS values.

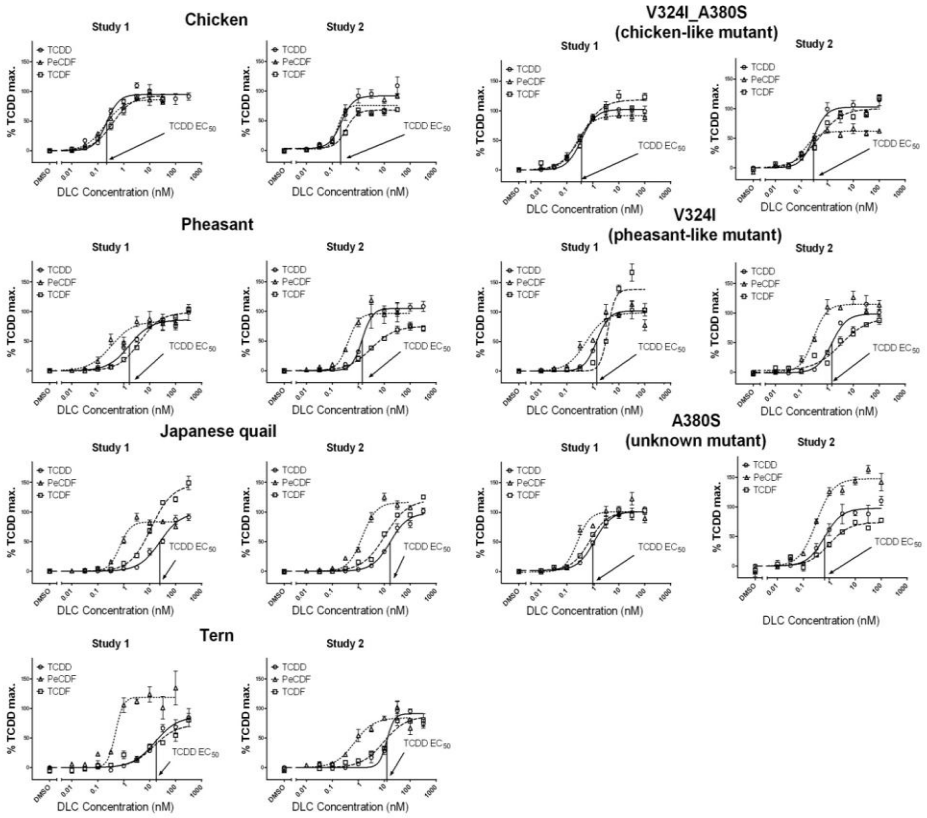


Figure 2.10. Concentration-dependent effects of TCDD, PeCDF and TCDF on AHRE-driven luciferase reporter gene activity in COS-7 cells. Full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail AHR1 or quail mutants (V324I [pheasant-like mutant], A380S [unknown mutant] or V324I_A380S [chicken-like mutant]) were transfected into COS-7 cells with cormorant ARNT, luciferase reporter vector and *Renilla* luciferase reporter vector. The cells were exposed to DMSO or serial dilutions (0–300 nM) of TCDD, PeCDF or TCDF for 18-20h and luciferase activity was determined. Luciferase ratio data were normalized to the TCDD% maximum response. Each data point represents the mean luciferase activity (firefly luminescence/*Renilla* luminescence) determined in four wells; bars represent SE of the mean values.

Table 2.4. Relative potency (ReP) values were calculated based on the ReP estimation methods similar to methods used by Villeneuve *et a.* (2000), Kim *et al.* (2011) and Mol *et al.* (2012). ReP is defined as $EC_{20,\ 50\ or\ 80\ (species\ A)}$ of TCDD ÷ three points of responses ($Y_{20,\ 50\ or\ 80}$) of the compound of interest that correspond to 20, 50 and 80% of TCDD maximum response. For compounds that elicited maximum responses that were less than 80% TCDD, ReP at the observed maximum response was calculated and replaced by the [a]$ReP_{75,}$ [b]$ReP_{65,}$ [c]$ReP_{60,}$ [d]ReP_{70} or [e]ReP_{72}. Three general classes of avian species (chicken-like, pheasant-like or quail-like) are highlighted in light, moderate or dark grey, respectively. The A380S genotype (unknown mutant) is not highlighted.

AHR1 construct	compound	ReP_{20}	ReP_{50}	ReP_{80}	$ReP_{average}$	ReP_{20-80}
Chicken	TCDD	1	1	1	1	1
	PeCDF	1.1	0.9	0.5[a]	0.84	0.5-1.1
	TCDF	0.7	0.4	0.3[b]	0.45	0.3-0.7
V324I_A380S (chicken-like)	TCDD	1	1	1	1	1
	PeCDF	1.6	1.1	0.6[c]	1.1	0.6-1.6
	TCDF	1.4	0.8	0.5	0.91	0.5-1.4
A380S (unknown mutant)	TCDD	1	1	1	1	1
	PeCDF	3.0	3.5	4.4	3.6	3-4.4
	TCDF	1.0	0.6	0.2[d]	0.58	0.2-1
Pheasant	TCDD	1	1	1	1	1
	PeCDF	4.0	4.0	3.0	3.7	3-4
	TCDF	0.6	0.3	0.2[d]	0.35	0.2-0.6
V324I (pheasant-like)	TCDD	1	1	1	1	1
	PeCDF	4.4	3.6	2.8	3.6	2.8-4.4
	TCDF	0.5	0.4	0.2	0.37	0.2-0.5
Tern	TCDD	1	1	1	1	1
	PeCDF	23	24	30	26	23-30
	TCDF	1.5	0.7	0.4[e]	0.89	0.4-1.5
Quail	TCDD	1	1	1	1	1
	PeCDF	13	20	26	20	13-26
	TCDF	3.0	3.0	4.0	3.4	3-4

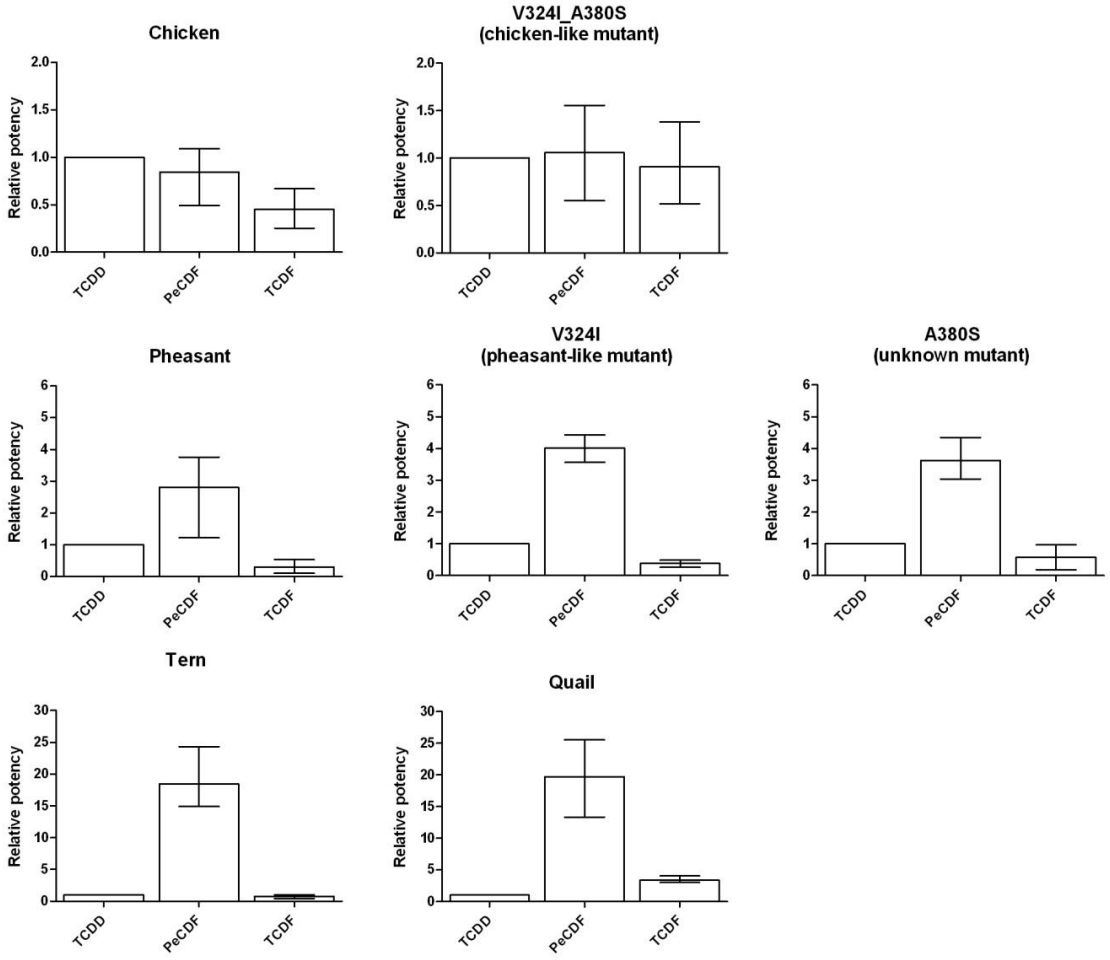


Figure 2.11 Relative potency (ReP) values of full-length and mutant AHR1 constructs transiently transfected into COS-7 cells when exposed to TCDD, PeCDF or TCDF. Large bars represent mean ReP values and error bars indicate the maximum and minimum ReP values.

2.3. DISCUSSION

Karchner *et al.* (2006) showed that key amino acids within the LBD of AHR1 elicit differential sensitivity to TCDD among avian species included studies on the *in vitro* functional analysis of AHR1 from one sensitive species (domestic chicken), one insensitive species (common tern) and selected mutants of common tern AHR1 (Karchner *et al.*, 2006). In the present report, the findings of the initial study with TCDD were confirmed and the work was broadened to include the effects of PeCDF and TCDF on AHR1-dependent gene expression for (a) full-length domestic chicken, ring-necked pheasant, Japanese quail and common tern AHR1 constructs and (b) three mutant constructs of Japanese quail AHR1. The concentration-dependent effects of TCDD, PeCDF and TCDF on *in vitro* expression of chicken, ring-necked pheasant and Japanese quail AHR1s were studied in order to compare the results from the present study to the results from the companion studies on the effects of TCDD, PeCDF and TCDF on embryotoxicity (Cohen-Barnhouse *et al.*, 2011) and CYP1A induction in primary cultures of embryonic hepatocytes (Herve *et al.*, 2010a; Herve *et al.*, 2010b). The present study not only confirms the earlier finding (Karchner *et al.*, 2006) that the chicken AHR1 is more sensitive than the common tern AHR1 to activation by TCDD, it extends that finding by providing a quantitative measure of the difference in sensitivity. For example, the concentration-response curves indicate that the chicken AHR1 is approximately 90- and 70-fold more sensitive to TCDD than the Japanese quail and common tern AHR1s, respectively (Table 2.3; $ReS_{average}$ values are 1.0, 0.011 and 0.014 for chicken, quail and tern,

respectively). Similarly, the ring-necked pheasant AHR1 is approximately 8-fold less sensitive (based upon comparisons of $ReS_{average}$ values) to TCDD than the chicken AHR1.

The LBDs of common tern and Japanese quail AHR1s both contain valine at the chicken site 324 and alanine at the chicken site 380 (chicken sites 324 and 380 are equivalent to tern sites 325 and 381). The present finding that common tern and Japanese quail AHR1s were relatively insensitive to AHR1-dependent gene expression upon exposure to TCDD is consistent with the hypothesis that Val324 and Ala380 impart characteristics to the AHR1 which confer a reduced sensitivity to activation by DLCs (Head *et al.*, 2008; Karchner *et al.*, 2006). The reduced sensitivity might be caused by a conformational change that affects the binding affinity of DLCs to the LBD. AHR1-binding assays and molecular modeling studies could be used to test this hypothesis. The amino acid at LBD site 257 is threonine in common tern AHR1 and alanine in Japanese quail AHR1, but the identity of the amino acid at site 257 appears to have no effect on AHR1-dependent gene activation by TCDD (Karchner *et al.*, 2006). There are several different amino acids within the DNA binding domain (DBD) and transactivation domain (TAD) of AHR1 (Figure 2.4), but Karchner and colleagues (Karchner *et al.*, 2006) found that differences in amino acids within those domains had no influence on differential sensitivity of chicken and common tern to TCDD.

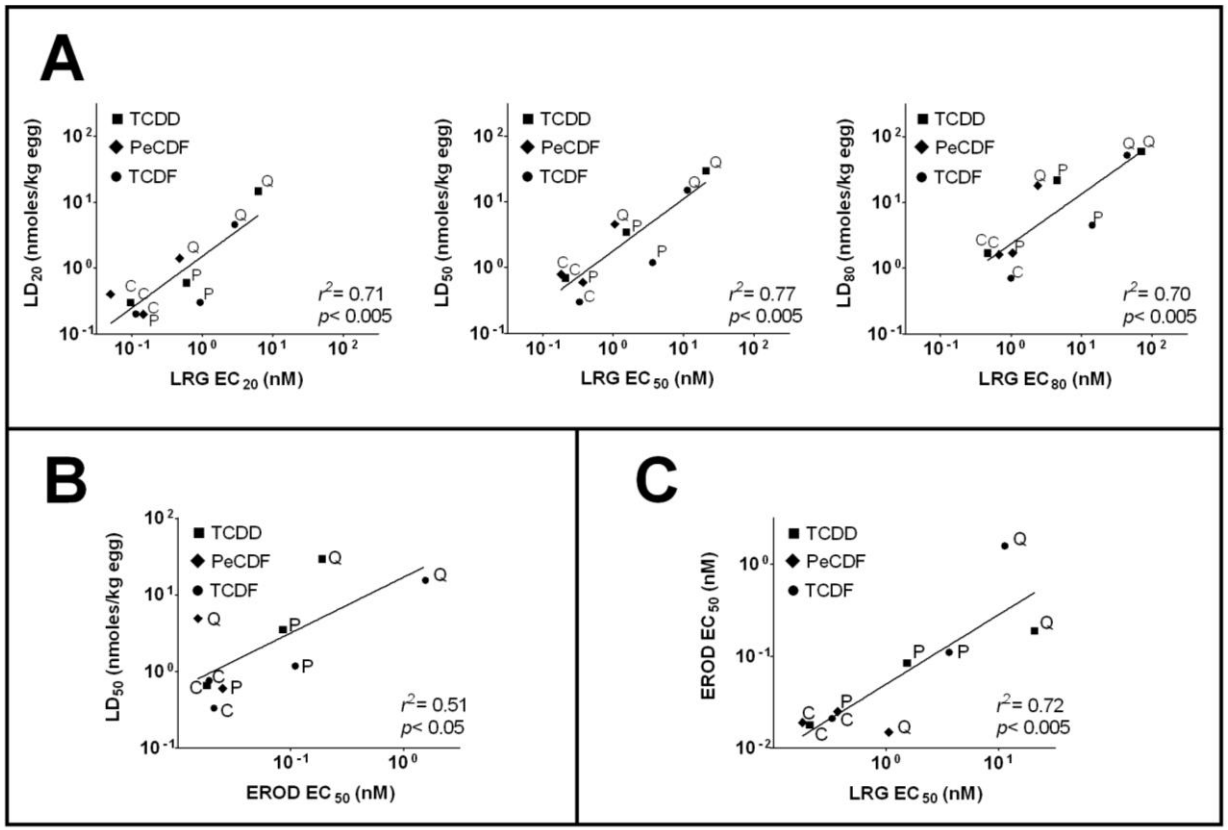


Figure 2.12. ***(A)*** Correlation between $LD_{20,\ 50\ and\ 80}$ data from egg injection studies (Cohen-Barnhouse *et al.*, 2011) and $EC_{20,\ 50\ and\ 80}$ data from the luciferase reporter gene assay (LRG assay; this paper) for chicken (C), ring-necked pheasant (P) and Japanese quail (Q). To my knowledge there are no LD_{50} values for common tern (the other species studied using the LRG assay) exposed *in* ovo to TCDD, PeCDF or TCDF. ***(B)*** Correlations between LD_{50} (Cohen-Barnhouse *et al.,* 2011) and ethoxyresorufin *O*-deethylase (EROD) EC_{50} in primary cultures of embryonic hepatocytes (Herve *et al.*, 2010b). ***(C)*** Correlations between EROD EC_{50} values (Herve *et al.*, 2010b) and LRG EC_{50} values (the present study).

Table 2.5. Summary of relative sensitivity and relative potency data obtained from the luciferase reporter gene (LRG; ReS_{20-80} and ReP_{20-80}), egg injection (LD_{20-80}) (Cohen-Barnhouse *et al.*, 2011) and primary hepatocyte cell culture studies (EC_{50}) (Herve *et al.*, 2010b). ReS_{20-80} and ReP_{20-80} values were calculated as indicated in the *Materials and Methods* section. Hepatocyte cell culture endpoints (EROD and CYP1A mRNA) were calculated based on EC_{50}. N/A; data not available.

Relative Sensitivity

				Primary hepatocyte cell culture		
Chemical	**Species**	**LD_{20-80}**	**LRG (ReS_{20-80})**	**EROD**	**CYP1A4**	**CYP1A5**
TCDD	Chicken	1	1	1	1	1
	Pheasant	0.077-0.47	0.1-0.2	0.2	0.7	1
	Quail	0.018-0.028	0.01-0.02	0.1	0.1	0.1
PeCDF	Chicken	1	1	1	1	1
	Pheasant	0.94-1.6	0.3-0.6	0.8	2	N/A
	Quail	0.089-0.26	0.1-0.3	1	0.8	0.7
TCDF	Chicken	1	1	1	1	1
	Pheasant	0.15-0.52	0.1	0.2	0.4	0.8
	Quail	0.013-0.035	0.02-0.04	0.01	0.02	0.03

Relative Potency

				Primary hepatocyte cell culture		
Species	**Chemical**	**LD_{20-80}**	**LRG (ReP_{20-80})**	**EROD**	**CYP1A4**	**CYP1A5**
Chicken	TCDD	1	1	1	1	1
	PeCDF	0.75-1.1	0.5-1.1	0.9	0.7	2
	TCDF	1.7-2.5	0.3-0.7	0.9	2	1
Pheasant	TCDD	1	1	1	1	1
	PeCDF	2.6-13	3-4	3	2	N/A
	TCDF	1.8-4.9	0.1-0.5	0.8	0.8	0.8
Quail	TCDD	1	1	1	1	1
	PeCDF	3.3-11	13-26	13	8	17
	TCDF	1.2-3.3	3-4	0.1	0.5	0.5

The importance of amino acids at AHR1 LBD sites 324 and 380 in birds is consistent with reports on the key role of corresponding sites within the AHR LBD in other vertebrates. For example, mutation of isoleucine at site 319 in mouse AHR to alanine (mouse site 319 corresponds to chicken site 324) resulted in the loss of transactivation activity (Goryo *et al.*, 2007). The importance of the amino acid at position 375 of mouse AHR and 381 of human AHR (mouse site 375 and human site 381 are equivalent to chicken site 380) has been shown in several studies. For example, mutation of valine 381 to aspartate completely abolished the ligand binding capacity of human AHR (Ema *et al.*, 1994). A study of two alleles of murine AHR showed that the reduced ligand binding affinity of one of the alleles could be attributed to valine at position 375. Changing this amino acid to alanine enhanced ligand binding affinity four-fold (Poland *et al.*, 1994). The human AHR has been shown to have a lower affinity for binding a photoaffinity-labelled TCDD analogue compared with the mouse AHR^{b-1} allele. A single amino acid (valine 381) in human AHR is responsible for the lower ligand binding affinity; mutating this residue to alanine restored high affinity ligand binding (Ramadoss and Perdew, 2004). When alanine 375 in mouse AHR was substituted with isoleucine or phenylalanine, both mutants failed to bind to ligands or to exhibit AHR activation in cells exposed to AHR ligands (Murray *et al.*, 2005). Mutation of mouse AHR at position 375 from alanine to valine reduced AHR DNA binding in gel retardation analysis and the A375L mutant was not able to bind to DNA (Pandini *et al.*, 2007; Pandini *et al.*, 2009). Finally, results of site-directed mutagenesis studies in rat AHR revealed the importance of the site that is equivalent to chicken site 380 in transactivation activity (Backlund and Ingelman-Sundberg, 2004).

The concentration-dependent effect of TCDD on luciferase activity in COS-7 cells transfected with chicken AHR1 or the chicken-like AHR1 mutant (V324I_A380S) yielded similar (< 1.5-fold differences) EC_x values. This finding supports the earlier report (Karchner *et al.*, 2006) that (1) the identities of amino acids at LBD sites 324 and 380 confer differential sensitivity to TCDD and (2) the major determinant of the sensitivity of avian AHR1 resides within the LBD and not the DBD or TAD. The concentration-dependent effect of TCDD on luciferase activity in cells expressing pheasant AHR1 and the pheasant-like AHR1 mutant (V324I) yielded almost identical EC_x values demonstrating the importance of the amino acid at site 324. The A380S mutant might have been slightly more sensitive to TCDD than the pheasant-like mutant (V324I), but as stated in the Results section, follow-up studies would be required to confirm or refute this possibility. I am not aware of any avian species with the V324_S380 genotype, but the results presented here suggest that species with this genotype would be equal to, or slightly more sensitive, than ring-necked pheasant.

PeCDF was more potent than TCDD in COS-7 cells expressing the ring-necked pheasant ($ReP_{average}$ = 3.7), Japanese quail ($ReP_{average}$ = 20), and common tern ($ReP_{average}$ = 26) AHR1s. This finding is important because TCDD has generally been considered the most potent of AHR agonists in all taxa, and has therefore been used as the reference DLC to which all other DLCs are compared to derive the Relative Potency Factors (RePs) and Toxicity Equivalency Factors (TEFs) (Van den Berg *et al.*, 1998). The finding that PeCDF was more potent than TCDD in COS-7 cells expressing the Japanese quail AHR1 is consistent with the finding that PeCDF is more potent than TCDD as an inducer of CYP1A mRNA and EROD activity in primary

cultures of Japanese quail hepatocytes (Herve *et al.*, 2010b). PeCDF is also more potent (20- to 30-fold) than TCDD in herring gull (*Larus argentatus*) hepatocytes (Herve *et al.*, 2010c). Japanese quail, common tern and herring gull (accession number: DQ371287) AHR1s all contain valine at site 324 and alanine at site 380. As such, it will be of interest to determine if PeCDF is more potent than TCDD in *all* species with this AHR1 genotype. It would also be of interest to conduct AHR1 binding studies and homology modeling studies to attempt to determine the molecular properties that cause PeCDF to be more potent than TCDD in some birds. Overall, the comparison of potencies of PeCDF and TCDD as activators of AHR1s from different avian species provides an example of ligand-dependent species differences in AHR affinities, similar to those found recently for other ligands in comparisons between human and mouse AHRs (Flaveny *et al.*, 2009).

The results of the present study with the LRG assay indicate a good correlation between *in vivo* (egg injections) and *in vitro* results ($r^2 = 0.77$; $p < 0.005$ for LD_{50}). If the TCDF data are removed from Figure 12, panel A, the correlation is even stronger ($r^2 = 0.93$; $p < 0.005$). I draw attention to this point because one might not expect as good a relationship when comparing *in vivo* and *in vitro* results for TCDF. TCDF is more rapidly eliminated from avian embryos compared to TCDD or PeCDF (Cohen-Barnhouse *et al.*, 2011), which could potentially reduce the predictive ability of *in vitro* studies for TCDF. The LRG assay might be an equal or better predictor of LD_{50} values than the primary hepatocyte EROD assay, but more *in vitro* and *in vivo* studies with other avian species and other DLCs would be needed to confirm this hypothesis.

This study was possible due to development of a rapid method that allows for determination of concentration-dependent effects of DLCs on activation of AHR1 in transiently transfected COS-7 cells. Unlike other reporter gene assays that use COS-7 cells to measure avian AHR activity (Karchner *et al.*, 2006; Lee *et al.*, 2009; Yasui *et al.*, 2007), I (a) seeded cells in 96-well plates rather than in Petri dishes or 48-well plates, (b) added the DLCs directly to the wells without changing the cell culture medium, and (c) used the same 96-well plates to measure luminescence without lysing the cells and transferring cells to another plate. The method allows generation of approximately 32 concentration-response curves per person-week and it could be easily automated to allow increased throughput and efficiency. Despite the efficiency and high throughput nature of the assay, it was less sensitive than the assay used by Iwata and colleagues (Lee *et al.*, 2009; Mol *et al.*, 2012). For example, I determined the EC_{50} of TCDD in COS-7 cells expressing chicken AHR1 to be 0.21 nM, but Iwata *et al*. reported a TCDD EC_{50} of 0.034 nM. I conducted several studies with the goal of improving the sensitivity of the assay (e.g. using serum-free medium, changing the time that cells were exposed to DLCs, changing the concentration of reporter and expression vectors, adding a cell lysis step to the procedure), but improvements in sensitivity were not obtained. Regardless of the reduced sensitivity, the assay was able to measure full concentration-response curves for all AHR1 constructs for the three compounds studied. One can prepare AHR1 constructs from any avian species (including wild species) and study sensitivity to any DLC using the approach described in the present paper.

The CYP1A5 promoter constructs and ARNT1 constructs (both from cormorant, and kindly provided by Dr. Iwata, Ehime University, Japan) were

kept consistent for all experiments. The rationale for this decision was based on two factors: (a) Iwata and colleagues demonstrated that structural differences between chicken and cormorant CYP1A5 did not appear to affect the response to TCDD (Lee *et al.*, 2009) and (b) it was necessary to keep the ARNT1 constant to test hypotheses regarding the importance of amino acids at sites 324 and 380 within AHR1 without introducing other forms of ARNT and CYP1A5 to the experiments. AHR1 might not be the only factor that affects the biochemical and toxic effects of DLCs in avian species and more studies are needed to elucidate the role(s) of the multiple isoforms of AHR or ARNT (i.e. AHR2, ARNT2). While future studies might indicate complex roles between AHRs and ARNTs in transfected cells, such work was beyond the scope of the present work; my findings support and expand the understanding of the importance of the amino acid residues at sites 324 and 380 within AHR1.

A practical goal of my research on avian AHR1 is to develop methods that can be used to predict the sensitivity of any avian species to the toxic effects of any DLC. This goal is of importance because the differences in sensitivity of wild avian species to DLCs make it difficult to make accurate assessments and predictions of adverse effects of DLCs in wild birds. Currently, the default assumption in risk assessments is to use the domestic chicken as a surrogate species for birds when risk assessments of DLCs are conducted. To my knowledge, the chicken is the most sensitive avian species to DLCs. Thus, the conventional approach may overestimate the potential risks of adverse effects and could lead to inappropriate decisions about remedial options at contaminated sites (Fredricks *et al.*, 2011a; Fredricks *et al.*, 2011b). The results of the present study suggest the possibility of an alternative approach to risk assessments that would involve the determination

of the AHR LBD genotype of species of concern. Using such molecular knowledge could help guide decision making regarding the potential impacts of DLC exposure with respect to avian wildlife.

CHAPTER 3 : AMINO ACID SEQUENCE OF THE LIGAND BINDING DOMAIN OF THE ARYL HYDROCARBON RECEPTOR 1 (AHR1) PREDICTS SENSITIVITY OF WILD BIRDS TO EFFECTS OF DIOXIN-LIKE COMPOUNDS

(Modified from Farmahin, *et al*., 2013. *Tox.Sci.,* **131**(1), 139-152)

3.1. ABSTRACT

The sensitivity of avian species to the toxic effects of dioxin-like compounds (DLCs) varies up to 1000-fold among species and this variability has been associated with inter-species differences in aryl hydrocarbon receptor 1 ligand binding domain (AHR1 LBD) sequence. I previously showed that LD50 values, based on *in ovo* exposures to DLCs, were significantly correlated *with in vitro* EC50 values obtained with a luciferase reporter gene (LRG) assay that measures AHR1-mediated induction of cytochrome P4501A in COS-7 cells transfected with avian AHR1 constructs. Those findings suggest that the AHR1 LBD sequence and the LRG assay can be used to predict avian species sensitivity to DLCs. In the present study, the AHR1 LBD sequences of 86 avian species were studied and differences at amino acid sites 256, 257, 297, 324, 337 and 380 were identified. Site-directed mutagenesis, the LRG assay and homology modeling highlighted the importance of each amino acid site in AHR1 sensitivity to 2,3,7,8-tetrachlorodibenzo-p-dioxin and other DLCs. The results of the study revealed that (1) only amino acids at sites 324 and 380 affect the sensitivity of AHR1 expression constructs of 86 avian species to DLCs and (2) *in vitro*

luciferase activity in AHR1 constructs containing only the LBD of the species of interest is significantly correlated (r^2 = 0.93, p<0.0001) with *in ovo* toxicity data for those species. These results indicate promise for the use of AHR1 LBD amino acid sequences independently, or combined with the LRG assay, to predict avian species sensitivity to DLCs.

3.2. INTRODUCTION

Dioxins and dioxin-like compounds (DLCs) share structural similarities and common mechanisms of biochemical and toxic action, but differences in sensitivity to DLCs exist among species and strains of animals. For example, sensitivity to the biochemical and toxic effects of some DLCs can vary up to 1000-fold among avian species (Brunstrom, 1988; Head *et al.*, 2008; Head and Kennedy, 2010; Hoffman *et al.*, 1998). Based on the available evidence, the domestic chicken (*Gallus gallus domesticus*) is the most sensitive species to the toxic effects of DLCs. For risk assessment purposes, the toxic potencies of individual DLCs are compared to the potency of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is generally considered to be the most potent compound within this class of chemicals (Van den Berg *et al.*, 1998; Van den Berg *et al.*, 2006). However, recent studies have shown that, in some species, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is more potent than TCDD (Cohen-Barnhouse *et al.*, 2011; Farmahin *et al.*, 2012; Herve *et al.*, 2010b; Herve *et al.*, 2010c).

Most, if not all, biochemical and toxic effects of DLCs are thought to be mediated by the aryl hydrocarbon receptor (AHR) (Denison *et al.*, 2011; Okey, 2007). While it has long been recognized that structural characteristics of the

AHR contribute to differential sensitivity among mammalian species (Okey, 2007; Poland *et al.*, 1976a), key characteristics of the AHR responsible for differential sensitivity among birds were not known until recently (Farmahin et al. 2012; Head et al. 2008; Karchner et al. 2006). Birds express at least two AHR paralogs (AHR1 and AHR2) (Yasui et al., 2004) *and* AHR1 is reported to be more transcriptionally active than AHR2 (Yasui et al. 2007). In addition, the basal level of expression of AHR1 is several-fold greater than that of AHR2 in liver of birds (Mol et al., 2012; Yasui et al., 2007).

The avian AHR1 contains three major domains - the DNA binding domain (DBD), the ligand binding domain (LBD) and the transactivation domain (TAD). By swapping the three domains of chicken and common tern (*Sterna hirundo*) AHR1 and making six chimeric constructs, Karchner *et al.* (1996) demonstrated that the LBD, and not the DBD or TAD, was responsible for the distinct functional properties of chicken and tern AHR1. This led to the hypothesis that the identity of amino acids within the LBD could determine the sensitivity of avian species to DLCs. The identities of amino acids at sites 324 and 380 within the LBD contributed to the differential sensitivity of chicken and tern AHR1 exposed to TCDD (Karchner *et al.*, 2006) and chicken, ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) exposed to TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and PeCDF (Farmahin *et al.* 2012). It has been proposed that birds can be classified into three main groups, for risk assessment purposes, based on the identities of these two amino acids: high sensitivity (type 1; Ile324_Ser380), moderate sensitivity (type 2; Ile324_Val380) and low

sensitivity (type 3; Val324_Ala380) (Farmahin *et al.,* 2012; Head *et al.,* 2008; Karchner *et al.* 2006; Manning *et al.* 2012).

The major goals of the present study were to (1) sequence the AHR1 LBD of a relatively large number of birds to identify amino acid residues that differ among species, (2) determine the influence of the identified amino acids, individually or in combination, on AHR1 activation by DLCs using a luciferase reporter gene assay (LRG) that measures transactivation of an aryl hydrocarbon response element (AHRE)-driven luciferase reporter gene, (3) determine if avian sensitivity to selected DLCs [TCDD, PeCDF and polychlorinated biphenyl 126 (PCB 126)] can be predicted from results of the LRG assay and/or knowledge of amino acids at sites 324 and 380 within the AHR1 and (4) attempt to understand why sites 324 and 380 play key roles in AHR1 activation by use of homology modelling studies.

3.3. MATERIALS AND METHODS

3.3.1. Sources of avian samples used for AHR1 LBD sequencing

Liver and blood were obtained from the Specimen Bank at the National Wildlife Research Centre (NWRC; Ottawa, ON), commercial suppliers in the Ottawa region or field-collected by our group in Michigan, USA (Table 3.1). All samples were collected, salvaged or donated in accordance with permit requirements. The samples obtained from Michigan were collected with either solvent-rinsed utensils (liver) or sterile syringes (blood) (Head *et al.*, 2010), preserved in RNAlater™ (Ambion, TX, USA) at -20ºC at Michigan State University (East Lansing, MI, USA) and shipped on wet or dry ice to the NWRC for RNA

isolation and AHR1 LBD sequencing. Albatross, great cormorant and red jungle fowl AHR1 LBD sequences were obtained from GenBank.

3.3.2. AHR1 LBD sequencing

RNA was isolated from liver (~ 3 mg) or blood (~ 300 µl) with TRIzol™ reagent (Invitrogen, Burlington, ON, Canada). Isolation of RNA, reverse transcription, polymerase chain reaction (PCR) and molecular cloning methods are described in detail elsewhere (Head *et al.*, 2008; Head *et al.*, 2010). Briefly, total RNA was reverse transcribed to cDNA and the AHR1 LBD was amplified by PCR and either (a) ligated into a vector, transformed into chemically-competent cells and purified (plasmid DNA) or, (b) separated on E-Gel 0.8% SYBR Safe™ pre-cast agarose gels using an E-Gel iBase system (Invitrogen). The latter procedure was used to reduce time and cost per sample (Gibson *et al.*, 2010). Purified plasmid DNA and E-Gel products were sequenced by use of an Applied Biosystems 3730 DNA Analyzer at the Ottawa Hospital Research Institute (OHRI, Ottawa, ON, Canada).

To ensure accuracy, most sequences of the LBD of AHR1 were obtained from two or more individuals per species and sequencing was conducted several times for each individual and on products from independent PCR reactions. Sequences of nucleotides were analyzed by use of Sequencher version 4.9 software (Gene Codes Corporation, Ann Arbor, MI, USA). Detailed analysis of each chromatogram ensured quality and accuracy of the sequence data. Amino acid sequences were translated from the consensus nucleotide alignments using

Sequencher. The AHR1 LBD sequences corresponded to amino acid residues 235-402 in chicken.

3.3.3. Expression constructs and site-directed mutagenesis of chicken AHR1

Full-length chicken, ring-necked pheasant and Japanese quail AHR1 constructs were prepared (Farmahin *et al.*, 2012) and the chicken AHR1 construct was used for the preparation of twelve constructs that were mutated at sites 256, 257, 297, 324, 337 or 380 by site-directed mutagenesis. The primers and templates are provided in Table 3.2 All of the mutations were constructed using PfuUltra High-Fidelity DNA polymerase (Stratagene, La Jolla, CA, USA). PCR conditions were as follows: 95 ˚C 1min followed by 21 cycles of 95 ˚C 30 sec, 55 ˚C 1 min, and 68˚C 18 min. PCR products were treated with *DpnI* endonuclease (New England Biolabs, Beverly, MA) and transformed into MAX Efficiency DH5α Competent Cells (Invitrogen). Full-length sequencing of each of the 12 mutant constructs was conducted to ensure correct sequence identity. Other constructs were cormorant ARNT1 (pcDNA-ccArnt1), reporter vector GL4-ccCYP1A5 (both were kindly provided by Dr. Hisato Iwata, Ehime University, Japan) (Lee *et al.*, 2009; Yasui *et al.*, 2007) and *Renilla* luciferase vector (phRL-CMV, Promega).

Table 3.1. Main types (**type 1**: Ile324_Ser380; **type 2**: Ile324_Ala380; **type 3**: Val324_Ala380) and sub-types (letter designations) of AHR1 LBDs determined in 86 species of birds. The identities of amino acids at sites 256, 257, 297, 324, 337 and 380 are indicated; sites 324 and 380 are in bold. The AHR1 LBD sequences were determined by our group or obtained from GenBank (black-footed albatross, great cormorant and red jungle fowl). The red jungle fowl and the domestic chicken are considered to be the same species. AHR1 LBD sequences were determined from liver or blood samples obtained from the National Wildlife Research Centre, Ottawa, ON (underlined), local suppliers (domestic chicken, Japanese quail, ring-necked pheasant, turkey and emu), the Tittabawassee River basin, Michigan, USA (bold) or approximately 100 km west of the Tittabawassee River basin (bold italic). Previously reported AHR LBD sequences were from *(Head, 2006; Head *et al.*, 2008), **(Karchner *et al.*, 2006).

sub-type	aa identity 256	257	297	324	337	380	name	scientific name	accession #
1A	A	A	T	I	V	S	domestic chicken	*Gallus gallus domesticus*	NM204118
1A							red jungle fowl	*Gallus gallus*	NC006089
1B	A	A	T	I	I	S	**European starling**	*Sturnus vulgaris*	FJ376538
1B							**ruby-throated hummingbird**	*Archilochus colubris*	FJ376541
1C	A	T	T	I	I	S	**gray catbird**	*Dumetella carolinensis*	FJ376509
2A	A	A	I	I	V	A	ruffed grouse*	*Bonasa umbellus*	FJ376525
2A							turkey*	*Meleagris gallopavo*	EU660874
2A							willow ptarmigan*	*Lagopus lagopus*	FJ376532
2B	A	A	T	I	I	A	***American redstart***	*Setophaga ruticilla*	JQ814789
2B							**Baltimore oriole**	*Icterus galbula*	FJ376487
2B							**black-and-white warbler**	*Mniotilta varia*	FJ376491
2B							**black-capped chickadee**	*Poecile atricapilla*	FJ376492
2B							**brown-headed cowbird**	*Molothrus ater*	FJ376495
2B							**cedar waxwing**	*Bombycilla cedrorum*	FJ376497
2B							**chipping sparrow**	*Spizella passerina*	FJ376498
2B							**common grackle**	*Quiscalus quiscula*	FJ376501
2B							**common yellowthroat**	*Geothlypis trichas*	FJ376503
2B							**indigo bunting**	*Passerina cyanea*	FJ376513
2B							**Northern cardinal**	*Cardinalis cardinalis*	FJ376516
2B							**ovenbird**	*Seiurus aurocapilla*	FJ376518
2B							**red-winged blackbird**	*Agelaius phoeniceus*	FJ376521
2B							**rose-breasted grosbeak**	*Pheucticus ludovicianus*	FJ376524
2B							***song sparrow***	*Melospiza melodia*	JQ824841
2B							**swamp sparrow**	*Melospiza georgiana*	FJ376528
2B							**tufted titmouse**	*Baeolophus bicolor*	FJ376536
2B							***white-throated sparrow***	*Zonotrichia albicollis*	JQ814794
2C	A	A	V	I	V	A	rock ptarmigan*	*Lagopus muta*	FJ376523
2D	A	P	T	I	V	A	**American woodcock**	*Scolopax minor*	JF969754
2D							**spotted sandpiper**	*Actitis macularius*	FJ376527
2E	A	T	T	I	I	A	***American crow***	*Corvus brachyrhynchos*	JQ814788
2E							**American goldfinch**	*Carduelis tristis*	FJ376484
2E							**American robin**	*Turdus migratorius*	FJ376485
2E							**bank swallow**	*Riparia riparia*	FJ376488
2E							**barn swallow**	*Hirundo rustica*	FJ376534
2E							**blue jay**	*Cyanocitta cristata*	FJ376493
2E							**cliff swallow**	*Petrochelidon pyrrhonota*	FJ376499
2E							**Eastern bluebird**	*Sialia sialis*	EU660870
2E							***hermit thrush***	*Catharus guttatus*	JQ814792
2E							**house finch**	*Carpodacus mexicanus*	FJ376510
2E							**house sparrow**	*Passer domesticus*	FJ376511
2E							**house wren**	*Troglodytes aedon*	FJ376512
2E							***Northern raven***	*Corvus corax*	JQ969022
2E							**red-eyed vireo**	*Vireo olivaceus*	FJ376519
2E							**tree swallow***	*Tachycineta bicolor*	FJ376530

sub-type	aa identity 256	257	297	324	337	380	name	scientific name	accession #
2E							***veery***	*Catharus fuscescens*	JQ814793
2E							**white-breasted nuthatch**	*Sitta carolinensis*	FJ376531
2F	A	T	T	I	V	A	black-footed albatross	*Phoebastria nigripes*	AB106109
2F							***brown thrasher***	*Toxostoma rufum*	JQ814790
2F							emu	*Dromaius novaehollandiae*	JF950300
2F							**mourning dove**	*Zenaida macroura*	FJ376515
2G	T	A	I	I	V	A	**bobwhite quail**	*Colinus virginianus*	FJ376494
2G							ring-necked pheasant*	*Phasianus colchicus*	EU660873
3A	A	A	T	V	V	A	great blue heron*	*Ardea herodias*	FJ376506
3A							Japanese quail*	*Coturnix japonica*	EU660871
3B	A	T	T	V	V	A	American kestrel*	*Falco sparverius*	EU660867
3B							arctic tern	*Sterna paradisaea*	HQ317441
3B							bald eagle*	*Haliaeetus leucocephalus*	FJ376486
3B							**barred owl**	*Strix varia*	FJ376489
3B							**belted kingfisher**	*Megaceryle alcyon*	FJ376490
3B							**common flicker**	*Colaptes auratus*	FJ376500
3B							**common loon**	*Gavia immer*	FJ376502
3B							common tern**	*Sterna hirundo*	AF192503
3B							***cooper's hawk***	*Accipiter cooperii*	JQ814791
3B							double-crested cormorant*	*Phalacrocorax auritus*	EU660869
3B							**downy woodpecker**	*Picoides pubescens*	FJ376504
3B							**Eastern kingbird**	*Tyrannus tyrannus*	FJ376505
3B							great cormorant	*Phalacrocorax carbo*	AB109545
3B							**great horned owl**	*Bubo virginianus*	FJ376507
3B							herring gull*	*Larus argentatus*	DQ371287
3B							ivory gull	*Pagophila eburnea*	FJ376540
3B							**killdeer**	*Charadrius vociferus*	FJ376514
3B							osprey*	*Pandion haliaetus*	FJ376517
3B							**red-tailed hawk**	*Buteo jamaicensis*	FJ376520
3B							ring-billed gull*	*Larus delawarensis*	FJ376522
3B							**sandhill crane**	*Grus canadensis*	FJ376535
3B							***Saw-whet owl***	*Aegolius acadicus*	JQ969021
3B							**screech owl**	*Megascops asio*	FJ376526
3B							***Sharp-shinned hawk***	*Accipiter striatus*	JQ969020
3B							thick-billed murre*	*Uria lomvia*	FJ376529
3B							**turkey vulture**	*Cathartes aura*	FJ376537
3C	T	T	T	V	V	A	brant goose	*Branta bernicla*	FJ376539
3C							Canada goose	*Branta canadensis*	FJ376496
3C							common eider*	*Somateria mollissima*	EU660868
3C							greater scaup	*Aythya marila*	FJ376508
3C							**mallard***	*Anas platyrhynchos*	EU660872
3C							wood duck*	*Aix sponsa*	EU660875
3C							**wood thrush**	*Hylocichla mustelina*	FJ376533

3.3.4. *In vitro* transcription and translation (IVTT) of AHR1 mutants

TnT Quick-Coupled Reticulocyte Lysate Systems kits (Promega, Madison, WI, USA) were used to synthesize AHR1 proteins for all mutants; proteins were labelled with FluroTect Green$_{lys}$ tRNA. Fluorescently labelled products were separated on 10% SDS polyacrylamide gels and scanned with a Typhoon 9210 Imager (Molecular Dynamics Inc., Sunnyvale, CA, USA; excitation 532 nm, emission 580 nm).

3.3.5. AHR1 protein expression in COS-7 cells

Western blot analysis was performed as described elsewhere (Farmahin *et al.*, 2012) with minor modifications. In brief, COS-7 cells transfected with AHR1 constructs were lysed and cell lysates were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes (GE Healthcare Bio-Sciences, Baie d'Urfé, QC, Canada) and anti-V5-HRP antibody (Invitrogen) was used at 1:2000 dilution for detecting V5-AHR1. Anti-β-actin-peroxidase (Sigma-Aldrich, Oakville, ON, Canada) was used at 1:10000 as a loading control. The blots were then visualized by enhanced chemiluminescence using a Typhoon 9210 Imager (Molecular Dynamics Inc).

Table 3.2. Templates and primers used to prepare mutated constructs within the chicken AHR1 LBD. Bold fonts in the primer sequences indicate the nucleotides that resulted in site-specific mutations in the amplicon.
[1]The 2A-1 mutant construct was not used in the LRG assay; it was used as a template for the synthesis of six other mutants.

type	*template*	*mutation*	*primers*
1B	1A	V337I	Forward: 5' GCTTTATTGTGCTGAAAATCAT**A**TCCGAATGATGAAGACGGGTG 3' Reverse: 5' CACCCGTCTTCATCATTCGGA**T**ATGATTTTCAGCACAATAAAGC 3'
1C	1B	A257T	Forward: 5' GAAAGGGAAGGATGGTGCT**A**CATTGTCTCCTCAGC 3' Reverse: 5' GCTGAGGAGACAATG**T**AGCACCATCCTTCCCTTTC 3'
2A-1[1]	1A	S380A	Forward: 5' GACCAGATTACATTATT**G**CCACACAAAGACCTCTTACAG 3' Reverse: 5' CTGTAAGAGGTCTTTGTGTGG**C**AATAATGTAATCTGGTC 3'
2A	2A-1	T297I	Forward: 5' CTGGATTTCACACCTA**T**TGGCTGTGATGCAAAAGG 3' Reverse: 5' CCTTTTGCATCACAGCCA**A**TAGGTGTGAAATCCAG 3'
2B	2A-1	V337I	Forward: 5' GCTTTATTGTGCTGAAAATCAT**A**TCCGAATGATGAAGACGGGTG 3' Reverse: 5' CACCCGTCTTCATCATTCGGA**T**ATGATTTTCAGCACAATAAAGC 3'
2C	2A-1	T297V	Forward: 5' CAAACTGGATTTCACACCT**GT**TGGCTGTGATGCAAAAGG 3' Reverse: 5' CCTTTTGCATCACAGCCA**AC**AGGTGTGAAATCCAGTTTG 3'
2D	2A-1	A257P	Forward: 5' GGAAGGATGGTGCT**C**CATTGTCTCCTCAGC 3' Reverse: 5' GCTGAGGAGACAATG**G**AGCACCATCCTTCC 3'
2E	2B	A257T	Forward: 5' GAAAGGGAAGGATGGTGCT**A**CATTGTCTCCTCAGC 3' Reverse: 5' GCTGAGGAGACAATG**T**AGCACCATCCTTCCCTTTC 3'
2F	2A-1	A257T	Forward: 5' GAAAGGGAAGGATGGTGCT**A**CATTGTCTCCTCAGC 3' Reverse: 5' GCTGAGGAGACAATG**T**AGCACCATCCTTCCCTTTC 3'
2G	2A	A256T	Forward: 5' GAAAGGGAAGGATGGT**A**CTGCATTGTCTCCTC 3' Reverse: 5' GAGGAGACAATGCAG**T**ACCATCCTTCCCTTTC 3'
3A	2A-1	I324V	Forward: 5' GAACAGGATACCAGTTC**G**TTCATGCAGCTGATATGC 3' Reverse: 5' GCATATCAGCTGCATGAA**C**GAACTGGTATCCTGTTC 3'
3B	3A	A257T	Forward: 5' GAAAGGGAAGGATGGTGCT**A**CATTGTCTCCTCAGC 3' Reverse: 5' GCTGAGGAGACAATG**T**AGCACCATCCTTCCCTTTC 3'
3C	3A	A256T_A257T	Forward: 5' GAAAGGGAAGGATGGT**A**CT**A**CATTGTCTCCTCAGCTGG 3' Reverse: 5' CCAGCTGAGGAGACAATG**T**AG**T**ACCATCCTTCCCTTTC 3'

3.3.6. LRG assays

3.3.6.1. Preparation of TCDD, PeCDF and PCB 126 solutions

Serial dilutions of TCDD and PeCDF were prepared from dimethyl sulfoxide (DMSO) stock solutions and concentrations of the stock solutions were determined by isotope dilution following EPA method 1613 (U.S.EPA, 1994) by high-resolution gas chromatography high-resolution mass spectrometry as described in detail elsewhere (Herve *et al.*, 2010b). PCB 126 (AccuStandard, New Haven, CT, USA; lot # 061204MS-AC; 99.7% purity) was weighed on an analytical balance and dissolved in 4 ml of DMSO to obtain a stock solution with a nominal concentration of 153 µg/mL. Serial dilutions of PCB 126 were subsequently prepared from this solution.

3.3.6.2. Cell culture, transfection and luciferase assay

A detailed description of the LRG assay is provided elsewhere (Farmahin *et al.*, 2012). Briefly, COS-7 cells (provided by Dr. R. Haché, University of Ottawa, Ottawa, ON, Canada) were plated at a concentration of 10,000 cells/well in 96-well plates and transfected after 18 hours. The amounts of transfected expression vectors were 8 ng of mutated avian AHR1, 1.55 ng of pcDNA-ccArnt1, 5 ng of pGL4-ccCYP1A5 and 0.75 ng of phRL-CMV. The total amount of transfected DNA was kept constant at 50 ng by the addition of salmon sperm DNA (Invitrogen). Cells were dosed 5 hours after transfection with DMSO or DMSO solutions of TCDD, PeCDF or PCB 126 at 0.05% final DMSO concentration. Cells were removed from the incubator 18–20 hours after dosing to measure luciferase

activity. Luminescence values are expressed as a ratio of firefly luciferase units to *Renilla* luciferase units.

3.3.6.3. Concentration-response curves

Two independent studies (referred to as Studies 1 and 2 below) were conducted and for each of the studies, four concentration-response curves were obtained for each DLC and each AHR1 construct. The four concentration-response curves were derived from four replicate wells/plate for each concentration. GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) was used for curve-fitting and data were fit to a four parameter logistic model (Head and Kennedy, 2007b). Statistical differences among the four EC_{50} values, derived from each of the two independent studies, were tested using one-way analysis of variance (ANOVA) for a representative DLC (TCDD).

3.3.7. Relative sensitivity and relative potency

A detailed description of the calculation of the relative sensitivity (ReS) of COS-7 cells to AHR1 activation by DLCs is provided elsewhere (Farmahin *et al.*, 2012). Briefly, EC_{20}, EC_{50} and EC_{80} values were determined for each concentration-response curve. ReS is defined as $EC_{20,\ 50\ or\ 80\ (compound\ A)}$ of chicken construct ÷ $EC_{20,\ 50\ or\ 80\ (compound\ A)}$ of construct *X*. The relative potency (ReP) of PeCDF or PCB 126 compared to TCDD for each AHR1 construct is defined as: $EC_{20,\ 50\ or\ 80}$ of TCDD determined for construct *X* ÷ $EC_{20,\ 50\ or\ 80}$ of PeCDF *or* PCB 126 determined for construct *X*.

3.3.8. Homology modeling

PSI-BLAST searches for chicken, ring-necked pheasant and Japanese quail AHR1 LBD sequences (residues 235-402) were performed against the Protein Data Bank (PDB) (Berman *et al.*, 2000). The sequences that produced the most significant alignments were identified and nuclear magnetic resonance (NMR) structures were obtained from the PDB. When different NMR structures were found in a PDB file, the most representative structure was determined by use of the NMRCLUST procedure from the On-Line Database Ensemble Representatives and Domains (OLDERADO) (Kelley and Sutcliffe, 1997) server. The model for the avian AHR1 LBD was generated by Easy Modeller version 2.1 (Kuntal *et al.*, 2010). The protein structure analysis (ProSA) validation method (Wiederstein and Sippl, 2007) (web-based version) was used to assess the quality of the model and the PROCHECK program (Laskowski *et al.*, 1993) was used to assess the stereochemical quality of the models. Templates and structural motifs of models were analysed with the Promotif program (Hutchinson and Thornton, 1996). The amino acid sequence and structure of models were aligned with templates using the DALI server (Holm and Rosenstrom, 2010). The Multiple Alignment of Protein Structures (MultiProt) (Shatsky *et al.*, 2004) server was used for the structural alignment of avian AHR1 models. Molegro Virtual Docker (Thomsen and Christensen, 2006) and UCSF chimera (Pettersen *et al.*, 2004) was used for three dimensional visualization and imaging of AHR1 LBD structures. The computed Atlas of Surface Topography of Proteins (CASTp) *server* (Dundas *et al.*, 2006) was used to analyze the ligand binding cavity. The

potential binding site was predicted using a grid-based cavity prediction algorithm by Molegro Virtual Docker (Thomsen and Christensen, 2006).

3.4. RESULTS

3.4.1. Avian AHR1 LBD sequences

Sequences of the AHR1 LBD (sites 235-402; 168 amino acids) of 86 avian species were determined by our group or obtained from GenBank (black-footed albatross, great cormorant and red jungle fowl). The species and Genbank accession numbers of the AHR1 LBD sequences are listed in Table 3.1. The nucleotide and amino acid identities of the AHR1 LBDs among species were greater than 91% and 96%, respectively. Species of birds were classified into three main types according to the identities of the amino acids at sites 324 and 380: a) type 1 [chicken-like species (Ile324_Ser380; 4 of 86)]; b) type 2 [pheasant-like species (Ile324_Ala380; 47 of 86)]; and c) type 3 [Japanese quail-like species (Val324_Ala380; 35 of 86)]. Four other amino acid sites within the AHR1 LBD (256, 257, 297 and 337) were variable among species and the identities of the amino acids at these sites were used to define 13 sub-types (Table 3.1). The full-length chicken AHR1 construct (sub-type 1A) was used to generate 12 mutant constructs representative of the other AHR1 sub-types. AHR1 protein expression levels in COS-7 cells transfected with chicken and 12 mutant constructs (1B, 1C, 2A, 2B, 2C, 2D, 2E, 2F, 2G, 3A, 3B, 3C) were similar (Figure 3.1, panel A). The mutant AHR1 proteins synthesized by IVTT migrated to similar positions on SDS polyacrylamide gels and were expressed to approximately the same degree (Figure 3.2).

3.4.2. Concentration-dependent effects of TCDD, PeCDF and PCB 126

3.4.2.1. Relative sensitivity (ReS) - inter-construct comparisons

TCDD, PeCDF and PCB 126 elicited concentration-dependent increases in luciferase expression in COS-7 cells containing full-length AHR1 constructs of chicken, pheasant, Japanese quail or mutant AHR1 constructs (Figure 3.1, panel B; Figures 3.3 and 3.4 [normalized luciferase ratios]; Figures 3.5 and 3.6 [raw luciferase ratios]). $EC_{20,\ 50,\ and\ 80}$ values were determined from the fitted curves (Table 3.3) and were used to calculate ReS_{20}, ReS_{50} and ReS_{80} values (Table 3.4).

The rank order of sensitivity of AHR1 constructs to TCDD and PCB 126 was type 1 > type 2 > type 3 (Table 3.5 and Figure 3.7). For example, in cells exposed to TCDD, ReS_{50s} ranged from 0.75 - 1.0 for the type 1 constructs, 0.061 - 0.14 for the type 2 constructs, and 0.0073 - 0.013 for the type 3 constructs (Table 3.5). In cells exposed to PCB 126, ReS_{50s} were 1.0 - 1.5 for type 1 constructs, 0.032 - 0.038 for type 2 constructs and 0.0060 - 0.0091 for type 3 constructs. The rank order of ReS_{50} values of cells exposed to PeCDF was also type 1 > type 2 > type 3, but the range was not as great as that observed in cells treated with TCDD or PCB 126. One-way analysis of variance (ANOVA) comparing the four EC_{50} values (derived from the four concentration-response curves/study) obtained for all AHR1 types and sub-types exposed to TCDD indicated that (a) there were significant differences between the main types and (b) within each type, there were no statistical differences among the sub-types (Figure 3.8).

3.4.2.2. Relative potency (ReP) - inter-compound comparisons

PeCDF was approximately equipotent to TCDD in cells transfected with type 1 AHR1s (ReP_{50} range = 1.2 - 1.9), slightly more potent than TCDD in cells transfected with type 2 AHR1s (ReP_{50} range = 2.3 - 6.8) and substantially more potent than TCDD in cells transfected with type 3 AHR1s (ReP_{50} range = 10 – 21; Table 3.5). PCB 126 was less potent than TCDD and PeCDF in cells transfected with all sub-types of AHR1 constructs. For example, PCB 126 was 7- to 14-fold less potent than TCDD in cells transfected with type 1 AHR1, 24- to 58-fold less potent than TCDD in cells transfected with type 2 AHR1 and 11- to 28-fold less potent than TCDD in the cells transfected with type 3 AHR1 (Table 3.5). The rank order of DLC potency based on RePaverage values (the mean value of $ReP_{20,\ 50\ and\ 80}$) of the three compounds studied was (a) PeCDF≈TCDD>PCB 126 for the type 1 constructs, (b) PeCDF≥TCDD>PCB 126 for the type 2 constructs and (c) PeCDF>TCDD>PCB 126 for the type 3 constructs (Table 3.6; Figure 3.9).

3.4.3. *In vitro - In ovo* comparisons

The LRG $EC_{20,\ 50\ and\ 80}$ values obtained in the present study for full-length and mutant AHR1s were compared to LD_{50} values obtained for all domestic and wild avian species that, to our knowledge, have been used for egg injection (*in ovo*) studies (Table 3.7). LD_{50} values, based on *in ovo* exposures, were significantly correlated (r^2 = 0.95, p < 0.0001) with *in vitro* EC_{50} values obtained with the LRG assay (Figure 3.10). The equation obtained from the linear regression was used to predict the sensitivity of type 1, 2 and 3 avian embryos to TCDD, PeCDF and PCB 126 (Table 3.8). Correlations between LD_{50} values and

LRG $EC_{20,\ 50\ and\ 80}$ values are presented (Figure 3.11); the results indicate that $EC_{20,\ 50\ and\ 80}$ values are all significantly correlated with LD_{50} values. LD_{50} values were similarly correlated with EC_{50} values for both wild-type AHR1 constructs (r^2=0.91, p=0.001; Figure 3.12, panel A) and mutant AHR1 constructs (r^2=0.93, p<0.0001; Figure 3.12, panel B) in the LRG assay.

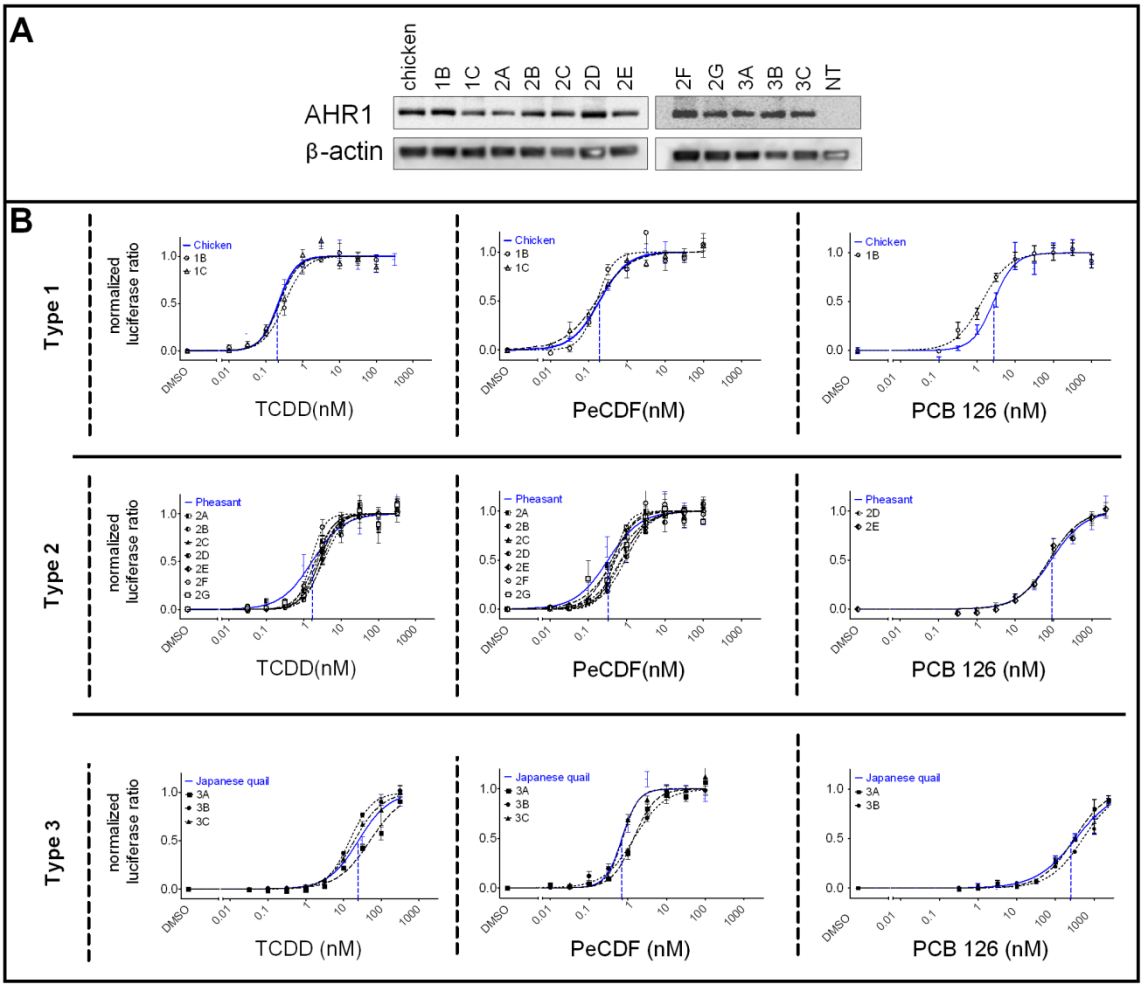


Figure 3.1. ***(A)*** Western blot analysis showing the expression of wild-type (chicken) and mutant (1B, 1C, 2A, 2B, 2C, 2D, 2E, 2F, 2G, 3A, 3B, 3C) avian AHR1 protein in COS-7 cells (upper panel). β-actin (lower panel) was used as an internal control. Non-transfected COS-7 cells (NT) were used as a negative control. Forty-eight hours after transfection, cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with anti-V5-HRP antibody for detection of AHR1 expression. The same blot was stripped and re-probed for β-actin using anti-β-actin-peroxidase antibody. ***(B)*** Representative example (study 1) of the concentration-dependent effects of TCDD, PeCDF and PCB 126 on aryl hydrocarbon response element (AHRE)-driven luciferase reporter gene activity in COS-7 cells transfected with either full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or mutant constructs of chicken AHR1 (designated 1B, 1C, etc; see Table 1 for details). Cells were exposed to DMSO or serial dilutions of TCDD, PeCDF or PCB 126 for 18-20 h, luciferase ratios (the ratio of firefly luminescence units to *Renilla* luminescence units) were determined, and data were normalized to the maximal response. Individual data points represent the mean ratio derived from four individual wells/concentration and bars represent SE. Each curve represents the average of four curves. The dashed vertical lines within each panel indicate EC_{50} values for the wild-type AHR1 constructs (chicken, ring-necked pheasant and Japanese quail).

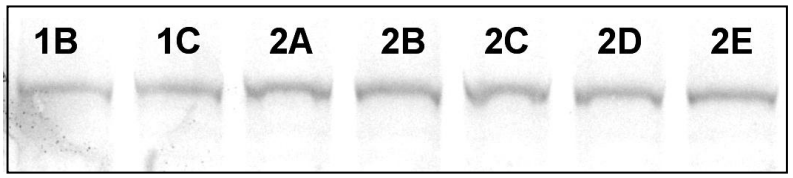
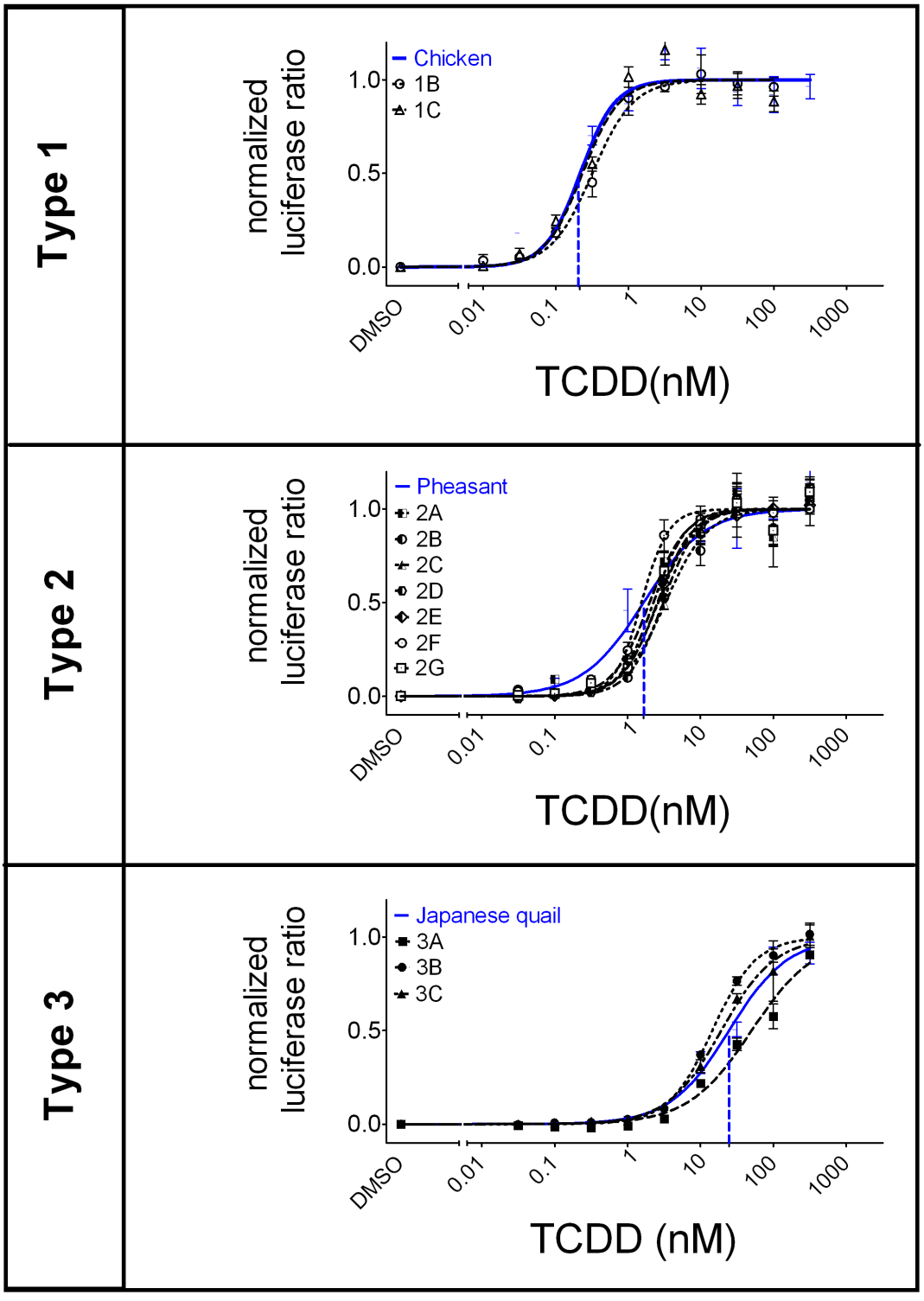
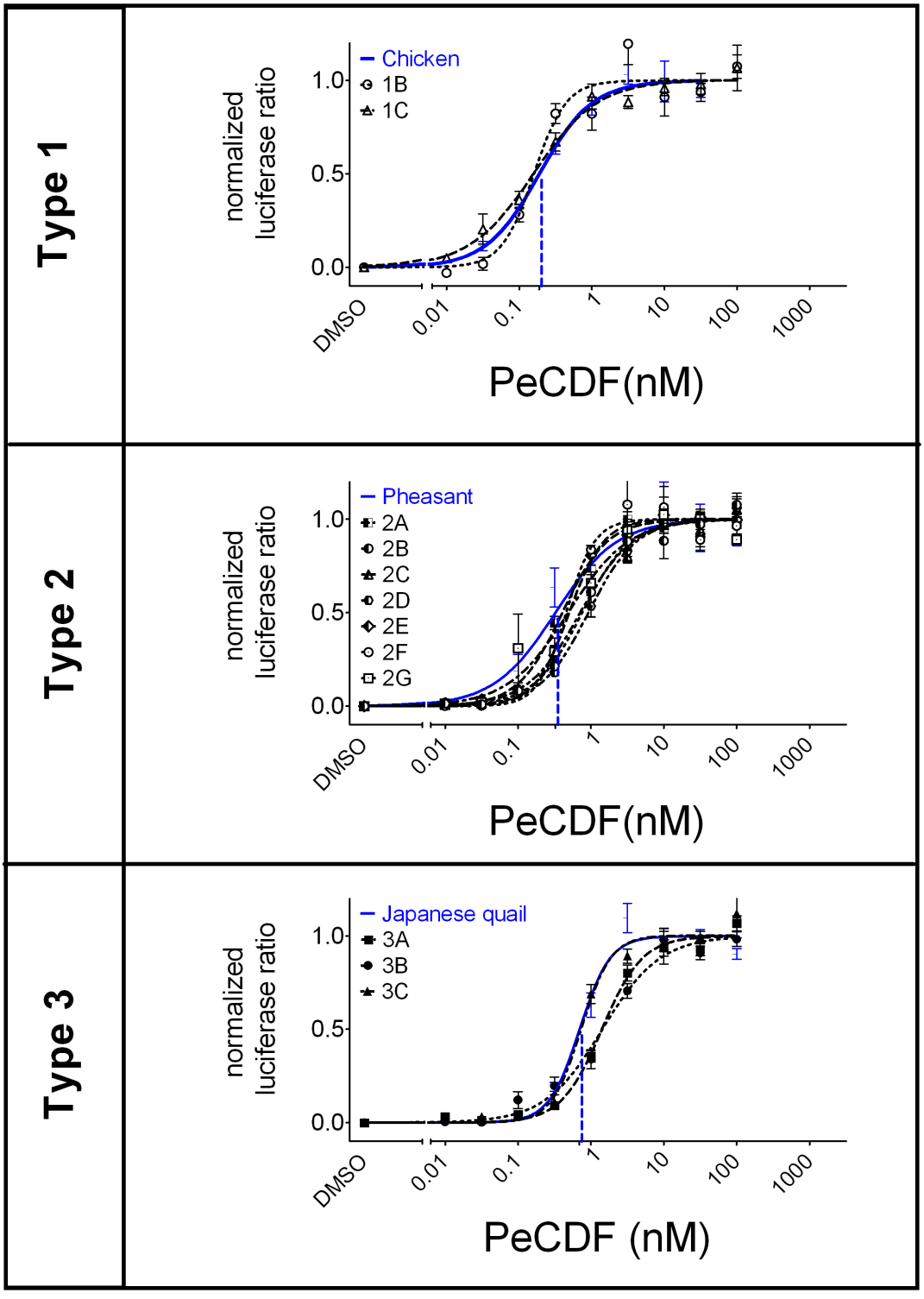


Figure 3.2 . Representative example of electrophoretic analysis of fluorescently-labelled AHR1 mutants that were expressed by *in vitro* transcription and translation (IVTT). In this example, AHR1 mutants 1B, 1C, 2B, 2C, 2D and 2E were labelled using FluoroTect Green$_{Lys}$ and loaded on a 10% SDS polyacrylaminde gel. A Typhoon 9210 Imager was used to detect the bands. Analysis of bands obtained from all full-length and mutant AHR1 constructs revealed no differences in the intensity among constructs, and all had similar migration properties

A


Type 1
normalized luciferase ratio
Chicken
1B
1C
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
TCDD(nM)
Type 2
normalized luciferase ratio
Pheasant
2A
2B
2C
2D
2E
2F
2G
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
TCDD(nM)
Type 3
normalized luciferase ratio
Japanese quail
3A
3B
3C
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
TCDD (nM)

B


Type 1
normalized luciferase ratio
Chicken
1B
1C
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
PeCDF(nM)
Type 2
normalized luciferase ratio
Pheasant
2A
2B
2C
2D
2E
2F
2G
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
PeCDF(nM)
Type 3
normalized luciferase ratio
Japanese quail
3A
3B
3C
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
PeCDF (nM)

C

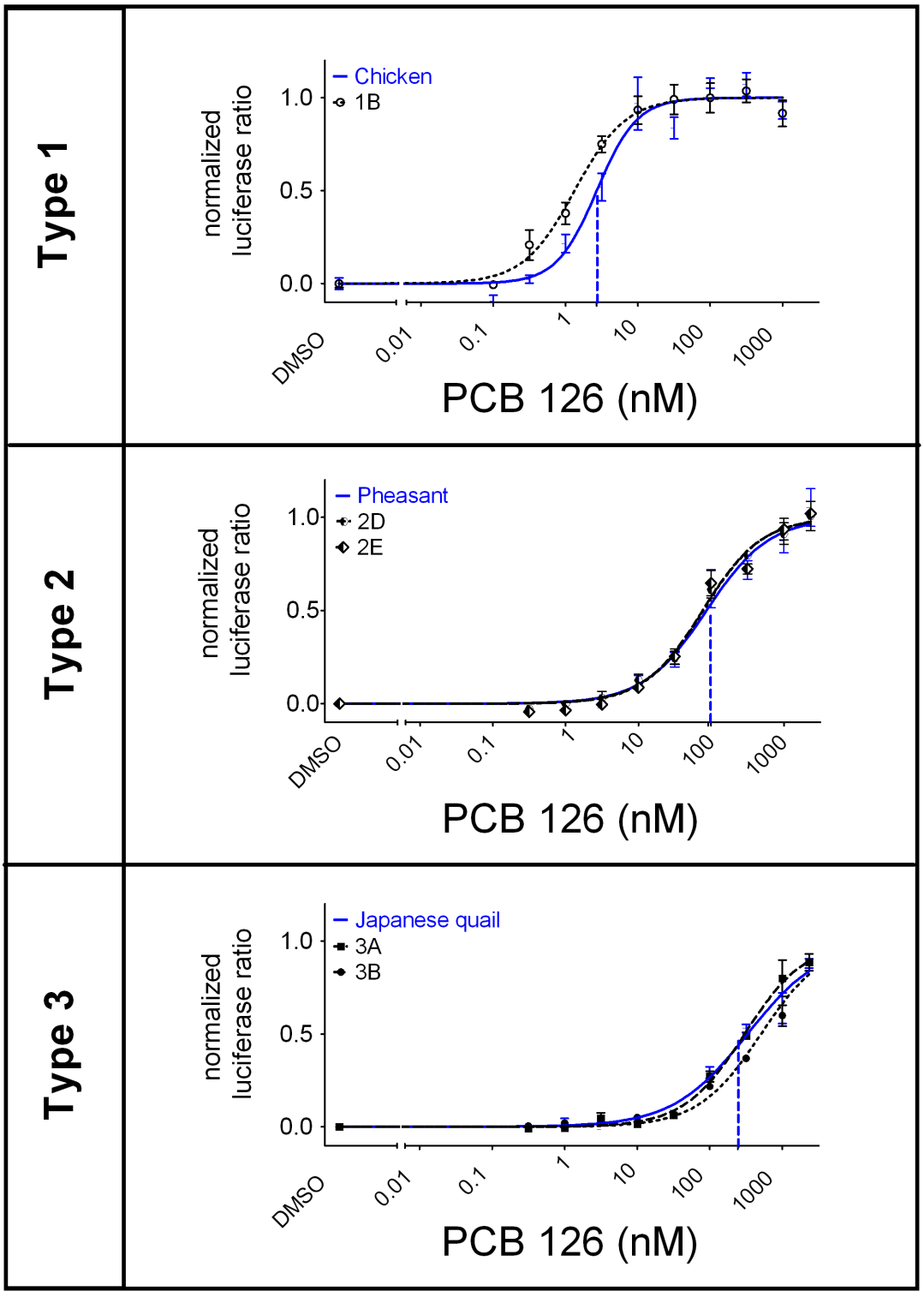

Type 1
normalized luciferase ratio
Chicken
1B
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
PCB 126 (nM)
Type 2
normalized luciferase ratio
Pheasant
2D
2E
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
PCB 126 (nM)
Type 3
normalized luciferase ratio
Japanese quail
3A
3B
1.0
0.5
0.0
DMSO
0.01
0.1
1
10
100
1000
PCB 126 (nM)

Figure 3.3. Representative example (study 1) of the concentration-dependent effects of TCDD (panel A), PeCDF (panel B) and PCB 126 (panel C) on aryl hydrocarbon response element (AHRE)-driven luciferase reporter gene activity in COS-7 cells transfected with either full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or mutant constructs of chicken AHR1 (designated 1B, 1C, etc; see Table 1 for details). Cells were exposed to DMSO or serial dilutions of TCDD, PeCDF or PCB 126 for 18-20 h, luciferase ratios (the ratio of firefly luminescence units to *Renilla* luminescence units) were determined, and data were normalized to the maximal response. Individual data points represent the mean ratio derived from four individual wells/concentration and bars represent SE. Each curve represents the average of four curves. The dashed vertical lines within each panel indicate EC_{50} values for the wild-type AHR1 constructs (chicken, ring-necked pheasant and Japanese quail).

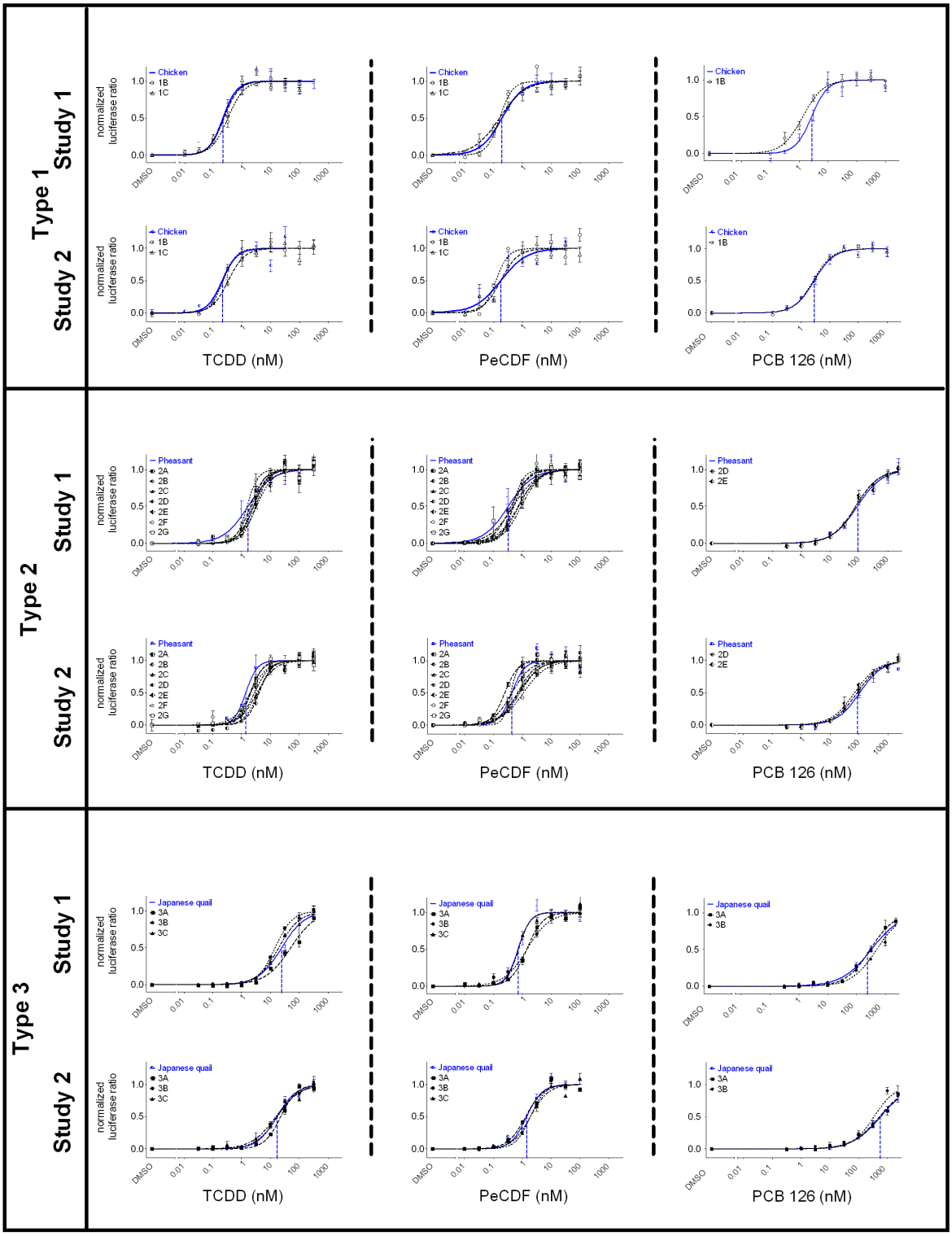

Type 1
Study 1
Study 2
Type 2
Study 1
Study 2
Type 3
Study 1
Study 2
normalized luciferase ratio
Chicken
1B
1C
Pheasant
2A
2B
2C
2D
2E
2F
2G
Japanese quail
3A
3B
3C
DMSO
0.01
0.1
1
10
100
1000
TCDD (nM)
PeCDF (nM)
PCB 126 (nM)

Figure 3.4. Concentration-dependent effects of TCDD, PeCDF and PCB 126 on aryl hydrocarbon response element (AHRE)-driven luciferase reporter gene activity in COS-7 cells transfected with either full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or mutant constructs of chicken AHR1 (designated 1B, 1C, etc; see Table 1 for details). Cells were exposed to DMSO or serial dilutions of TCDD, PeCDF or PCB 126 for 18-20 h, luciferase ratios (the ratio of firefly luminescence units to *Renilla* luminescence units) were determined, and data were normalized to maximal response. Individual data points represent the mean ratio derived from four individual wells/concentration and bars represent SE. Each curve represents the average of four curves. The dashed vertical lines within each panel indicate EC_{50} values for the wild-type AHR1 constructs (chicken, ring-necked pheasant and Japanese quail).

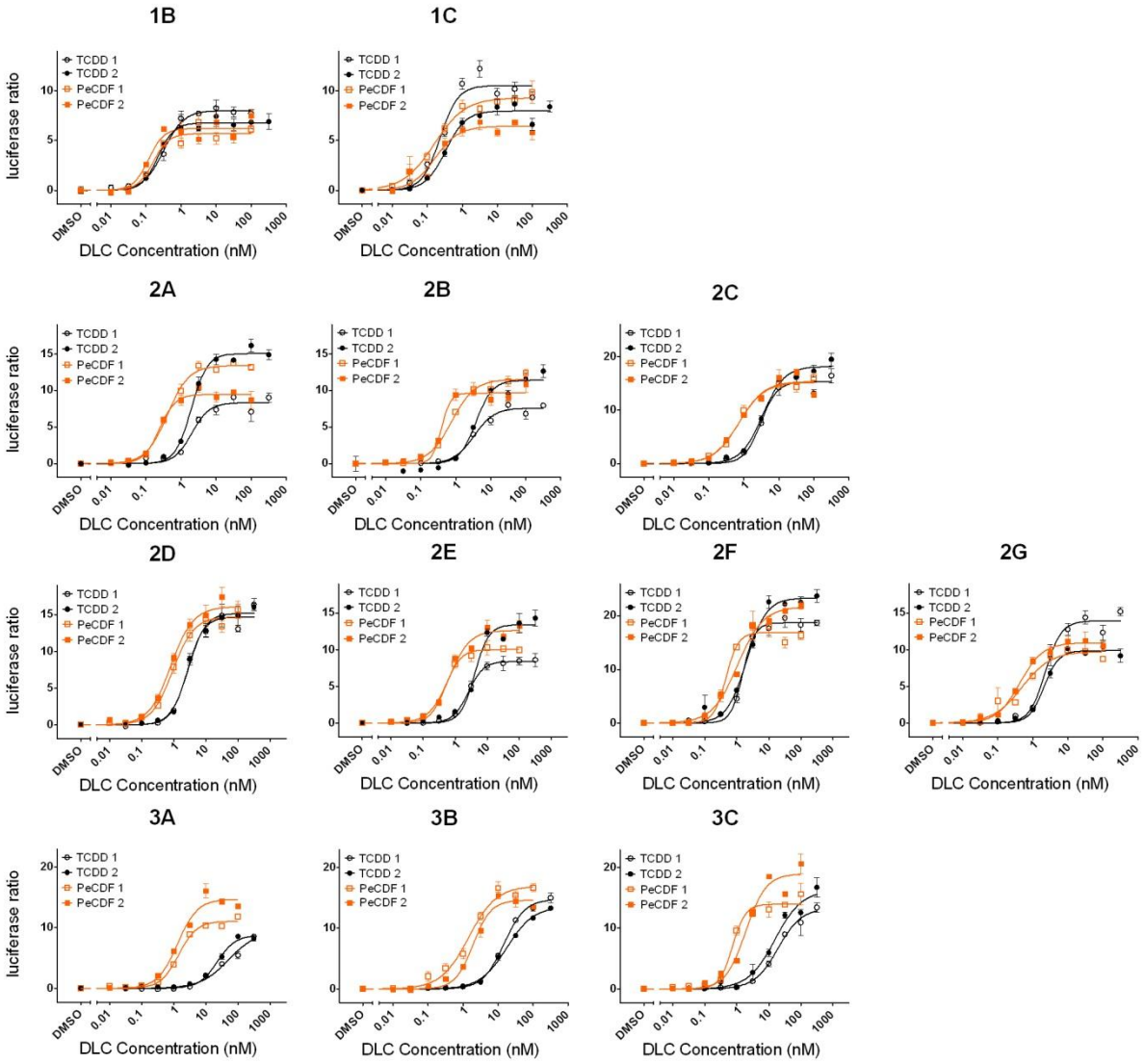


Figure 3.5. Concentration-dependent effects of TCDD and PeCDF on aryl hydrocarbon response element (AHRE)-driven luciferase reporter gene activity in COS-7 cells transfected mutant AHR1 constructs. Cells were exposed to DMSO or serial dilutions of TCDD or PeCDF for 18-20 h and luciferase ratios (the ratio of firefly luminescence units to *Renilla* luminescence units) were determined. Two studies were conducted for each compound and each construct. Individual data points represent the mean ratio derived from four individual wells/concentration and bars represent SE. Each curve represents the average of four curves.

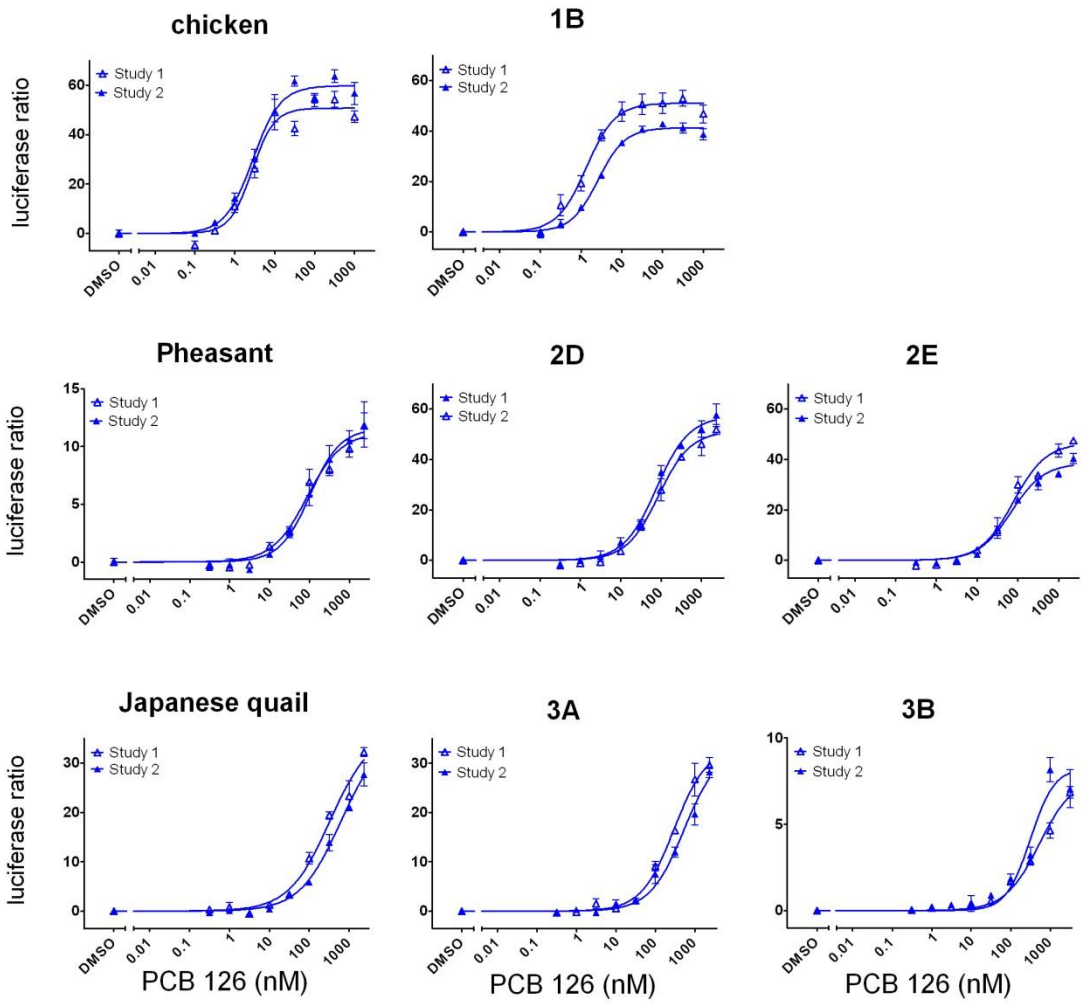


Figure 3.6. Concentration-dependent effects of PCB 126 on aryl hydrocarbon response element (AHRE)-driven luciferase reporter gene activity in COS-7 cells transfected with either (a) full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or (b) mutant AHR1 constructs. Cells were exposed to DMSO or serial dilutions of PCB 126 for 18-20 h and luciferase ratios (the ratio of firefly luminescence units to *Renilla* luminescence units) were determined. Two studies were conducted for each construct. Individual data points represent the mean ratio derived from four individual wells/concentration and bars represent SE. Each curve represents the average of four curves.

Table 3.3. $EC_{20,\ 50\ and\ 80}$ values that were calculated from concentration-response curves of COS-7 cells transfected with either (a) full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or (b) mutant AHR1 constructs and exposed to TCDD, PeCDF or PCB 126 for 18-20 h. Two separate studies were performed and in each study four $EC_{20,\ 50\ and\ 80}$ values were calculated from four concentration response curves. The EC values reported represent the mean of the four curves for each study. Three general classes of avian species (chicken-like, pheasant-like and quail-like) are indicated by white, light grey or dark grey, respectively. Data presented for chicken, pheasant and quail exposed to TCDD and PeCDF are from our previous report (Farmahin *et al.*, 2012).

chemical	*AHR1 construct*	*EC_{20} (nM)*		*EC_{50} (nM)*		*EC_{80} (nM)*	
		Study 1	*Study 2*	*Study 1*	*Study 2*	*Study 1*	*Study 2*
TCDD	Chicken	0.10	0.09	0.22	0.21	0.47	0.47
	1B	0.14	0.12	0.33	0.22	0.81	0.42
	1C	0.11	0.14	0.23	0.34	0.51	0.85
	Pheasant	0.44	0.74	1.7	1.4	6.2	2.6
	2A	0.91	0.92	2.3	2.0	5.8	4.9
	2B	1.4	1.8	3.4	3.6	8.7	7.5
	2C	1.5	1.4	3.3	3.8	7.5	12
	2D	1.2	1.3	2.8	3.2	6.6	9.0
	2E	1.4	2.1	2.5	4.0	4.6	7.9
	2F	0.85	0.71	1.6	2.0	3.4	6.1
	2G	1.2	1.1	2.4	2.1	4.9	4.1
	Quail	6.8	5.5	25	17	90	50
	3A	10	9	35	24	152	73
	3B	6	5	15	18	40	63
	3C	7	5	19	17	69	65
PeCDF	Chicken	0.060	0.040	0.19	0.17	0.61	0.74
	1B	0.080	0.082	0.19	0.11	0.51	0.15
	1C	0.039	0.094	0.18	0.18	1.0	0.36
	Pheasant	0.08	0.21	0.33	0.42	1.3	0.85
	2A	0.16	0.16	0.43	0.29	1.3	0.58
	2B	0.27	0.25	0.65	0.39	1.6	0.64
	2C	0.24	0.23	0.72	0.74	2.3	2.4
	2D	0.33	0.27	0.89	0.76	2.5	2.2
	2E	0.24	0.25	0.55	0.71	1.3	2.2
	2F	0.25	0.30	0.48	1.1	0.9	4.0
	2G	0.23	0.15	0.58	0.44	1.5	1.3
	Quail	0.36	0.59	0.70	1.4	1.4	3.4
	3A	0.62	0.49	1.4	1.4	3.5	3.8
	3B	0.43	0.88	1.4	1.9	5.0	4.5
	3C	0.39	0.73	0.88	1.8	2.3	4.5
PCB 126	Chicken	1.2	1.1	2.9	3.0	7.5	8.6
	1B	0.44	0.85	1.4	2.6	4.9	8.0
	Pheasant	23	34	95	91	464	301
	2D	19	25	70	90	335	273
	2E	22	24	82	75	182	261
	Quail	64	96	288	410	1356	1785
	3A	73	110	243	409	815	1581
	3B	107	108	620	347	3851	1061

Table 3.4. $ReS_{20,\ 50\ and\ 80}$ values were calculated based on the $EC_{20,\ 50\ and\ 80}$ values as follows: $EC_{20,\ 50\ and\ 80}$ (compound A) in chicken ÷ $EC_{20,\ 50,\ or\ 80}$ (compound A) in the AHR1 constructs of interest. Three general classes of avian species (chicken-like, pheasant-like and quail-like) are shown in white, light grey or dark grey, respectively.

	AHR1 construct	*ReS20*	*ReS_{50}*	*ReS_{80}*	*$ReS_{average}$*	*ReS_{20-80}*
	Chicken	1	1	1	1	1
	1B	0.66	0.77	0.82	0.75	0.66 - 0.82
	1C	0.77	0.75	0.81	0.78	0.75 - 0.81
	Pheasant	0.16	0.14	0.079	0.13	0.079 - 0.16
	2A	0.11	0.10	0.084	0.10	0.084 - 0.11
	2B	0.066	0.061	0.057	0.061	0.057 - 0.066
TCDD	2C	0.073	0.061	0.053	0.062	0.053 - 0.073
	2D	0.087	0.072	0.066	0.075	0.066 - 0.09
	2E	0.057	0.066	0.080	0.068	0.057 - 0.080
	2F	0.13	0.12	0.10	0.12	0.10 - 0.13
	2G	0.087	0.10	0.11	0.10	0.09 - 0.11
	Quail	0.016	0.010	0.0057	0.011	0.01 - 0.02
	3A	0.010	0.0073	0.0039	0.0072	0.0039 - 0.010
	3B	0.019	0.013	0.010	0.014	0.010 - 0.019
	3C	0.017	0.012	0.0060	0.012	0.0060 - 0.017
	Chicken	1	1	1	1	1
	1B	0.68	1.2	1.5	1.1	0.68 - 1.5
	1C	0.8	0.99	0.79	0.86	0.79 - 1.0
	Pheasant	0.34	0.48	0.46	0.43	0.34 - 0.48
	2A	0.34	0.50	0.72	0.52	0.34 - 0.72
	2B	0.21	0.35	0.61	0.39	0.21 - 0.61
PeCDF	2C	0.24	0.25	0.30	0.26	0.24 - 0.30
	2D	0.18	0.22	0.28	0.23	0.18 - 0.28
	2E	0.24	0.29	0.39	0.30	0.24 - 0.39
	2F	0.19	0.23	0.31	0.24	0.19 - 0.31
	2G	0.28	0.35	0.50	0.38	0.28 - 0.50
	Quail	0.11	0.17	0.31	0.20	0.11 - 0.31
	3A	0.10	0.13	0.20	0.14	0.10 - 0.20
	3B	0.08	0.11	0.16	0.12	0.08 - 0.16
	3C	0.10	0.14	0.18	0.14	0.10 - 0.18
	Chicken	1	1	1	1	1
	1B	1.8	1.5	1.3	1.5	1.3 - 1.8
	Pheasant	0.042	0.032	0.015	0.030	0.015 - 0.042
PCB 126	2D	0.052	0.037	0.020	0.036	0.020 - 0.052
	2E	0.052	0.038	0.032	0.041	0.032 - 0.052
	Quail	0.015	0.0085	0.0051	0.0094	0.0051 - 0.015
	3A	0.014	0.0091	0.0071	0.010	0.0071 - 0.014
	3B	0.011	0.006	0.0025	0.006	0.0025 - 0.011

Table 3.5. EC_{50}, ReS_{50} and ReP_{50} values calculated from the concentration-response curves obtained after exposure of COS-7 cells transfected with avian AHR1 constructs to TCDD, PeCDF or PCB 126. Two separate studies were performed and in each study four replicate wells/DLC concentration were included. The four concentration response curves and resulting EC50 values were calculated based on the four replicate wells. The mean EC_{50} values were derived from the four concentration-response curves/study. ReS_{50s} and ReP_{50s} were calculated using the mean EC_{50} values from Study 1 and Study 2. ReS is defined as $EC_{50\ (compound\ A)}$ of chicken construct ÷ $EC_{50\ (compound\ A)}$ of construct *X*. ReP is defined as EC_{50} of TCDD determined in construct *X* ÷ EC_{50} of PeCDF or PCB 126 determined for construct *X*. The data for wild-type chicken, pheasant and quail AHR1 constructs exposed to TCDD and PeCDF are from Farmahin *et al.* (2012).

chemical	AHR1 construct	$EC_{50\ (nM)}$ Study 1	$EC_{50\ (nM)}$ Study 2	ReS_{50}	ReP_{50}
TCDD	Chicken	0.22	0.21	1.0	1.0
	1B	0.33	0.22	0.77	1.0
	1C	0.23	0.34	0.75	1.0
	Pheasant	1.7	1.4	0.14	1.0
	2A	2.3	2.0	0.10	1.0
	2B	3.4	3.6	0.061	1.0
	2C	3.3	3.8	0.061	1.0
	2D	2.8	3.2	0.072	1.0
	2E	2.5	4.0	0.066	1.0
	2F	1.6	2.0	0.12	1.0
	2G	2.4	2.1	0.10	1.0
	Quail	25	17	0.010	1.0
	3A	35	24	0.0073	1.0
	3B	15	18	0.013	1.0
	3C	19	17	0.012	1.0
PeCDF	Chicken	0.19	0.17	1.0	1.2
	1B	0.19	0.11	1.2	1.9
	1C	0.18	0.18	0.99	1.6
	Pheasant	0.33	0.42	0.48	4.1
	2A	0.43	0.29	0.50	6.0
	2B	0.65	0.39	0.35	6.8
	2C	0.72	0.74	0.25	4.8
	2D	0.89	0.76	0.22	3.6
	2E	0.55	0.71	0.29	5.2
	2F	0.48	1.1	0.23	2.3
	2G	0.58	0.44	0.35	4.4
	Quail	0.70	1.4	0.17	20
	3A	1.4	1.4	0.13	21
	3B	1.4	1.9	0.11	10
	3C	0.88	1.8	0.14	14
PCB 126	Chicken	2.9	3.0	1.0	0.072
	1B	1.4	2.6	1.5	0.14
	Pheasant	95	91	0.032	0.017
	2D	70	90	0.037	0.038
	2E	82	75	0.038	0.042
	Quail	288	410	0.0085	0.060
	3A	243	409	0.0091	0.090
	3B	620	347	0.006	0.035

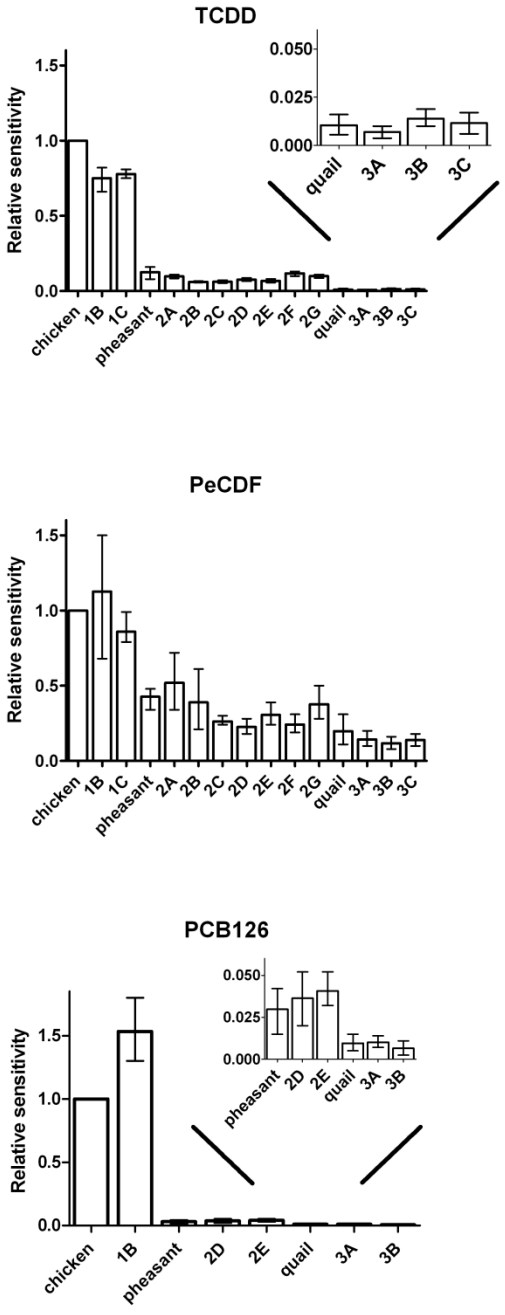


Figure 3.7. Relative sensitivity (ReS) values of COS-7 cells transfected with either (a) full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or (b) mutant AHR1 constructs and exposed to TCDD, PeCDF or PCB 126 for 18-20 h.

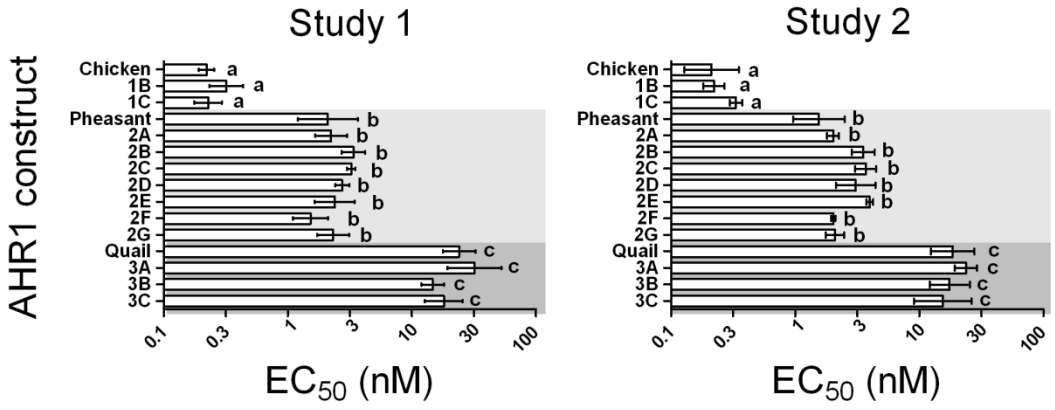


Figure 3.8. Comparison of EC_{50} values (mean ± SD), derived from four concentration-response curves/study, obtained in COS-7 cells transfected with either (a) full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or (b) mutant AHR1 constructs and exposed to TCDD for 18-20 h. Letters indicate significant differences (one-way ANOVA) among AHR1 constructs (n=4; $p < 0.001$). Three general classes of avian species (chicken-like, pheasant-like and quail-like) are indicated in white, light grey or dark grey, respectively.

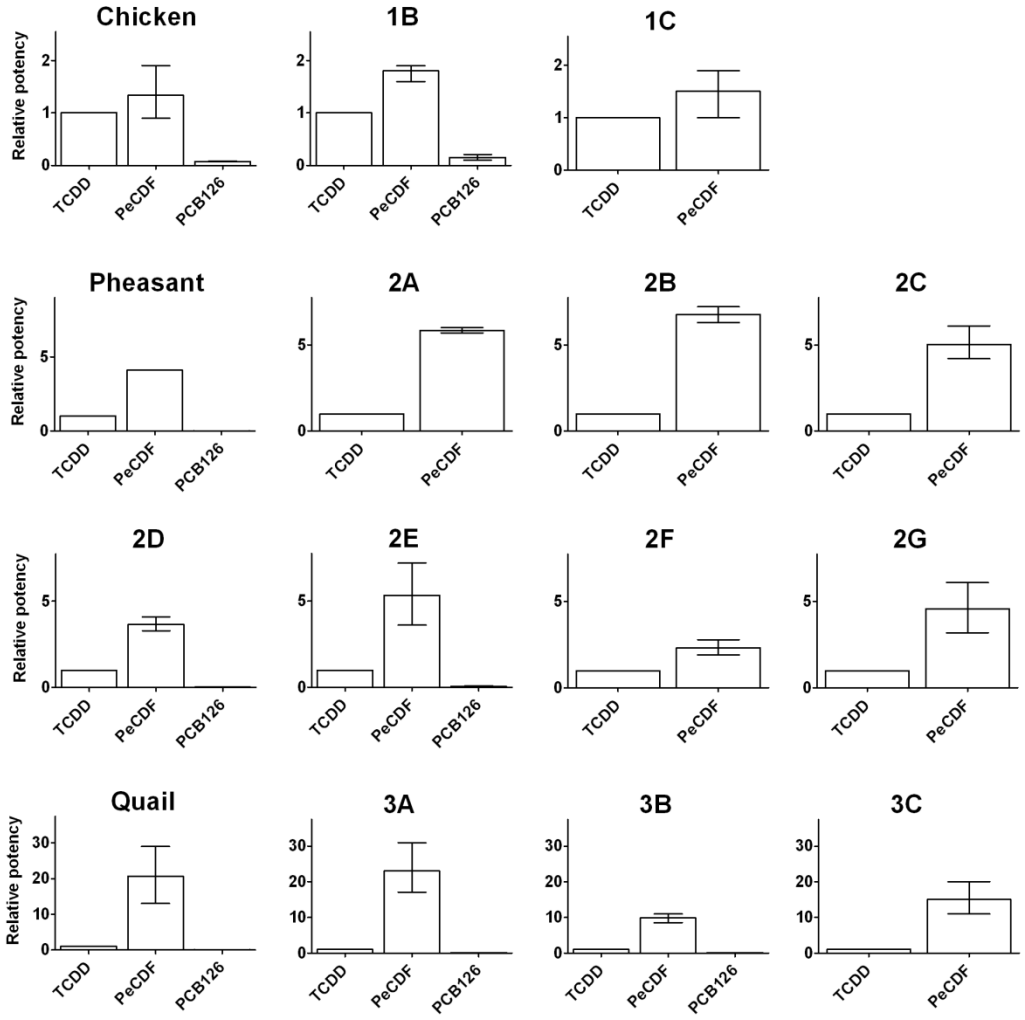


Figure 3.9. Relative potency (ReP) values of TCDD, PeCDF and PCB 126 in COS-7 cells transfected with either (a) full-length AHR1 constructs of chicken, ring-necked pheasant, Japanese quail or (b) mutant AHR1 constructs and exposed to TCDD, PeCDF or PCB 126 for 18-20 h. Bars represent $ReP_{(average)}$ (the average of $ReP_{20,\ 50,\ and\ 80}$ values) and error bars indicate the maximum and minimum $ReP_{20,\ 50,\ and\ 80}$ values.

Table 3.6. $ReP_{20,\ 50\ and\ 80}$ values were calculated as follows: $EC_{20,\ 50\ or\ 80}$ (species A) of TCDD ÷ $EC_{20,\ 50\ and\ 80}$ (species A) of PeCDF (or PCB 126). Three general classes of avian species (chicken-like, pheasant-like and quail-like) are indicated by white, light grey or dark grey, respectively.

AHR1 construct	*chemical*	*ReP_{20}*	*ReP_{50}*	*ReP_{80}*	*$ReP_{average}$*	*$ReP_{20\text{-}80}$*
Chicken	TCDD	1	1	1	1	1
	PeCDF	1.9	1.2	0.9	1.3	0.90 - 1.9
	PCB 126	0.08	0.072	0.075	0.077	0.07 - 0.1
1B	TCDD	1	1	1	1	1
	PeCDF	1.6	1.9	1.9	1.8	1.6 - 1.9
	PCB 126	0.21	0.14	0.10	0.15	0.10 - 0.21
1C	TCDD	1	1	1	1	1
	PeCDF	1.9	1.6	1.0	1.5	0.99 - 1.9
Pheasant	TCDD	1	1	1	1	1
	PeCDF	4.1	4.1	4.1	4.1	4.1 - 4.1
	PCB 126	0.021	0.017	0.012	0.016	0.012 - 0.021
2A	TCDD	1	1	1	1	1
	PeCDF	5.7	6.0	5.8	5.8	5.7 - 6.0
2B	TCDD	1	1	1	1	1
	PeCDF	6.3	6.8	7.2	6.7	6.3 - 7.2
2C	TCDD	1	1	1	1	1
	PeCDF	6.1	4.8	4.2	5.0	4.2 - 6.1
2D	TCDD	1	1	1	1	1
	PeCDF	4.1	3.6	3.3	3.7	3.3 - 4.1
	PCB 126	0.056	0.038	0.026	0.040	0.026 - 0.056
2E	TCDD	1	1	1	1	1
	PeCDF	7.2	5.2	3.6	5.3	3.6 - 7.2
	PCB 126	0.076	0.042	0.028	0.049	0.028 - 0.076
2F	TCDD	1	1	1	1	1
	PeCDF	2.8	2.3	1.9	2.4	1.9 - 2.8
2G	TCDD	1	1	1	1	1
	PeCDF	6.1	4.4	3.2	4.6	3.2 - 6.1
Quail	TCDD	1	1	1	1	1
	PeCDF	13	20	29	21	13 - 29
	PCB 126	0.077	0.060	0.045	0.061	0.045 - 0.077
3A	TCDD	1	1	1	1	1
	PeCDF	17	21	31	23	17 - 31
	PCB 126	0.10	0.090	0.094	0.096	0.090 - 0.10
3B	TCDD	1	1	1	1	1
	PeCDF	8.5	9.9	11	10	8.5 - 11
	PCB 126	0.052	0.035	0.021	0.036	0.021 - 0.052
3C	TCDD	1	1	1	1	1
	PeCDF	11	14	20	15	11 - 20

Table 3.7. Comparison of EC_{50} values (nM) derived using the LRG assay and LD_{50} values (pmol/g egg) reported from egg injection studies for various avian AHR1 constructs and species.
* The Eastern bluebird LD_{50} is between 3.1 pmol TCDD/g egg (no observable adverse effect level) and 31 pmol TCDD/g egg (100% mortality).
** The chicken LD_{50} value was calculated based on a review of several egg injection studies (Head *et al.*, 2008).

chemical	*AHR1 construct*	*LRG assay*	*species*	*Egg injection study*
		EC_{50} (nM)		*LD_{50} (pmol/g egg)*
TCDD	chicken [a]	0.21	chicken[c]	0.66
	pheasant[a]	1.5	pheasant[c]	3.5
	2E[b]	3.3	Eastern bluebird[d]	3.1-31*
	2G[b]	2.2	pheasant[c]	3.5
	Japanese quail[a]	21	Japanese quail[c]	30
	3A[b]	30	Japanese quail[c]	30
	3B[b]	17	cormorant[e]	12.4
PeCDF	chicken [a]	0.18	chicken[c]	0.75
	pheasant[a]	0.37	pheasant[c]	0.60
	2G[b]	0.51	pheasant[c]	0.60
	Japanese quail[a]	1.1	Japanese quail[c]	4.9
	3A[b]	1.4	Japanese quail[c]	4.9
PCB 126 [a]	chicken[b]	2.8	chicken[f]	3.4**
	3B[b]	483	American kestrel[g]	197
	3B[b]	483	common tern[g]	351
	3B[b]	483	cormorant[e]	542

(Farmahin *et al.*, 2012); [b] Current Study; [c] (Cohen-Barnhouse *et al.*, 2011);
[d] (Thiel *et al.*, 1988); [e](Powell *et al.*, 1998) ; [f](Head *et al.*, 2008) ; [g](Hoffman *et al.*, 1998)

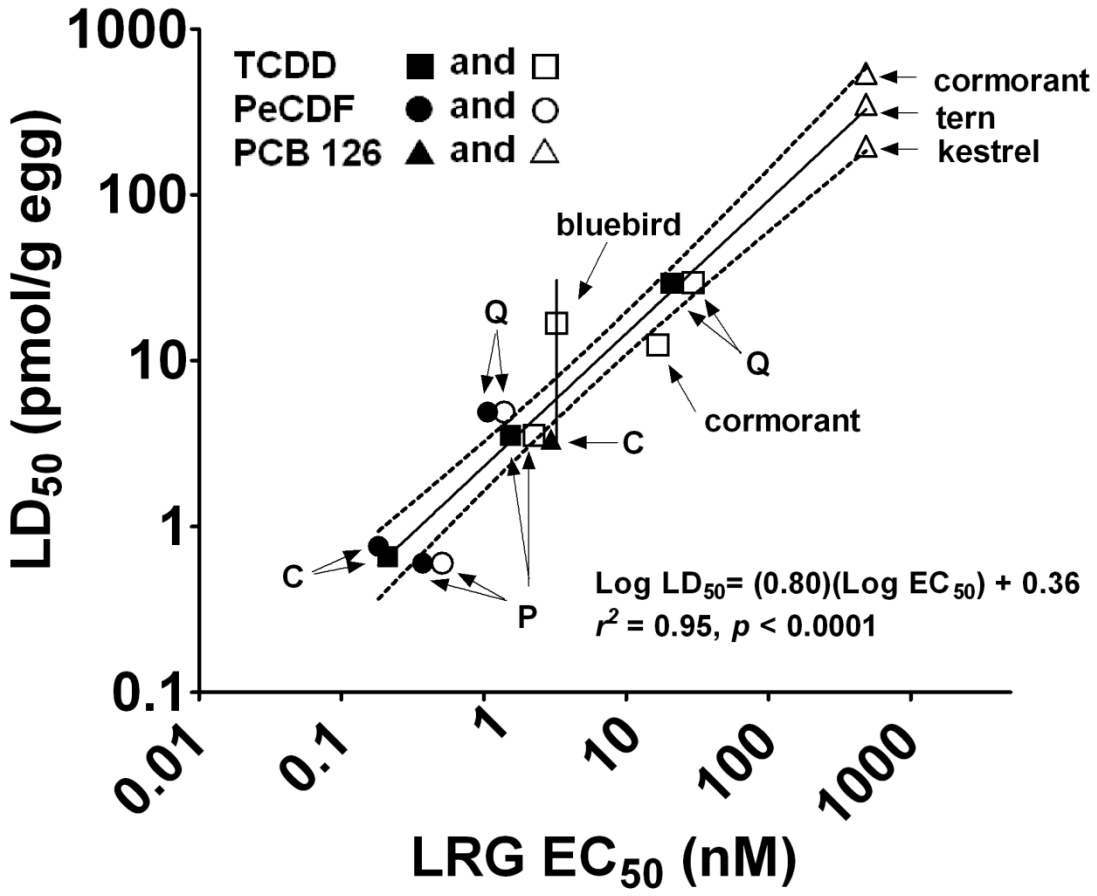


Figure 3.10. Correlation between LD_{50} data for TCDD, PeCDF and PCB 126 obtained from egg injection studies (sources for LD_{50} data are indicated in Table S5) and EC_{50} data from the luciferase reporter gene (LRG) assay. Closed symbols represent EC_{50} data for full-length (wild-type) AHR1 constructs for chicken (C), ring-necked pheasant (P) and Japanese quail (Q). Open symbols represent EC_{50} data for mutant constructs of chicken AHR1 (cormorant, tern, kestrel, and bluebird). For example, the open symbol for bluebird represents the EC_{50} for construct chicken DBD_2E LBD_chicken TAD (DBD, DNA-binding domain; LBD, ligand-binding domain; TAD, transactivation domain). For pheasant and quail, there are both closed and open symbols; open symbols represent the chicken AHR1 mutant that includes the pheasant or quail LBD sequence. The dotted lines represent the 95% confidence intervals.

Table 3.8. Predicted LD_{50s} (95% confidence intervals) of TCDD, PeCDF and PCB 126 for the 3 main avian types calculated from the regression line shown in Figure 3.

compound	*avian type*	*predicted LD_{50} (pmol/g egg)*	*predicted LD_{50} (ng/g egg)*
TCDD	Type 1	0.78 (0.40 - 1.4)	0.26 (0.13 - 0.45)
	Type 2	5.0 (2.2 - 10)	1.7 (0.73 - 3.4)
	Type 3	27 (11 - 71)	9 (3.8 - 24)
PeCDF	Type 1	0.56 (0.29 - 1.0)	0.19 (0.10 - 0.34)
	Type 2	1.5 (0.64 - 2.8)	0.51 (0.22 - 1.0)
	Type 3	2.9 (1.7 - 5.2)	1.0 (0.58 - 1.8)
PCB 126	Type 1	4.7 (2.6 - 8.7)	1.5 (0.86 - 2.8)
	Type 2	80 (33 - 203)	26 (11 – 66)
	Type 3	273 (90 - 911)	89 (29 - 297)

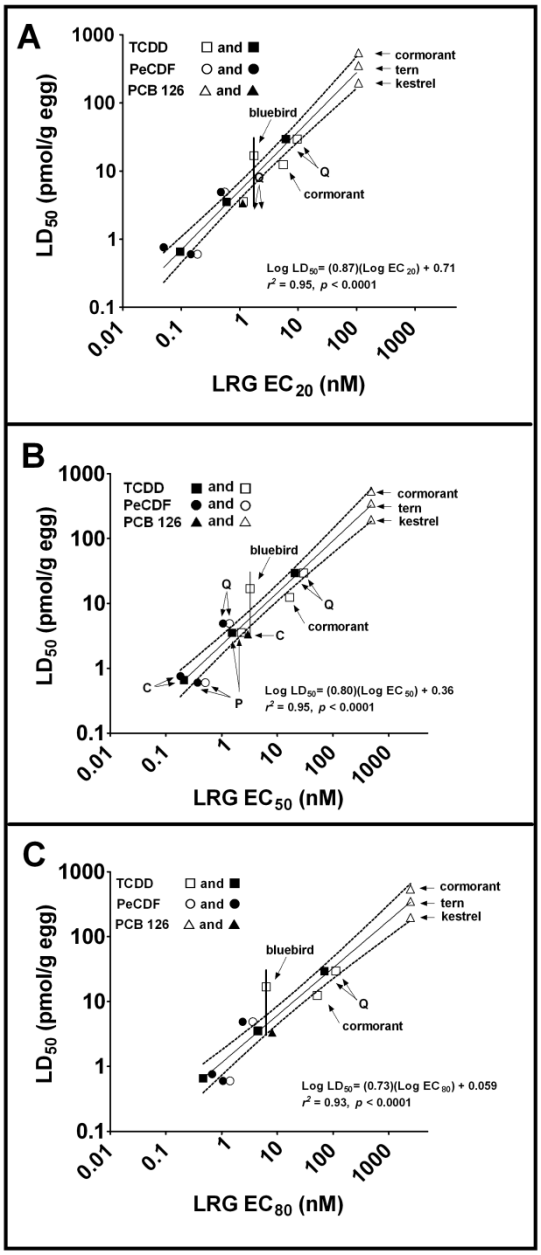


Figure 3.11. Correlation between LD_{50} data from avian egg injection studies and $EC_{20,}$ $EC_{50\ and}$ EC_{80} data from the luciferase reporter gene (LRG) assay for TCDD, PeCDF and PCB 126. EC_{50} values obtained from cells transfected with wild AHR1 constructs (closed symbols) and mutant AHR1 constructs (open symbols) are indicated.

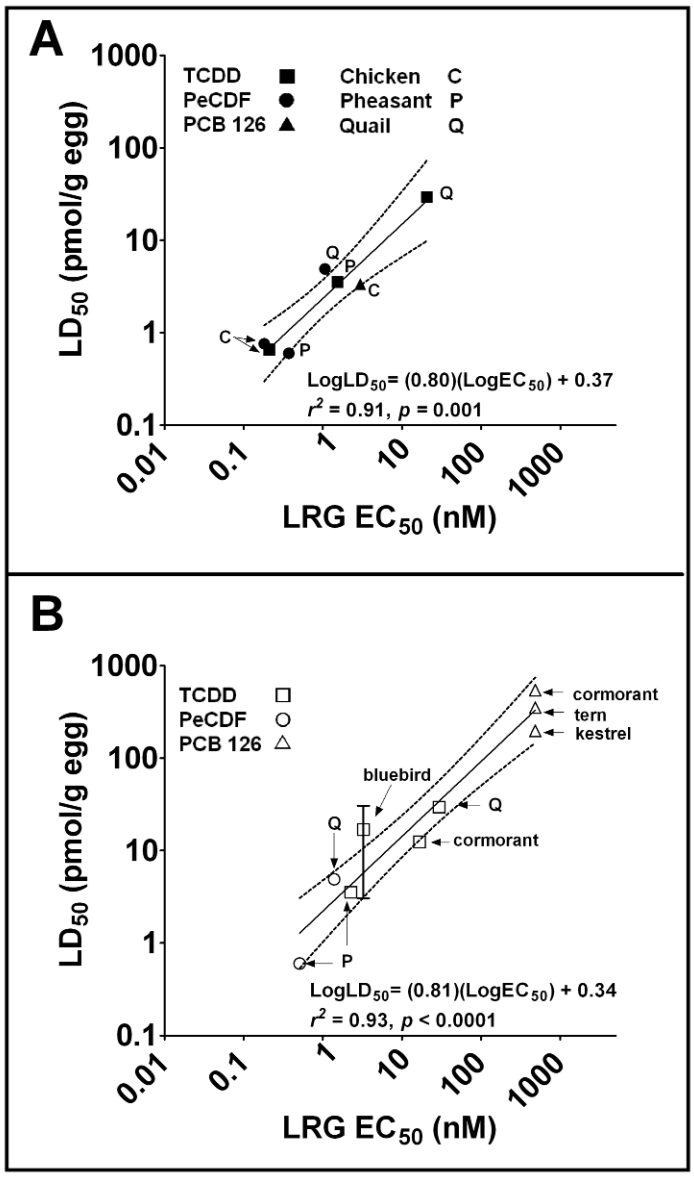


Figure 3.12. Correlation between LD_{50} data from avian egg injection studies and EC_{50} data from the luciferase reporter gene (LRG) assay using wild-type AHR1 constructs (panel A) and mutant AHR1 constructs (panel B) for TCDD, PeCDF and PCB 126.

3.4.4. Homology modeling

Homology modeling of avian AHR1 LBD resulted in the models shown in Figures 3.13 and 3.14. PSI-BLAST searches revealed that of all of the proteins in the database, the ones with greatest sequence identity with chicken, ring-necked pheasant and Japanese quail AHR1 were (a) HIF-2α (PDB ID: 1P97) and (b) a docked complex containing HIF-2α and ARNT (PDB ID: 2A24). Pairwise sequence identities among avian AHR1 LBDs and 1P97 and 2A24 (Table 3.9) were similar to those reported for mammalian AHRs and HIF-2α (Pandini et al., 2009). The NMR structures of 1P97 and 2A24 were selected as templates to construct three-dimensional structures of avian AHR1 LBD. These templates were also used by others to construct AHR LBD models for mouse (Miyagi et al., 2012) and rat (Yoshikawa et al., 2010). The region between amino acids 283 and 389 of avian AHR1 was used to develop two-template models for chicken, pheasant and quail AHR1 LBDs. The 1P97 and 2A24 PBD files each contained 20 different candidate structures. The most representative candidate structures determined by ORLANDO were structure 17 for 1P97 and structure 11 for 2A24.

The ProSA z-scores were -4, -3.5 and -3.9 for chicken, pheasant and quail, respectively (Figure 3.15, panel A). The z-score indicates overall model quality and measures the deviation of the total energy of the structure from an energy distribution derived from random conformations (Wiederstein and Sippl, 2007). The z-scores for the three avian AHR1 LBD structures are within the range of z-scores typically found for protein NMR structures of similar size. The three avian models passed all criteria implemented by PROCHECK (which determines the

stereo-chemical quality of models). Approximately 93%, 88% and 89% of amino acid residues in chicken, pheasant, and quail, respectively, reside in the “most favored” areas of the Ramachandran plots (90% for structures solved at a resolution of 2.0 Å), with only one residue (HIS325) in a “disallowed” region (Figure 3.15, panel B). The overall G-factors, which measure stereochemical quality, were -0.12, -0.15 and -0.19 (from -0.5 to 0.3 for structures solved at 1.5 Å resolution) for chicken, pheasant and quail, respectively.

The structures of chicken, pheasant and quail AHR1 LBD models were aligned with 1P97 and 2A24 by use of the DALI (Holm and Rosenstrom, 2010) server (Figure 3.13, panel A). Chicken, pheasant and quail models each contained five β-sheets and a central helix. The models of the AHR1 LBDs of quail, chicken and pheasant contained one, two or three short helices, respectively (Figure 3.13, panel A). The nomenclature (Gong et al., 1998) for helices and beta sheets of the FixL protein PAS domain was used for avian AHR1 LBD models. The avian AHR1 LBD models were aligned using (1) the Multiprot server for visual comparison (Figure 3.16) and (2) the DALI server for obtaining average pairwise root-mean-square deviation (RMSD) values. The results showed high similarity between chicken, pheasant and quail with RMSD values ranging from 0.5 - 0.7 Å. RMSD is the measure of the average distance between the atoms of superimposed proteins and shows the similarity between two structures. For two perfectly identical structures, the RMSD value would be 0 Å; for two randomly chosen dissimilar proteins, the RMSD would likely be 10 Å or greater. The side chains of amino acids at positions 324 and 380 in chicken, pheasant and quail models face within the binding pocket, but the side chains of

amino acids at positions 297 and 337 face away from the binding pocket (Figure 3.13, panel B).

Potential binding site locations were identified by use of the Molegro cavity detection algorithm, and ligand-binding sites for chicken, pheasant and quail were located close to the centre of the side chains of amino acids 324 and 380 (Figure 3.14). The main cavities, identified by use of CASTp, were buried in the core of the avian models, and were delimited by the helices, β-sheets and their connecting loops. A list of amino acid residues with side chains that contribute to the internal cavity surface in the avian models was extracted from the CASTp output and compared (Figure 3.13, panel C). The cavity volumes were found to be 399, 501 and 542 $Å^3$ for chicken, pheasant and quail, respectively. *In silico* mutagenesis was performed and the chicken AHR1 LBD model was targeted at positions 324 and 380 to generate three mutant models (I324V, S380A and I324_S380A). CASTp analysis showed that mutation of amino acid 324 from Ile to Val (I324V) increased the cavity volume of the chicken AHR1 LBD model from 399 $Å^3$ to 485 $Å^3$. My results also show that the amino acid at position 380 contributes to cavity volume. For example, mutation at position 380 (serine to alanine; S380A) caused expansion of the cavity volume of chicken from 399 $Å^3$ to 465 $Å^3$. When both positions 324 and 380 were changed (I324V_S380A), the cavity volume of mutant chicken AHR1 LBD was 528 $Å^3$ (Table 3.10).

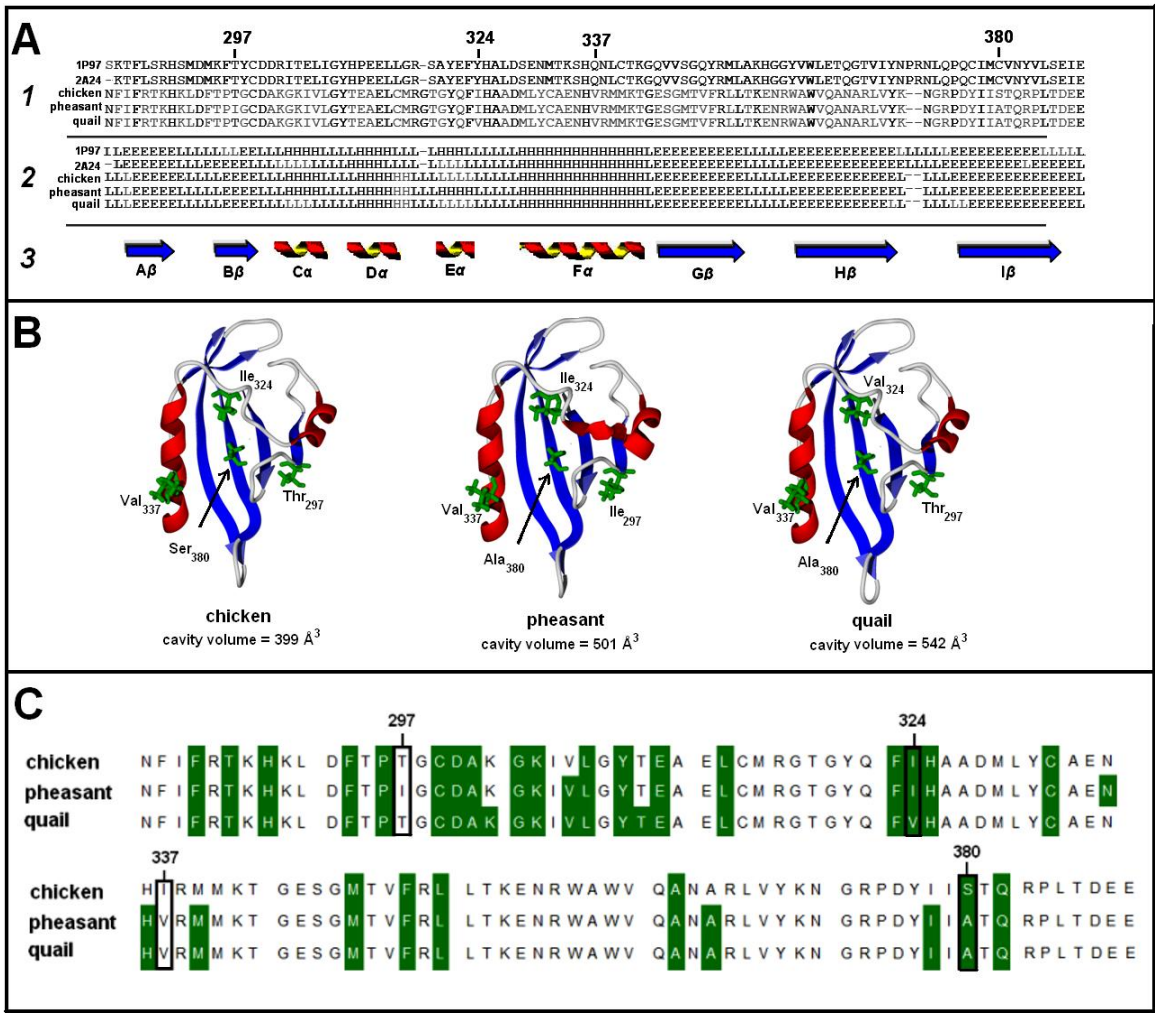


Figure 3.13. ***(A)*** Alignments of chicken, ring-necked pheasant and Japanese quail AHR1 LBD sequences with HIF-2α secondary structure templates 1P97A and 2A24 that were obtained using the DALI server. *Sub-section 1*: The amino acid sequence alignments of 1P97A (structure 17), 2A24 (structure 11), chicken, pheasant and quail. *Sub-section 2:* The secondary structure assignments obtained by use of the Define Secondary Structure of Proteins (DSSP) algorithm (H/h: helix, E/e: strand, L/l: coil). *Sub-section 3:* Illustration of the secondary structure labelled with conventional PAS domain structure nomenclature (Gong *et al.*, 1998). ***(B)*** Cartoon representations of chicken, ring-necked pheasant and Japanese quail structural models of the AHR1 LBD. The amino acids at positions 297, 324, 337 and 380 are indicated, and those at 324 and 380 point into the cavity, while amino acids 297 and 337 point away from the cavity. The volumes of the main cavities in the Connolly's molecular surface calculated by CASTp are indicated for the three avian species. ***(C)*** Identification of amino acids with side chains that contribute to the AHR1 ligand binding cavity. The amino acid residues that contribute to the internal cavities (highlighted in green) of chicken, pheasant, and quail AHR1 LBD models were identified by use of CASTp. Amino acids at position 297, 324, 337 and 380 are shown within black boxes.

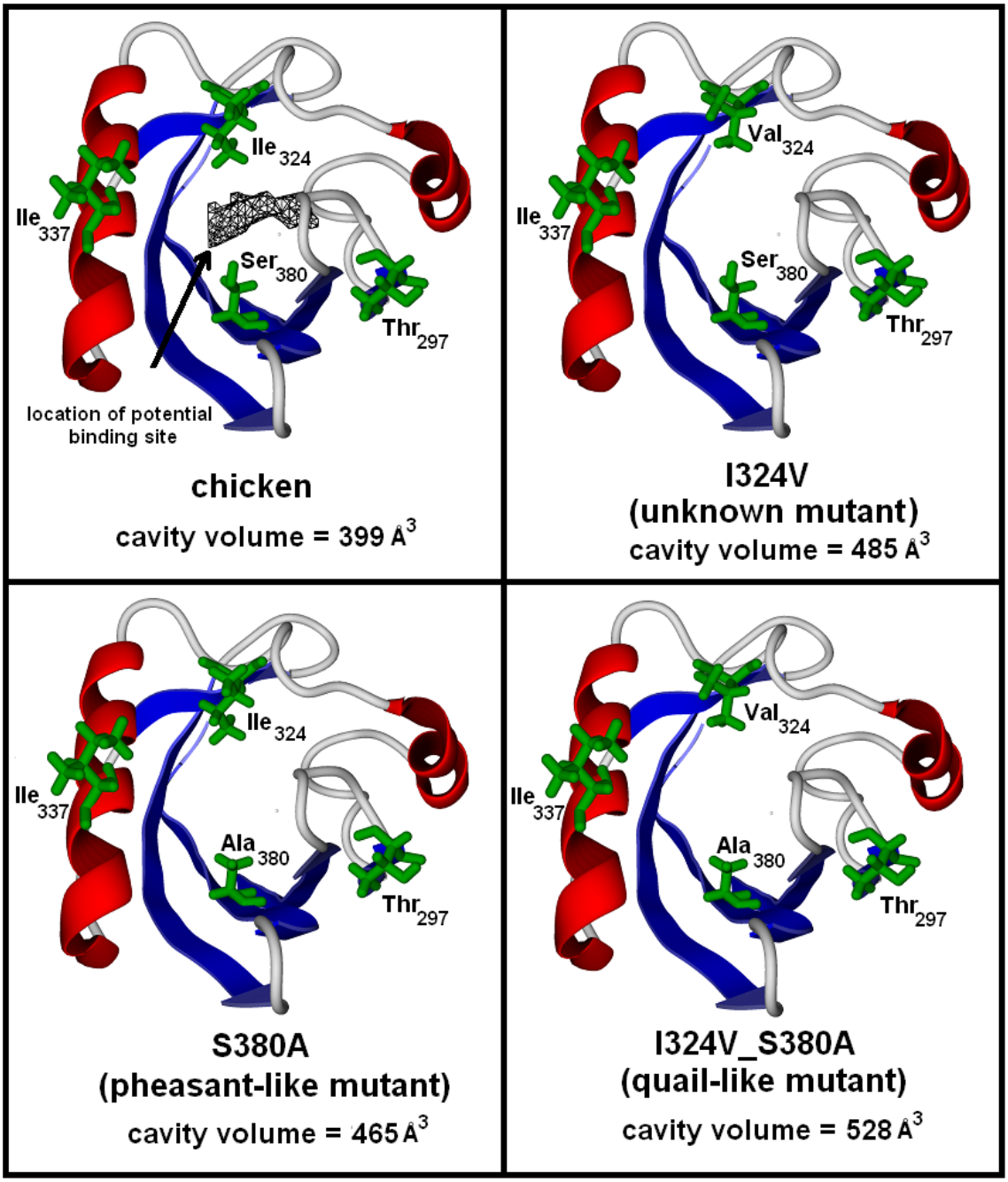


Figure 3.14. Cartoon representations of structural models of chicken and three mutant (I324V, S380A and I324V_S380A) AHR1 LBDs. The location of the potential binding site in the chicken AHR1 LBD model was determined by use of the Molegro software. Volumes of the main cavities in the Connolly's molecular surface, calculated by CASTp, are indicated. The four key amino acid locations are shown; the amino acids at positions 324 and 380 point inward and those at positions 297 and 337 point outward.

Table 3.9. The percent identity and E-values among avian AHR1 LBDs and 1P97 and 2A24. For example, the E-value for chicken AHR1 LBD alignment to 1P97, which indicates the probability that the alignment occured by chance, is 8.9×10^{-42}. Chicken, ring-necked pheasant and Japanese quail AHR1 LBD sequences (residues 235-402) were searched against the PDB using PSI-BLAST to identify the templates. The most significant alignments were HIF-2α (PDB ID: 1P97) and the complex of HIF-2α + ARNT (PDB ID: 2A24).

	1P97		**2A24**	
	Identity (%)	*E*-value	Identity (%)	*E*-value
Chicken	26	8.9×10^{-42}	26	8.9×10^{-42}
Pheasant	25	4.4×10^{-40}	25	5.1×10^{-40}
Quail	26	3.1×10^{-40}	26	3.1×10^{-40}

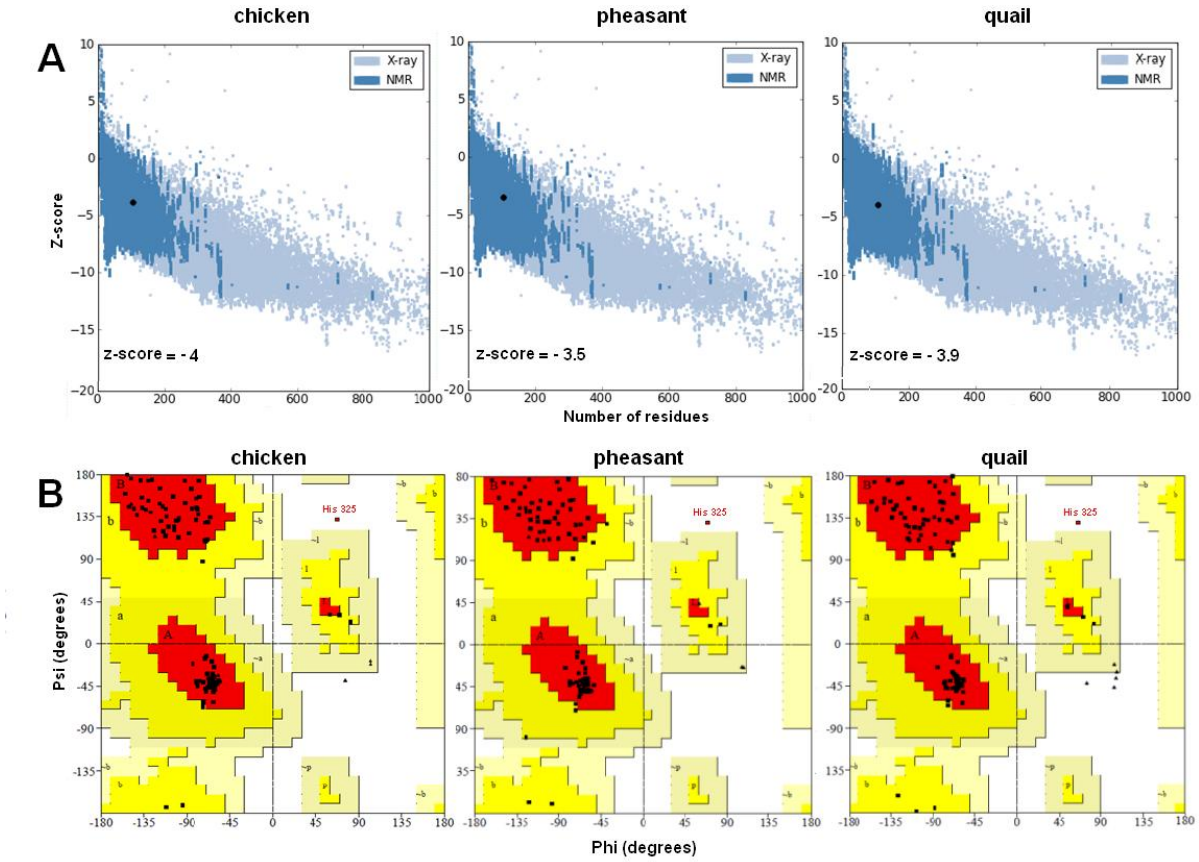


Figure 3.15. ***(A)*** ProSA-web analysis of overall Z-scores for chicken, pheasant and quail AHR1 LBD models. ***(B)*** Ramachandran plots of the phi-psi torsion angles for all residues in the structures of chicken, pheasant and quail AHR1 LBDs models determined using PROCHECK. Triangles represent glycine and proline residues, and squares indicate all other amino acids. 92.6%, 88.3% and 89.4% of all amino acids have torsion angles in the most favoured region (red background) for chicken, pheasant and quail AHR1 LBDs, respectively. The percent amino acids in the additional allowed region (yellow background) were 6.4%, 10.6% and 9.6 for chicken, pheasant and quail AHR1 LBDs, respectively. There were no amino acids in the generously allowed region (light yellow background) and only 1.1% in the disallowed region (white background).

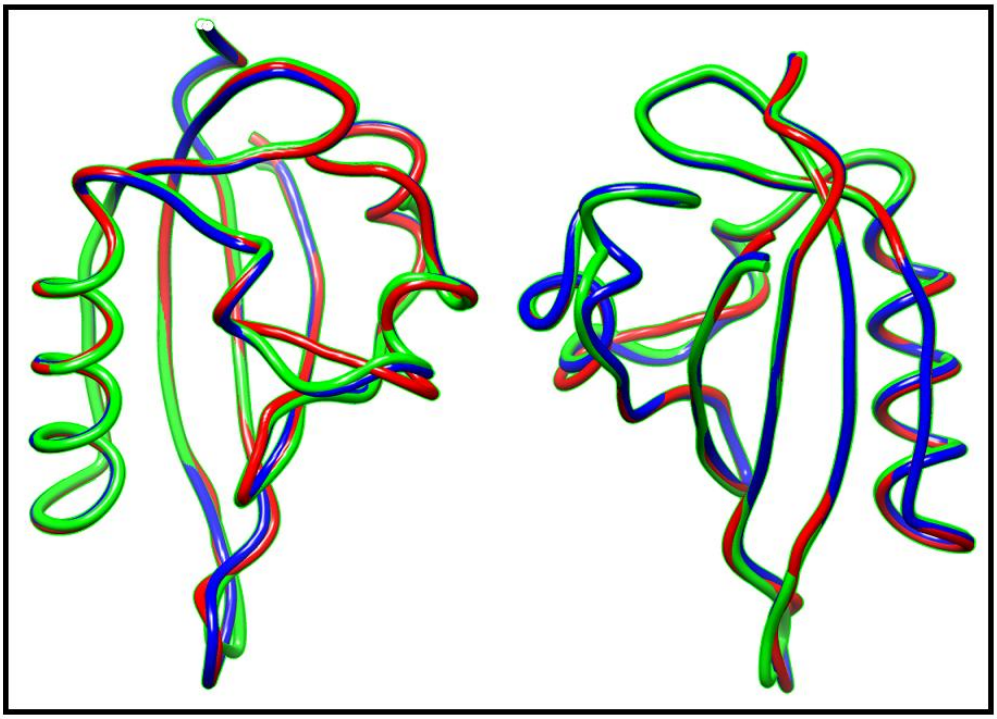


Figure 3.16. Two orientations of the structural alignments of chicken (blue), pheasant (red) and quail (green) that were obtained by use of the MultiProt web server and visualized by use of UCSF chimera.

Table 3.10. Surface area and volume measurements of the main cavity in the Connolly's molecular surface, as obtained by CASTp for wild-type (chicken, pheasant, quail) and mutant (I324V, S380A, and I324V_S380A) AHR1 LBD constructs. The chicken AHR1 LBD was used to generate mutants by use of in silico mutagenesis.

	surface (Å^2)	*volume (Å^3)*
chicken AHR1	421	399
I324V (unknown mutant)	535	485
pheasant	500	501
S380A (pheasant-like)	477	465
quail	572	542
I324V_S380A (quail-like)	519	528

3.4.5. The six variable amino acids within AHR1 LBD

3.4.5.1. Site 256

Site 256 was not within the templates (1P97 and 2A24) used to generate avian AHR1 LBD homology models. The amino acid residue at this site was either alanine (11 sub-types) or threonine (2G and 3C). The identity of the amino acid at site 256 does not appear to be associated with differential AHR1 transactivation after exposure of transfected cells to DLCs. In support of this conclusion, the only difference between the 2A and 2G AHR1 LBDs is the identity of the amino acid at site 256 (2A, alanine; 2G, threonine), and cells containing these constructs did not differ in sensitivity to AHR1 activation by TCDD (Figure 3.8). Similarly, the only difference between 3B and 3C is the amino acid at site 256 (3B, alanine; 3C, threonine), and cells containing these constructs did not differ in sensitivity to AHR1 activation by TCDD (Figure 3.8).

3.4.5.2. Site 257

The amino acid residue at site 257 was alanine (7 sub-types), threonine (5 sub-types) or proline (2D). Similar to site 256, site 257 was not within the templates used to generate models, nor did the identity of the amino acid at this site affect the sensitivity of AHR1 to transactivation in the LRG assay. For example, the only difference between 2B and 2E is the amino acid at site 257 (2B, alanine; 2E, threonine), and cells containing these constructs did not differ in sensitivity to AHR1 activation by TCDD (Figure 3.8). Similarly, 2D and 2F, which differed only at site 257 (2D, proline; 2F, threonine) did not differ in sensitivity to AHR1 activation by TCDD. Importantly, alteration of threonine-258 to alanine-258 in common tern AHR1 (site 258 in tern corresponds with site 257 in most other

avian species) did not alter the binding affinity to AHR1 or AHR1-mediated reporter gene activity in COS-7 cells exposed to TCDD (Karchner *et al.*, 2006).

3.4.5.3. Site 297

The amino acid residue at site 297 was threonine in the three sub-types of type 1 species and also in the three sub-types of type 3 species. In type 2 species, site 297 was isoleucine (2A and 2G), threonine (4 sub-types) or valine (2C). The identity of the amino acid at site 297 did not affect the sensitivity of AHR1 to transactivation in the LRG assay. For example, the only difference between 2A and 2C is the identity of the amino acid at site 297 (2A, isoleucine; 2C, valine), and there was no statistically significant difference between concentration-response effects of TCDD in cells expressing these sub-types (Figure 3.8). Homology modeling showed that amino acid position 297 in chicken, pheasant and quail AHR1 is located within one of the beta sheets (Bβ, Figure 3.13, panel A) and the side chain of this amino acid in the three avian models is directed away from the ligand binding pocket (Figures 3.13 and 3.14) and does not contribute to the cavity surface (Figure 3.13, panel C).

3.4.5.4. Site 324

Site 324 is occupied with either isoleucine (type 1 and type 2 species) or valine (type 3 species). The results of the present study showed that the identity of the amino acid at site 324 has a key effect on AHR1 activation by DLCs; this is in agreement with the results of earlier studies (Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006). For example, the only difference between 2F and 3B AHR1 LBDs is the identity of the amino acid at site 324 (2F, isoleucine; 3B, valine), and there was a statistically significant difference (9-fold; $p<0.001$)

between the concentration-dependent effect of TCDD on AHR1 activation in cells expressing these sub-types (Figure 3.8). The study by Farmahin *et al.* (2012) found that alteration of valine in Japanese quail AHR1 to isoleucine (V324I) was responsible for a 12–fold increase in AHR-dependent luciferase activity in cells exposed to TCDD. The isoleucine and valine side chains are both hydrophobic but the isoleucine side chain (-CH-(CH3, CH2-CH3)) is longer than the valine side chain (-CH-(CH3)2). Interestingly, a study that used site-directed mutagenesis to change isoleucine to alanine at site 319 in mouse AHR (mouse site 319 and avian site 324 are equivalent) resulted in complete loss of the ability of AHR to bind to TCDD and DNA (Pandini *et al.*, 2009). As such, one might predict that site-directed mutagenesis of valine to alanine (-CH3) in avian type 3 AHR1 LBD constructs might also result in complete loss of activity in the LRG assay.

Homology modeling showed that amino acid residue 324 in chicken, pheasant and quail is located in a loop between Eα and Fα (Figure 3.13, panel A) of the AHR1 LBD, and the side chain of this residue contributes to the cavity surface (Figure 3.13, panel C). *In silico* mutagenesis of this residue from Ile-324 to Val-324 in chicken AHR1 resulted in an increase in the cavity volume from 399 $Å^3$ to 485 $Å^3$. Taken together, these results suggest that Val-324 is responsible for reducing the sensitivity of type 3 AHR1, perhaps by increasing the binding cavity volume and weakening the ligand-receptor interaction. Previous studies have shown the importance of this position in mouse (Goryo *et al.*, 2007) and tern (Karchner *et al.*, 2006).

3.4.5.5. Site 337

The amino acid residue at site 337 was valine (9 sub-types) or isoleucine (4 sub-types), and based on the results of the present study, the identity of the amino acid at this site does not contribute to differences in concentration-dependent effects of DLCs. For example, the only difference between 2E and 2F is the identity of the amino acid at this site (2E, isoleucine; 2F, valine), but there was no statistically significant difference in the concentration-dependent effects of TCDD on AHR1 activation for constructs containing either amino acid residue in the LRG assay (Figure 3.8). Similarly, the only difference between types 1A and 1B is the amino acid at site 337 (1A, valine; 1B, isoleucine), and cells containing these constructs did not differ in sensitivity to AHR1 activation by TCDD (Figure 3.8).

Homology modeling showed that Val-337 in chicken, pheasant and quail AHR1 LBD is located in the helical connector (Fα). The finding that the identity of the amino acid residue at site 337 does not affect sensitivity to AHR1 activation is not surprising because the amino acid side chain of this residue does not contribute to the cavity surface (Figure 3.13, panel C) and points towards the outside of the binding cavity (Figure 3.13, panel B).

3.4.5.6. Site 380

The amino acid residue at site 380 was either serine (type 1 species) or alanine (type 2 and type 3 species). The identity of the amino acid at site 380 has a significant effect on differential sensitivity of AHR1 activation by DLCs, in agreement with the results of earlier studies (Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006). For example, the only difference between 1B and

2B is the identity of the amino acid at site 380 (1B, serine; 2B, alanine), and there was a statistically significant difference (13-fold; $p<0.001$) between the concentration-dependent effects of TCDD on AHR1 activation in cells expressing these sub-types (Table 3.5, Figure 3.8). The results of a previous study (Farmahin *et al.*, 2012) showed that changing Ala-380 to Ser-380 in Japanese quail AHR1 increased sensitivity to AHR1 activation 25-fold and 3.5-fold with TCDD and PeCDF, respectively. It has also been shown that mutation of alanine to serine at this site in common tern AHR1 increased the binding affinity of TCDD to AHR1 and the ability to transactivate a luciferase reporter gene (Karchner *et al.*, 2006). The importance of this amino acid in other vertebrates has been shown in several other studies (Backlund and Ingelman-Sundberg, 2004; Ema *et al.*, 1994; Murray *et al.*, 2005; Pandini *et al.*, 2007; Pandini *et al.*, 2009; Poland *et al.*, 1994; Ramadoss and Perdew, 2004).

Homology modeling showed that the amino acid residue 380 in avian AHR1 forms part of the antiparallel β-sheet (I β) and the amino acid side chain at this site contributes to the binding cavity of the AHR1 (Figures 3.13, panel B and C). The reason that serine at position 380 enhances the AHR1 transactivation ability is possibly due to hydrogen-bonding interactions of the hydroxyl group of the serine side chain with the dioxygen bridge or chlorine atom of the ligand, which could stabilize the ligand-receptor interaction.

3.5. DISCUSSION

In this study, amino acid sequences of the AHR1 LBD from 86 avian species were determined and compared. Twelve mutant AHR1 constructs were

generated and the sensitivity of the constructs to AHR1 activation by TCDD, PeCDF and PCB 126 was determined by use of the LRG assay. The results revealed that AHR1 activation is controlled by the identity of amino acids at sites 324 and 380 in the AHR1 LBD. A statistically significant correlation between *in vitro* AHR1 activation and *in ovo* toxicity of DLCs provided conclusive evidence that the identity of amino acids 324 and 380 in the AHR1 LBD can be used to predict the relative sensitivity of avian species to DLC toxicity (LD_{50s}).

Among avian species, the AHR1 LBD was first shown to impart distinct *in vitro* functional properties in chicken and common tern (Karchner *et al.*, 2006). Only two amino acids, located at sites 324 and 380 in chicken AHR1 LBD, are responsible for differences observed in TCDD binding affinity between tern and chicken AHR1. These two amino acids are also responsible for the distinct abilities of chicken, pheasant and quail AHR1 to transactivate a luciferase reporter gene in cells exposed to TCDD, PeCDF or TCDF (Farmahin *et al.*, 2012). In addition, the LBD amino acid sequence and *in vitro* function of chicken, ring-necked pheasant and Japanese quail AHR1 predict *in ovo* toxicity (LD_{50}) of these three species to TCDD, PeCDF and TCDF (Farmahin *et al*., 2012). To our knowledge, there are no studies which demonstrate that the identities of amino acids within the DBD or TAD of avian AHR1 cause differential sensitivity to DLCs. However, based on findings in mammals (Ishiniwa *et al.*, 2010; Minsavage *et al.*, 2004; Pohjanvirta *et al.*, 1998), it is conceivable that amino acid deletions in an important segment of the DBD or TAD of avian AHR1 or a mutation in a critical and conservative amino acid position in these domains could affect avian

sensitivity to DLCs. The determination of such potential differences in the DBD and TAD of avian AHR1 was beyond the scope of this study.

The objective of the current study was to establish a method to predict *in ovo* sensitivity to the toxic effects of DLCs for a range of birds from diverse taxa and feeding guilds, based on their AHR1 LBD sequence. Here, I showed that AHR1 LBDs from 86 avian species belong to one of 13 different sub-types, and that, when these AHR1s are expressed in COS-7 cells, the sensitivity of each AHR1 to activation by TCDD, PeCDF or PCB-126 is (1) determined by the identity of amino acids 324 and 380 and (2) significantly correlated with the sensitivity of each species to *in ovo* toxicity (LD_{50}). The current study confirmed our previous finding (Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006) that birds can be classified into three major types based on their sensitivity to the toxic and biochemical effects of DLCs (chicken-like, pheasant-like and quail-like), and that the assignment to these three classes can be predicted from the identity of amino acids at sites 324 and 380 in the AHR1 LBD. The results reported here also revealed that the other four variable amino acid sites within the LBD (256, 257, 297 and 337) do not affect the ability of AHR1 to transactivate the AHRE-driven reporter gene when exposed to DLCs. Among avian AHR1 constructs, the rank order in sensitivity to induce AHRE-driven reporter gene activity was: type 1(1A, 1B and 1C) > type 2 (2A, 2B, 2C, 2D, 2E, 2F and 2G) > type 3 (3A, 3B and 3C). These results are consistent with those of a similar study conducted in our laboratory that employed the LRG assay to determine avian species sensitivity to AHR1 activation by several PCB congeners (Manning *et al.*, 2012).

The ReP of PeCDF was very similar to the ReP of TCDD (i.e., approximately 1.0) for AHR1 activation in cells expressing various mutants of type 1 AHR1s. However, PeCDF was more potent than TCDD in cells expressing type 2 AHR1s (ReP_{50}=2.3 to 6.8) and type 3 AHR1s (ReP_{50}=10 to 28). This observation is similar to previous results showing that PeCDF was more potent than TCDD in cells containing full-length ring-necked pheasant (2G), Japanese quail (3A) or common tern (3B) AHR1 constructs (Farmahin *et al.*, 2012). The ReP values obtained from the LRG assay are consistent with ReP values that have been calculated by other studies such as *in ovo* toxicity (Cohen-Barnhouse *et al.*, 2011) and induction of ethoxyresorufin *O*-deethylase (EROD) and expression of CYP1A mRNA in primary hepatocyte cultures of chicken, pheasant, quail and herring gull (Herve *et al.*, 2010b; Herve *et al.*, 2010c). Taken together, the data from all of these studies are important because the current assumption in risk assessments is to consider PeCDF to be as potent as TCDD, which is correct only for type 1 birds.

Alteration of amino acids at sites 324 and 380 resulted in larger differences in sensitivity between AHR1 constructs exposed to TCDD, compared to the differences in sensitivity observed between AHR1 constructs exposed to PeCDF. These apparent ligand-dependent differences are perhaps due to the larger molecular size of PeCDF. Type 2 and 3 AHR1 LBDs contain larger binding cavity volumes than type 1 AHR1 LBDs. As such, PeCDF might conform better to the binding cavity of type 2 and type 3 AHR1s than TCDD. In addition, PeCDF contains an additional electronegative Cl group that might contribute to hydrogen bonding with Val-324 and Ala-380. Detailed docking studies of TCDD, PeCDF

and other AHR agonists with avian AHR1 LBDs might be able to determine why PeCDF is more potent than TCDD in type 2 and type 3 avian species.

A significant and positive correlation (r^2=0.77, p<0.005) was previously observed between LD_{50} values for DLCs in chicken, pheasant, and Japanese quail embryos, and EC_{50} values from the LRG assay (Farmahin *et al.*, 2012). In the current study, mutant AHR1s that contained LBD sequences matching all 13 sub-types of avian species were developed by use of site-directed mutagenesis. Determination of EC_{50} values in cells expressing mutant AHR1s allowed comparisons with *in ovo* results for other species. A significant, positive correlation (r^2=0.95, p<0.0001; Figure 3.10) was observed between the *in vitro* LRG assay and *in ovo* toxicity data. This strong correlation was used to predict the sensitivity of a larger group of birds to TCDD, PeCDF and PCB 126 (Table 3.8). These predicted LD_{50} values could be used to estimate the embryotoxic effects of DLCs for wild birds in site-specific risk assessments. My findings also suggest that amino acids within the DBD and TAD of AHR1s have no effect on the sensitivity of birds to the DLCs studied because the linear regression equations between wild-type or mutant AHR1 LRG EC_{50} data and *in ovo* LD_{50s} had similar slopes and Y-intercepts. Use of the EC_{20}, EC_{50} or EC_{80} gives similar correlations with LD_{50} data, thus any of these endpoints could be used to predict the embryotoxic effects of DLCs (Figure 3.11). Taken together, the results of the present study have (1) confirmed and extended the results of Karchner *et al.* (2006) showing that differential sensitivity of chicken and common tern AHR1 to activation by TCDD reside in the ligand binding domain, (2) confirmed and extended the results of Farmahin *et al.* (2012) that LD_{50s} can be predicted from

the LRG assay in cells expressing wild-type AHR1 and (3) suggested that LD50s can be predicted using the LRG assay for cells expressing mutant AHR1. The predictive relationship was valid for all avian species for which LRG assay and in ovo toxicity data are available.

In conclusion, the findings from this study suggest that the sequence of the AHR1 LBD can predict the sensitivity of all avian species to DLCs. This approach, unlike other in vitro methods such as induction of EROD activity and measurement of CYP1A mRNA expression (Head and Kennedy, 2007b; Kennedy *et al.*, 1996), does not require the bird of interest to be euthanized, because the AHR1 LBD sequence can be determined by using a drop of blood (Head *et al.*, 2010). Sequencing of the AHR1 LBD is likely to be useful in identifying the most susceptible avian species in ecological risk assessments.

CHAPTER 4 : 2,3,4,7,8-PENTACHLORODIBENZOFURAN HAS A HIGHER AFFINITY THAN 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN FOR RING-NECKED PHEASANT AND JAPANESE QUAIL ARYL HYDROCARBON RECEPTOR 1

(Farmahin, *et al*., manuscript in preparation for publication)

4.1. ABSTRACT

The World Health Organization (WHO) established toxic equivalency factors (TEFs) for dioxin-like compounds (DLCs) for avian species to facilitate environmental risk assessments for wild birds exposed to complex mixtures of DLCs. The data available at the time of the WHO report indicated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) were equipotent. As such, avian WHO-TEFs for these two compounds were set to 1.0. Results of recent egg-injection studies (*in ovo* studies) and *in vitro* studies confirm that the toxic potencies and the potencies of TCDD and PeCDF in domestic chicken (*Gallus gallus domesticus*) are approximately similar. However, PeCDF is more potent than TCDD in ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*). To elucidate the mechanism(s) underlying differences in potency of PeCDF among avian species, we tested the hypothesis that this is due to differential binding affinity interactions of TCDD and PeCDF to the AHR1 among species. Here, we modified a cell-based assay that allowed us for measurement of the binding affinity of TCDD, PeCDF and tetrachlorodibenzofuran (TCDF) to chicken, pheasant and quail AHR1 expressed in COS-7 cells. The results of the binding affinity studies

showed (1) PeCDF and TCDD bind with equal affinity to chicken AHR1, but PeCDF binds to pheasant and Japanese quail AHR1 with greater affinity than TCDD; (2) the modified cell-based binding assay can be used as an alternative to a well-known method, the hydroxyapatite (HAP) binding assay. The results increase our understanding of the mechanism(s) underlying differential potencies of DLCs among species of birds.

4.2. INTRODUCTION

To aid environmental and human health risk assessments of complex mixtures of dioxins and dioxin-like compounds (DLCs), the World Health Organization (WHO) established toxic equivalency factors (TEFs) for mammals, fish and birds for several polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and polychlorinated biphenyls (PCBs). TEFs were assigned by an international panel of scientific experts that considered all available data on the toxic and biochemical potencies of DLCs published in peer-reviewed scientific journals (Van den Berg *et al.*, 1998), and TEFs are used to calculate toxic equivalent (TEQ) concentrations by use of the concentration of each DLC and its TEF value. The TEQ approach assumes that the TEF value for each DLC is the same for all avian species. For example, the WHO-TEFs for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) are 1.0 in birds.

The relative potency (ReP) values that were used to derive TEFs for birds were obtained from a small number of *in vivo* and *in vitro* studies, and generally from data for only one avian species – the domestic chicken (*Gallus gallus*

domesticus). However, recent studies indicate that the ReP values of some DLCs vary among avian species. For example, PeCDF and TCDD are approximately equipotent activators of aryl hydrocarbon receptor 1 (AHR1) in primary cultures of domestic chicken hepatocytes (Herve *et al.*, 2010b) and in COS-7 cells transfected with chicken AHR1 (Farmahin *et al.*, 2012). In contrast, PeCDF is a more potent AHR1 activator than TCDD in primary cultures of ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) hepatocytes and in COS-7 cells transfected with pheasant or quail AHR1 (Farmahin *et al.*, 2012; Herve *et al.*, 2010b). The results from these *in vitro* studies are in general agreement with the results of egg injection studies (Cohen-Barnhouse *et al.*, 2011), and comparisons between *in vitro* and *in vivo* results for chicken, pheasant and Japanese quail are presented in an earlier report (Farmahin *et al.*, 2012) .

Data from AHR1 binding studies could enhance our understanding of mechanism(s) underlying differential potencies of DLCs among species of birds. Here, we compared the binding affinities of TCDD, PeCDF and TCDF to chicken, ring-necked pheasant and Japanese quail AHR1 and we tested the hypothesis that the differential potency of PeCDF among avian species is due to differences in the binding affinities of these compounds to AHR1 expressed in each species.

To do this work, we modified a cell-based binding assay developed by others (Dold and Greenlee, 1990) so that it could be used with COS-7 cells transfected with avian AHR1. The method measures binding affinities of DLCs to AHR1 expressed in cells after exposure to DLCs. Data obtained with the cell-based assay were compared with data obtained with a hydroxyapatite (HAP)

binding assay. One of the drawbacks of the HAP binding assay is the inability to detect weak ligand-receptor interactions due to a detergent-washing step that disrupts weak interactions between TCDD and AHR (Karchner *et al.*, 2006; Nakai and Bunce, 1995)

There are other AHR binding assays; however, none were appropriate for our current research. For example, (1) the charcoal-adsorption assay has high levels of non-specific binding that complicate the calculation of specific binding (Manchester *et al.*, 1987a; Okey *et al.*, 1979); and (2) velocity sedimentation on sucrose gradients (Okey *et al.*, 1979; Tsui and Okey, 1981) is very labour-intensive and time-consuming. The cell-based binding assay that was developed and used for the work described in this study allowed for the measurement of TCDD, PeCDF and TCDF binding affinities to chicken, ring-necked pheasant and Japanese quail AHR1 expressed in COS-7 cells.

4.3. MATERIALS AND METHODS

4.3.1. Cloning of AHR1 cDNA and expression constructs

The methods for cloning, sequence analysis and construction of expression vectors for chicken, ring-necked pheasant and Japanese quail AHR1 are described elsewhere (Farmahin *et al.*, 2012). In brief, full-length cDNAs of pheasant and Japanese quail AHR1 were obtained by use of Marathon cDNA amplification kits (Clontech, Foster City, CA USA); the methods for chicken AHR1 cloning and sequencing are described elsewhere (Karchner *et al.*, 2006). The full-length cDNAs were ligated into pENTRE/D-TOPO vector (Invitrogen, Burlington, ON, Canada) and subcloned into pcDNA 3.2/V5-DEST vector (Invitrogen).

4.3.2. Cell culture and transfection

COS-7 cells, provided by Dr. R. Haché (University of Ottawa, Ottawa, ON, Canada), were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS; Wisent, St. Bruno, QC, Canada), 1% MEM nonessential amino acids (Invitrogen), and 1% penicillin-streptomycin (Invitrogen; 10,000 unit/ mL penicillin, 10,000 µg/ mL streptomycin) at 37°C under 5% CO_2. Cells were seeded in 6-well plates at a concentration of 300,000 cells/well in dextran-coated charcoal-treated DMEM (Invitrogen), supplemented with 10% charcoal stripped FBS and 1% penicillin-streptomycin (Invitrogen). Transfection was performed 18 h after plating. DNA and Fugene 6 transfection reagent (Roche, Laval, QC, Canada) were diluted in OPTI-MEM (Invitrogen). Avian AHR1 (chicken, ring-necked pheasant or Japanese quail; 250 ng quantities) and 750 ng salmon sperm DNA (Invitrogen) were transfected in each well. DNA was complexed with 4 µl of Fugene 6 transfection reagent (Roche) and this mixture (100 µl) was added to each well.

4.3.3. Chemicals

[^{3}H]TCDD (2,3,7,8-tetrachloro[1,6-^{3}H]dibenzo-*p*-dioxin; specific activity 27.7 Ci/mmol, purified to 99% by HPLC) was purchased from American Radiolabeled Chemicals Inc. (ARC, St. Louis, MO, USA). Details concerning the preparation of un-labelled TCDD, PeCDF and TCDF solutions can be found elsewhere (Herve *et al.*, 2010b). In brief, stock solutions of TCDD, PeCDF and TCDF were prepared in dimethyl sulfoxide (DMSO) and concentrations were

determined by isotope dilution following EPA method 1613 (U.S.EPA, 1994) by high-resolution gas chromatography high-resolution mass spectrometry. Serial dilutions of each chemical were prepared from their respective stocks in DMSO.

4.3.4. Saturation binding analysis

Saturation binding was performed using two methods (1) a hydroxyapatite (HAP) adsorption assay and (2) the cell-based binding assay developed for the current project. The HAP assay was conducted according to methods described by Gasiewicz and Neal (1982), as modified by Hahn and colleagues (Karchner et al., 2006).

4.3.4.1. HAP assay

Avian AHR1 proteins were synthesized by *in vitro* transcription/translation (IVTT) as described in detail elsewhere (Farmahin *et al.*, 2012). The IVTT lysate was diluted in MEEDG buffer [25 mM MOPS, 1 mM EDTA, 5 mM EGTA, 0.02% NaN_3, 10% vol/vol glycerol, 1 mM DTT, protease inhibitor cocktail tablet (PI tablet; Roche ; 1 tablet/ 25 ml buffer) ; pH 7.5]. DTT and PI tablets were added to the MEEDG buffer on the day of each experiment. Diluted IVTT lysate was incubated with different concentrations of [^{3}H]TCDD for 2 h, and shaken gently at room temperature. A 5 µl aliquot from each incubation tube was used to determine the actual total concentration of [^{3}H]TCDD. After 2 h incubation, aliquots (200 µl) of 10% DNA grade HAP (Bio-Rad, Mississauga, ON, Canada) in MEEDMG (MEEDG buffer + 20 mM Na_2MoO_4) were distributed to glass incubation tubes. The tubes were placed on ice for 15 to 30 min, and mixed vigorously every 5 min.

The HAP suspension was transferred onto a 25 mm GF/F filter (Whatman, Florham Park, NJ, USA) in a sampling manifold (Millipore, Billerica, MA, USA). After application of a vacuum, the filter was washed three times with 800 µl MEEDGT buffer (MEEDG buffer + 0.15% tween-20). The filters were then transferred to a scintillation vial containing 2.5 mL scintillation cocktail (Scintiverse II; Fisher Scientific., Don Mills, Ont., Canada) and the radioactivity was measured using a 1450 MicroBeta Trilux scintillation counter (PerkinElmer, Waltham, MA, USA).

4.3.4.2. Cell-based binding assay

Modifications were made to a cell-based binding assay procedure that was previously established for measuring AHR binding in mouse and human cell lines (Dold and Greenlee, 1990). An important modification was that binding to AHR1 was determined in cells transfected with AHR1s, while the method by Dold and Greenlee measured endogenous AHR in cell lines. Transfected COS-7 cells were incubated for 24 h at 37°C in 5% CO_2 and dosed with six concentrations of [^{3}H]TCDD (0.1, 0.25, 0.8, 2.5, 8 and 14 nM) for 2 h at 37°C in a 5% CO_2 atmosphere. A 10 µl aliquot was taken from each well to determine the total concentration of [^{3}H]TCDD. After incubation, the medium was aspirated and the cells were washed with ice-cold PBS and ice-cold 10% fetal calf serum in PBS. The cells were lifted by incubation with 700 µl trypsin-EDTA (0.05%; Invitrogen) for 5 min at 37°C. DME medium (Invitrogen) (700 µl) was then added to the wells to deactivate the trypsin. The cell suspension was transferred onto 25 mm GF/F filters (Whatman) that were presoaked with PBS in a sampling manifold

(Millipore). The filters were washed twice with 2.5 ml/filter of acetone that had been pre-cooled to -80°C. The filters were dried by applying a vacuum for 5 min, and radioactivity was measured as described above.

4.3.5. Competitive binding analysis

4.3.5.1. HAP assay

Minor modifications were made to the HAP assay described elsewhere (Jensen *et al.*, 2010; Karchner *et al.*, 2006). In brief, 16.5 µl IVTT lysate diluted with 33.5 µl MEEDG buffer was incubated in glass tubes with one of ten concentrations of unlabeled TCDD, PeCDF or TCDF. Incubation tubes were mixed using a plate shaker at 220 rpm at room temperature for 15 min. [^{3}H]TCDD (1.0 nM nominal concentration) was added to incubation tubes, and the tubes were mixed at 220 rpm at room temperature for 105 min. The tubes were transferred to ice and a 5 µl aliquot was taken from each to determine the total concentration of [^{3}H]TCDD. The re-suspended HAP (200 µl) was added to each tube and incubated on ice for 15 to 30 min. Finally, the HAP was washed and the radioactivity was measured as described above.

4.3.5.2. Cell-based binding assay

Twenty four hours after transfection, cells were incubated with one of ten concentrations of unlabeled TCDD, PeCDF or TCDF for 15 min followed by addition of [^{3}H]TCDD (1nM nominal concentration) for 105 min at 37°C in a 5% CO_2 atmosphere. A 10 µl aliquot was taken from each well to determine the [^{3}H]TCDD concentration. The medium was then aspirated, and the cells were

washed and lifted using trypsin. The cell suspensions were filtered and washed with acetone, and the radioactivity was measured as described above.

4.3.6. Concentration-response curves

Specific binding is receptor-bound [^{3}H]TCDD, and is defined as the difference between total and non-specific binding (NSB). For the HAP and cell-based assays, NSB was determined by using (1) IVTT lysate that did not have AHR expression vector (unprogrammed lysate; UPL) and (2) non-transfected cells (NT), respectively. The binding detected by the use of the UPL or NT was similar to the minimum levels of binding detected when a 200-fold excess of displacer was used.

The specific binding data were fit to a one-site binding hyperbola curve with the following equation:

$$Y = \frac{B_{max} \times X}{K_d + X}$$

where Bmax is the maximum specifically bound [^{3}H]TCDD bound receptor, X is the concentration of free [^{3}H]TCDD, and K_d is the equilibrium dissociation constant. Nonlinear regression analysis was performed by the use of GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA).

To determine the IC_{50} values, the fractional specific binding (SB) of [^{3}H]TCDD was calculated with the following equation:

$$Fractional\,SB = SBA \div SB_{max}$$

where SBA is SB in the presence of a given concentration of compound A, and SBmax is the SB of [^{3}H]TCDD in the absence of a competitor. The calculated fractional SB data were then analyzed by non-linear regression, using a one-site competition equation:

$$Y = Bottom + \frac{(TOP - Bottom)}{1 + 10^{(X - LogIC_{50})}}$$

where top is the fraction of [^{3}H]TCDD specific binding in the absence of a competitor; bottom is the fraction of [^{3}H]TCDD binding observed when specific binding sites are occupied with an unlabeled competitor (UPL in the HAP assay and NT in cell-based assay). X is the log of the concentration of the competitor in nM, and Y is the fractional SB at each competitor concentration. Data were fit by unweighted non-linear regression using GraphPad.

4.4. RESULTS

4.4.1. HAP binding assay

HAP assays were conducted to determine dissociation constants (K_d) of [^{3}H]TCDD with chicken or Japanese quail AHR1. The mean values (±SD) for K_d and B_{max} for chicken were 0.64 (±0.2) nM and 98 (±11) fmol, respectively, but specific binding of [^{3}H]TCDD to quail AHR1 was undetectable (Figure 4.1, panel A). HAP competitive binding assays were used to determine IC_{50} values for TCDD, PeCDF and TCDF for *in vitro*-expressed chicken AHR1 (Figure 4.1, panel B), and no significant differences among IC_{50} values were found ($p<0.05$). IC_{50} values were 3.0 nM, 2.9 nM and 2.9 nM and the ReP values were 1, 1.1 and 1.1 for TCDD, PeCDF and TCDF, respectively (Table 4.1).

4.4.2. Cell-based binding assay

4.4.2.1. Optimization of assay conditions and saturation binding of TCDD to Japanese quail AHR1

Acetone or ethanol can be used for the washing step of this assay. Following incubation of COS-7 cells with [^{3}H]TCDD, in the presence or absence of displacer (un-labelled TCDF), the cells were detached from the plate with trypsin, filtered, then washed once or twice with 2.5 ml cold acetone (-80°C) or ethanol (-80°C). They were also washed twice with 2.5 ml of different concentrations of Tween-20 (0.1% or 0.25%) in PBS (4°C). The results indicated that washing the filters twice with cold ethanol (Figure 4.2, condition 4: total/non-specific ratio= 7.4, specific binding= 446 CCPM) can be used as an alternative to the previously suggested method by Dold and Greenlee (1990) where filters were washed twice with cold acetone (condition 2: total/non-specific ratio=5.5, specific binding= 611 CCPM). Tween-20 in PBS (0.25%) might also be a useful washing reagent (condition 6: total/non-specific ratio=3.4, specific binding=702; Figure 4.2).

Saturation binding of [^{3}H]TCDD to Japanese quail AHR1, which was not detectable in the HAP assay, was assessed with the cell-based assay, and the mean K_d value was 2.1 nM (Figure 4.3).

4.4.2.2. Relative potency (ReP)

Competitive binding curves of TCDD, PeCDF and TCDF to chicken, pheasant and quail AHR1 were obtained with the cell-based assay and IC_{50}

values were determined (Figure 4.4; Table 4.2). The ReP value for the DLCs in COS-7 cells transfected with the chicken AHR1 (1.0 for TCDD, 1.5 for PeCDF and 1.1 for TCDF) showed that the three DLCs had similar binding affinity to chicken AHR1. In cells expressing pheasant AHR1, PeCDF (ReP = 3) was slightly more potent than TCDD, but TCDF (ReP = 0.4) showed a somewhat lower affinity to pheasant AHR1 than TCDD. PeCDF was more potent than TCDD in COS-7 cells expressing Japanese quail AHR1 (ReP = 5; Table 4.2).

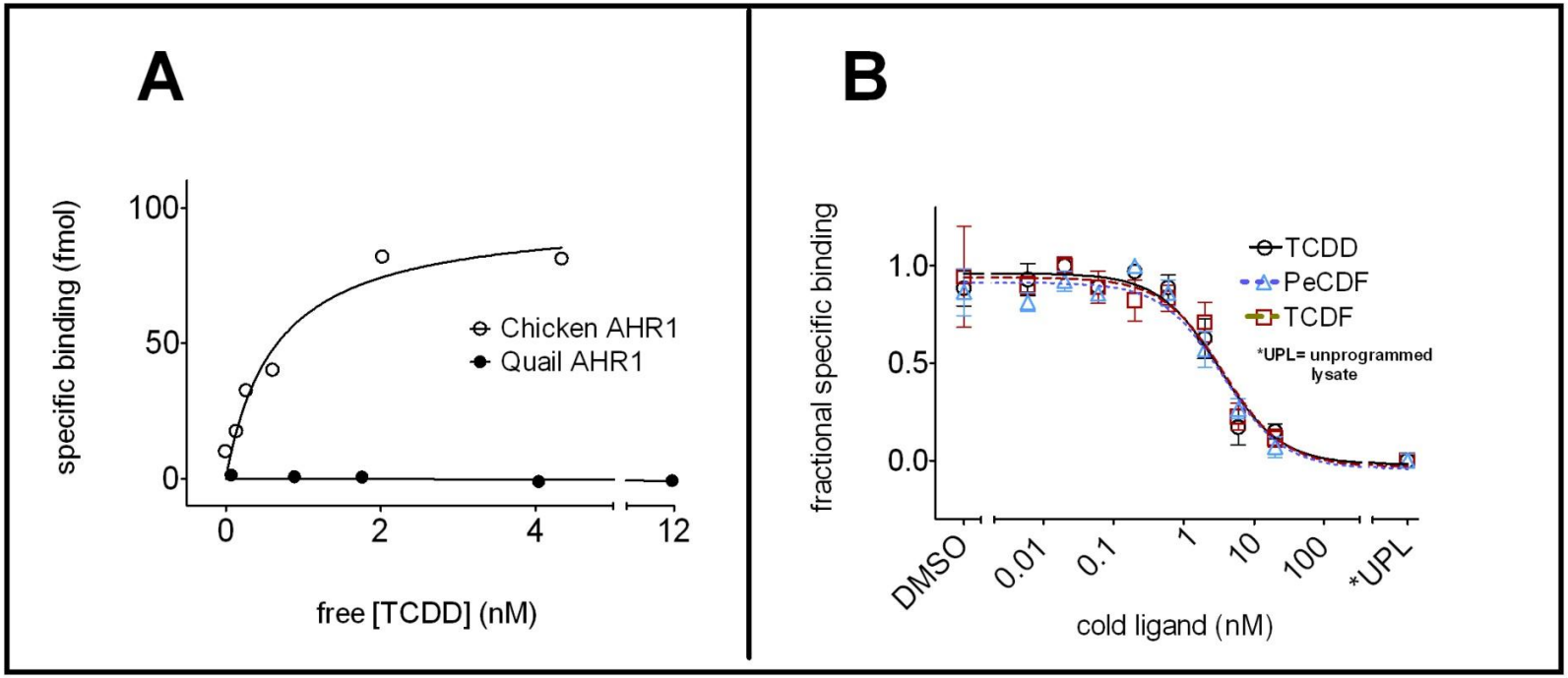


Figure 4.1. Saturation and competitive binding assessed with the HAP assay. *(A)* Saturation analysis of binding of [^{3}H]TCDD to chicken and quail AHR1s. AHR1s were expressed by IVTT, incubated with different concentrations of [^{3}H]TCDD for 2h at room temperature, and analyzed using the HAP assay as described in *Materials and Methods*. Specific binding (SB) was defined as the difference between total binding and non-specific binding (NSB). Mean values (±SD) for K_d were 0.64 ± 0.2 nM for chicken (n=4), and specific binding of [^{3}H]TCDD by the quail AHR was undetectable. *(B) C*ompetitive binding curves for chicken AHR1 for various DLCs. Chicken AHR1s were expressed by IVTT, incubated with a single concentration of hot ligand ([^{3}H]TCDD) in the presence of various concentrations of TCDD, PeCDF or TCDD, incubated for 2h at room temperature, and analyzed according to the filtered HAP assay described in *Materials and Methods*. Each symbol represents the mean value of four replicates; bars indicate standard error.

Table 4.1. Summary of 50% inhibitory concentration (IC_{50}) ± standard error (SE) and the relative potency (ReP) values determined for chicken AHR1 using the HAP assay. Data points from the average values obtained from four experiments were used to generate concentration-response curves of inhibition of [^{3}H]TCDD binding .There were no statistical differences among IC_{50} values [ANOVA ($p < 0.05$) followed by Tukey's post-hoc test ($p < 0.05$)].

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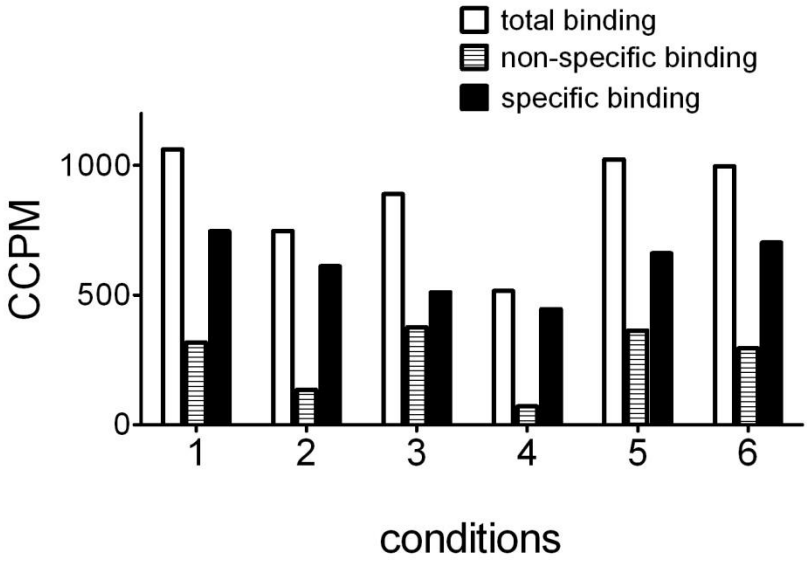
compound	Mean IC_{50} ± SE (nM)	ReP
TCDD	3.1 ± 1.0	1.0
PeCDF	2.9 ± 0.9	1.1
TCDF	2.9 ± 1.0	1.1



	specific binding (CCPM)	total/non-specific binding ratio
condition 1	745	3.4
condition 2	611	5.5
condition 3	512	2.4
condition 4	446	7.4
condition 5	661	2.8
condition 6	702	3.4

Figure 4.2. The effect of washing reagents on total, non-specific and specific binding. Acetone and ethanol (pre-cooled to -80°C in a freezer); and Tween-20 (4°C) were used for washing the cells. Cells were incubated for 2h at 37°C with 1nM [^{3}H]TCDD in the absence or presence of a 200-fold molar excess of TCDF. The cells were washed with pre-cooled acetone once (condition 1), pre-cooled acetone twice (condition 2), pre-cooled ethanol once (condition 3), pre-cooled ethanol twice (condition 4), 0.1% Tween-20 twice (condition 5), or 0.25% Tween-20 twice (condition 6) then collected on the filter membrane as described in *Materials and Methods.* The values indicated in the graph are the total/non-specific binding ratios (n=1). The results of total and non-specific binding are reported as corrected counts per minute (CCPM).

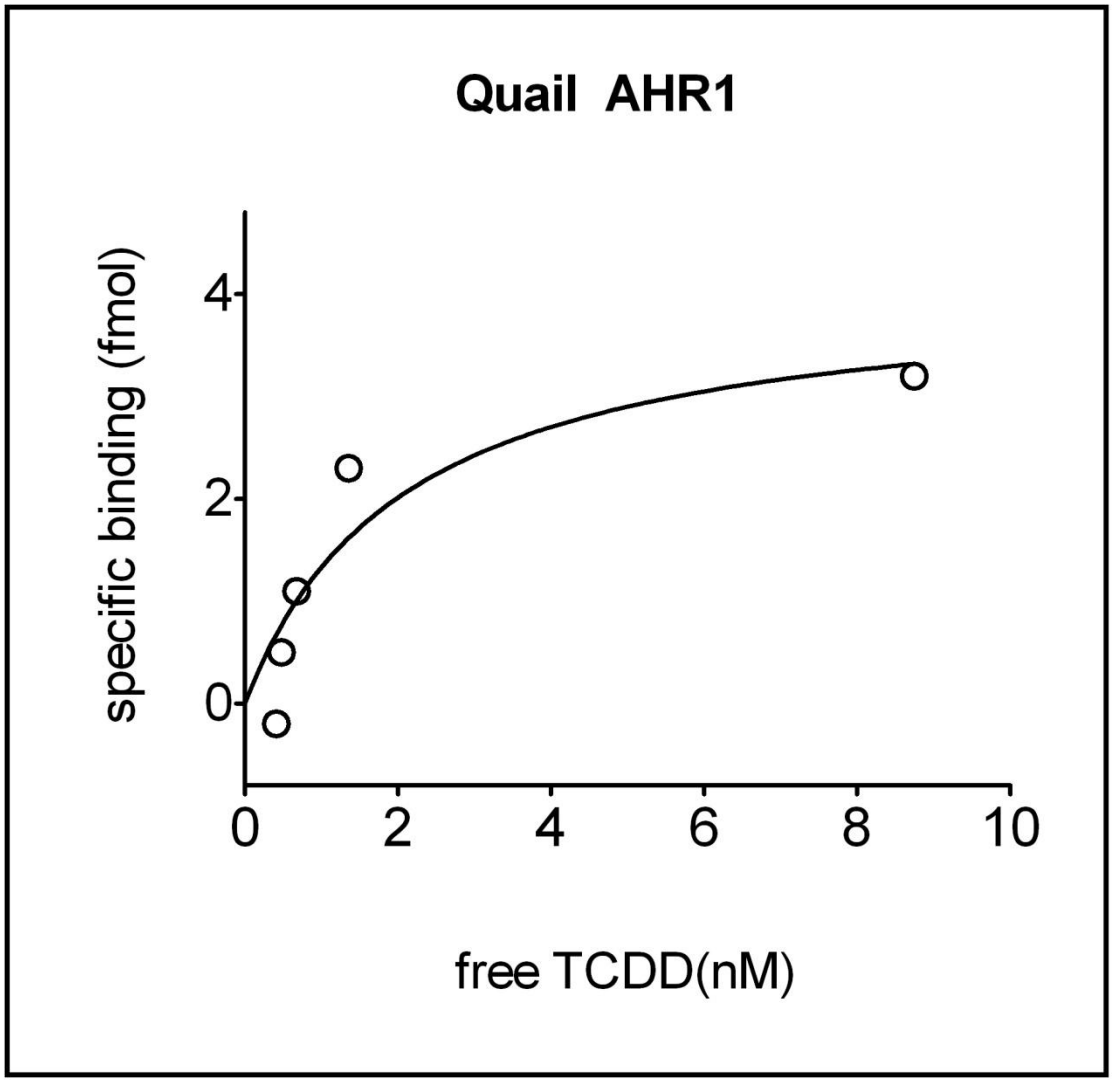


Figure 4.3. Saturation binding assessed with the cell-based binding assay. COS-7 cells expressing quail AHR1 were incubated with [^{3}H]TCDD for 2h at 37°C, then analyzed. Specific binding (shown) was calculated as the difference between total binding and non-specific binding. The values for K_d and B_{max} were 2.1 nM and 4.1 fmol/ well

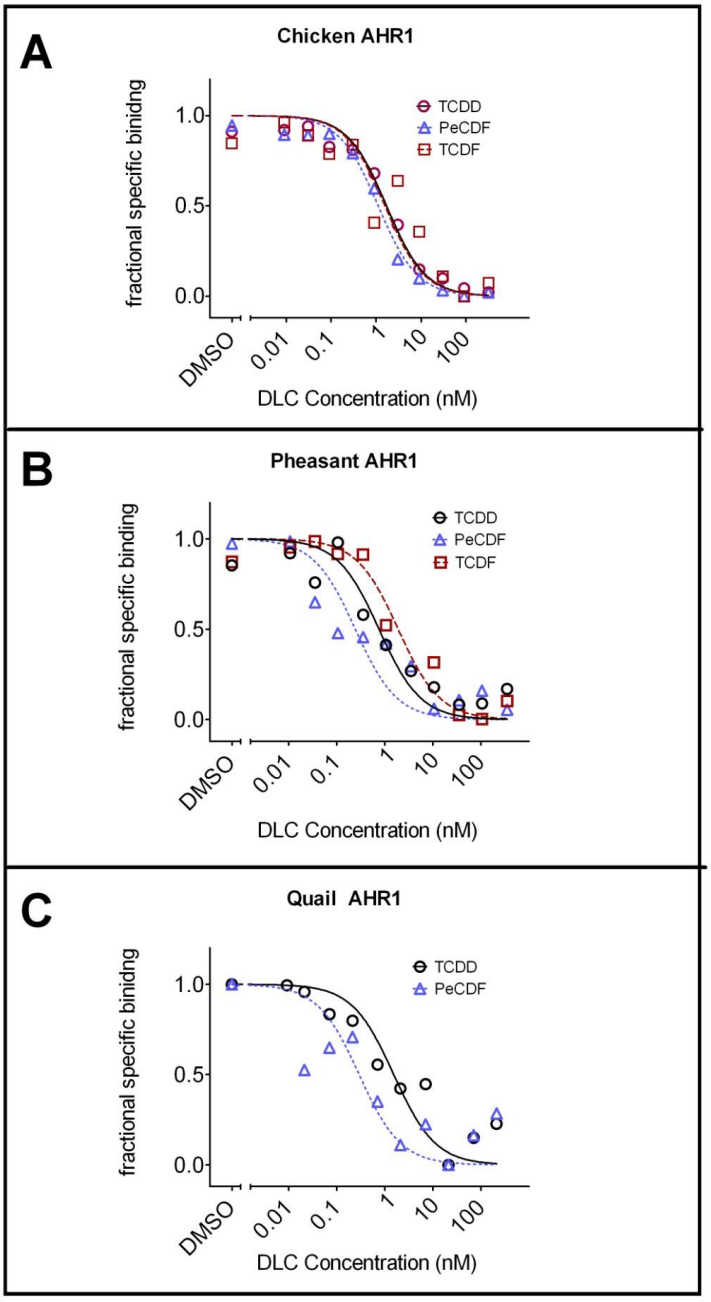


Figure 4.4. Panels ***A***, ***B*** and ***C*** show the results obtained with the cell-based binding assay for chicken, pheasant and quail AHR1. Inhibition of binding of [^{3}H]TCDD (single concentration) by various concentrations of TCDD, PeCDF and TCDF in COS-7 cells expressing chicken AHR1 (panel **A**), pheasant AHR1 (panel **B**), and quail AHR1 (panel **C**) were determined as described in *Materials and Methods*. Curves were fit to a one-site competition model. Each symbol represents the mean value of at least two replicates.

Table 4.2. Summary of inhibitory concentration 50% (IC_{50}) and the relative potency (ReP) values determined for chicken, pheasant and Japanese quail AHR1 using a cell-based assay. Data from the average of two to six experiments was used to generate dose-response curves for inhibition of [^{3}H]TCDD binding . Statistical tests could not be performed on the IC_{50} values because only one curve fit was generated and only one IC_{50} value was derived for each dioxin-like chemical.

AHR1 construct	compound	IC_{50} (nM)	Binding ReP	LRG ReP[a]
Chicken	TCDD	1.7 (1.1 - 2.7)	1	1
	PeCDF	1.1 (0.80 - 1.5)	1.5	1
	TCDF	1.6 (0.78 – 3.1)	1.1	0.4
Pheasant	TCDD	0.76 (0.51 – 1.1)	1	1
	PeCDF	0.24 (0.14 -0.41)	3	4
	TCDF	1.9 (1.1 – 3.3)	0.4	0.3
Quail	TCDD	1.5 (0.84 – 2.8)	1	1
	PeCDF	0.29 (0.11 – 0.78)	5	20

[a]Based on *in vitro* EC_{50} values for luciferase reporter gene assay from Farmahin *et al.* (2012)

4.5. DISCUSSION

PeCDF is a more potent inducer of EROD, CYP1A4 mRNA and CYP1A5 mRNA than TCDD in ring-necked pheasant embryo and Japanese quail embryo hepatocyte cultures, but PeCDF and TCDD are equipotent inducers in chicken embryo hepatocytes (Herve *et al.*, 2010b). Similarly, the results of a luciferase reporter gene (LRG) assay revealed that PeCDF is more potent than TCDD in COS-7 cells expressing ring-necked pheasant or Japanese quail AHR1. However, PeCDF and TCDD are equipotent inducers in cells containing chicken AHR1 (Farmahin *et al.*, 2012). The results of the cell-based binding assay and HAP binding assay presented in this study show the same trend as observed with hepatocytes and the LRG assay; PeCDF binds to pheasant and Japanese quail AHR1 with greater affinity than TCDD, and PeCDF and TCDD bind with equal affinity to chicken AHR1. However, comparison of receptor occupancy and induction of CYP1A5 reporter gene did not appear to reveal a direct relationship. For example, the ReP value obtained from cell-based assay in this study showed PeCDF had 5-fold stronger binding affinity than TCDD to Japanese quail AHR1 while ReP value from LRG assay (Farmahin *et al.*, 2012) suggested that PeCDF was 20-fold stronger CYP1A5-mediated reporter gene inducer than TCDD (Table 4.2). Because, the potency of AHR-ligands to induce CYP1A gene or EROD do not show a direct relationship to their receptor binding affinity (Hestermann Eli V. *et al.*, 2000; Karchner *et al.*, 2006).

It would be useful to perform further saturation binding studies to determine the K_ds for chicken and pheasant. While the results from such studies would allow comparison of quail AHR1 affinity for DLCs to that of chicken and

pheasant AHR1 (i.e., to obtain relative sensitivity (ReS) values), such studies were beyond the scope of this research.

There are various methods to measure the binding affinity of DLCs to AHR1. A charcoal-adsorption assay was reported to be useful for measuring binding affinity of DLCs to AHR in mouse hepatic cytosol (Poland *et al.*, 1976). However, there are practical problems associated with this assay due to very high levels of [^{3}H]TCDD nonspecific binding, which complicates the calculation of specific binding (Okey *et al.*, 1979). In addition, it is difficult to determine the correct amount of charcoal to use for each species of animal (Manchester *et al.*, 1987b). Other methods suggest adsorbing the proteins and washing the free ligand away, rather than adsorbing it with charcoal, such as HAP (Gasiewicz and Neal, 1982) or protamine sulfate (Denison *et al.*, 1984). These batch assays are very useful for kinetic studies that require the processing of a large number of samples. However, they are not able to detect weak ligand-receptor interaction due to a detergent-washing step that disrupts weak interactions between TCDD and AHRs of some species. For example, the HAP assay was not able to detect specific binding to human (Nakai and Bunce, 1995), common tern (Karchner *et al.*, 2006) or Japanese quail AHR (the current study).

Another classical AHR binding assay is conducted using velocity sedimentation on sucrose gradients, where ligand-receptor complexes are separated based on their sedimentation coefficient (Okey *et al.*, 1979; Tsui and Okey, 1981). Although velocity sedimentation has been useful due to its ability to detect weak ligand-receptor interactions and it provides secondary data regarding the size of the receptor, the method is quite labour-intensive and time-consuming.

Thus, this method may not be suitable for the analysis of large numbers of samples (normally a requirement in our laboratory). Gel electrophoresis (Poland *et al.*, 1986) can also be used to separate free and bound ligand if there is covalent binding between the ligand and the AHR (for example, through use of a photoaffinity ligand). Gel electrophoresis was also employed to detect binding affinity between photoaffinity ligands and native AHR in hepatic cytosol (Poland *et al.*, 1986), native AHR in cell culture (Swanson and Perdew, 1991), or exogenous AHR expressed in transfected cell (Ramadoss and Perdew, 2004). The cells were lysed and cytosol fractions were obtained for gel electrophoresis.

Cell-based binding assays measure the binding affinity of endogenous AHR to DLCs in whole cells. In earlier versions of cell-based assays, glass-fiber membranes were used to retain proteins while washing away the free ligands (Dold and Greenlee, 1990). For the work described in this study, we modified the cell-based binding assay which was previously developed by Dold and Greenlee (1990). An important modification was to use COS-7 cells as the host cells and subsequent transfection of the cells with avian AHR1. In the modified assay, COS-7 cells are transfected with avian AHR1 constructs and relative binding affinities between DLCs and expressed avian AHR1 proteins are measured.

Transfected cells have been used in previous studies to produce high-levels of AHR expression to detect the binding affinity between AHR expressed in a host cell and ligand. However, unlike our assay, transfected cells were lysed and the cytosol fraction was extracted to analyze AHR binding to the ligand through charcoal adsorption or HAP assay (Fan *et al.*, 2009) or gel electrophoresis (Ramadoss and Perdew, 2004). Our cell-based assay is

particularly useful for species that have low AHR1 affinity to DLCs (e.g., Japanese quail) probably because (1) washes with the cold organic inhibit denaturation of proteins, so the ligand-binding complex remains intact during the washes or (2) the ligand-receptor complexes are protected by the cell membrane; the washing step (unlike the washing step used in the HAP assay) does not disrupt the receptor-ligand binding. The assay modified in this study, similar to the HAP assay, is suitable for the analysis of a large number of samples. The IC_{50s} for TCDD, PeCDF and TCDF obtained with the cell-based assay agree with those obtained with the HAP assay (Figure 4.1, panel B; Figure 4.4, panel A). As such, the modified cell-based binding assay can be used as an alternative to the HAP assay. This assay can provide a much more realistic assessment of non-specific binding than that obtained by use of an excess of unlabeled ligand as is done with the Dold and Greenlee (Dold and Greenlee, 1990) method. This advantage of the modified assay is due to the expression of protein in host cells, which allows parallel assays to be performed in the same system in the absence of a receptor.

Using cells instead of cytosol (or *in vitro* lysate) may result in higher non-specific binding due to the binding of ligands to cell membranes. To minimize this potential problem, a cold organic washing step suggested in previous reports was used (Ashendel and Boutwell, 1981; Dold and Greenlee, 1990; Hergenhahn and Hecker, 1981). It is possible that the use of transfected COS-7 cells with avian AHR1 results in heterologous binding with exogenous avian AHR1 and endogenous COS-7 cell AHR. However, COS-7 cells express no or very little AHR (Ema *et al.*, 1994), so the use of such cells is unlikely to be a concern. Using COS-7 cells expressing exogenous AHR1 might raise concern for different

characteristics of AHRs in COS-7 cells compared to the native species or species-specific interactions with other proteins, but we are not aware of reports of such potential effects.

The use of pre-chilled acetone was recommended by others for measurement of specific binding of a highly lipophilic ligand to epidermal membranes (Ashendel and Boutwell, 1981; Hergenhahn and Hecker, 1981) because the majority of the lipophilic ligand partitions into the lipids of cell membranes. Acetone dissolves lipophilic compounds, which bind to lipid membranes and reduces non-specific binding. Furthermore, it was shown that specific binding increased when cold acetone was used, which is likely due to slower protein denaturation at colder temperatures (Ashendel and Boutwell, 1981). Our results suggest that cold ethanol could be used in the washing process as an alternative to acetone. Ethanol is a versatile general purpose solvent, and is miscible with many organic solvents. Using ethanol would allow us to modify the method, and achieve faster output by performing all the steps of the assay in one cell culture plate. This modification is not practical with acetone, because it damages cell culture plates.

The results obtained from the binding affinity study in this study enhance our understanding of the mechanism(s) underlying previous findings from egg injection studies (Cohen-Barnhouse *et al.*, 2011), mRNA expression, EROD and reporter gene expression studies.

CHAPTER 5 : CYTOCHROME P4501A INDUCTION IN PRIMARY CULTURES OF EMBRYONIC EUROPEAN STARLING HEPATOCYTES EXPOSED TO TCDD, PeCDF AND TCDF

(Farmahin, *et al*., manuscript in preparation for publication)

5.1. ABSTRACT

Novel methods that predict the sensitivity of avian embryos to the toxic effects of dioxin-like compounds (DLCs) using either (1) knowledge of the identity of amino acids at key sites within the ligand binding domain (LBD) of aryl hydrocarbon receptor 1 (AHR1) or (2) a luciferase reporter gene assay that measures AHR1 activation were recently reported. Results from both methods predict that European starling (*Sturnus vulgaris*) and domestic chicken (*Gallus gallus domesticus*) embryos have similar sensitivity to the biochemical and toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). Chicken embryos are highly sensitive to DLC toxicity, and the prediction that starlings are equally sensitive is surprising given their widespread distribution and large population size. In an attempt to learn more about starling sensitivity to DLCs, we determined concentration-dependent effects of TCDD, PeCDF and TCDF on cytochrome P4501A4 and 1A5 (CYP1A4 and 1A5) mRNA levels in primary cultures of hepatocytes prepared from embryonic European starlings. The sensitivity of avian hepatocytes to CYP1A4/5 induction can be used to estimate LD_{50} values of DLCs in avian embryos. The results indicate that

European starling hepatocytes are indeed as sensitive as chicken hepatocytes to CYP1A4/5 induction after exposure to TCDD. However, they are less sensitive to CYP1A4/5 induction by PeCDF and TCDF.

5.2. INTRODUCTION

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and dioxin-like compounds (DLCs) cause adverse effects in birds, including embryonic and chick mortality, endocrine disruption, developmental abnormalities, cardiovascular toxicity and uroporphyria (Brunstrom *et al.*, 1990; Giesy *et al.*, 1994; Gilbertson *et al.*, 1991; Hoffman *et al.*, 1987; Kennedy *et al.*, 1998; Nosek *et al.*, 1993; Peterson *et al.*, 1993; Walker and Catron, 2000). Many of the effects are mediated by the aryl hydrocarbon receptor (AHR), a ligand-activated nuclear transcription factor (Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997; Schmidt *et al.*, 1993). Birds possess at least two AHRs, designated AHR1 and AHR2 (Hahn, 2002), and hepatic AHR1 is more transcriptionally active than AHR2. DLCs bind to avian AHR1 and induce the expression of multiple dioxin-responsive genes, including liver cytochrome P4501A4 and P4501A5 (CYP1A4 and CYP1A5).

Avian embryos can differ in sensitivity to the toxic effects of DLCs. For example, the TCDD LD_{50} of domestic chicken (*Gallus gallus domesticus*) embryos is approximately 45-fold lower than the LD_{50} observed for Japanese quail (*Coturnix japonica*) (Cohen-Barnhouse *et al.*, 2011). The LD_{50} of 3,3',4,4'-tetrachlorobiphenyl (PCB 77; IUPAC nomenclature) in the chicken is less than 1/200 of the LD_{50} in the herring gull (*Larus argentatus*) (Brunstrom, 1988; Head *et al.*, 2008).

For several years, our group has attempted to develop *in vitro* and molecular approaches to predict the sensitivity of avian embryos to DLC toxicity. The work is of importance for environmental risk assessments because it is often not possible to conduct whole animal studies (e.g., feeding or egg injection studies) due to the cost of such studies and the challenges of working with rare or endangered species. The sensitivity of primary cultures of avian hepatocytes to induction of CYP1A4 (as measured with the ethoxyresorufin *O*-deethylase [EROD] assay) by DLCs has been correlated with embryotoxicity (Herve *et al.*, 2010a; Herve *et al.*, 2010b; Herve *et al.*, 2010c; Kennedy *et al.*, 1996; Kennedy *et al.*, 2003; Lorenzen *et al.*, 1997; Manning *et al.*, 2013; Mundy *et al.*, 2010; Mundy *et al.*, 2012; Sanderson *et al.*, 1998) and this collection of work was recently reviewed (Head and Kennedy, 2010).

Genotyping of the ligand binding domain (LBD) of AHR1 in birds (Farmahin *et al.*, 2013; Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006; Manning *et al.*, 2012) revealed that avian species can be classified into three main groups of relative sensitivity to DLCs based on the identity of the amino acids at sites 324 and 380: high sensitivity (type 1; Ile324_Ser380), moderate sensitivity (type 2; Ile324_Val380) and low sensitivity (type 3; Val324_Ala380). Current knowledge indicates that the domestic chicken, a type 1 species, is more sensitive than any other avian species to the embryotoxic effects of TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and non-*ortho* substituted PCBs (Cohen-Barnhouse *et al.*, 2011; Farmahin *et al.*, 2013; Farmahin *et al.*, 2012; Head *et al.*, 2008; Head and Kennedy, 2010; Manning *et al.*, 2012; Manning *et al.*, 2013).

Within the past century, the North American population of European starlings (*Sturnus vulgaris*) has expanded from the 60 originally introduced to over 200,000,000 (one-third of the world's European starling population) (Feare, 1984). Not only are starlings abundant, they are also exposed to DLCs in the environment (Arenal *et al.*, 2004; Halbrook and Arenal, 2003; Van den Steen *et al.*, 2007) and thus one would predict low sensitivity to this class of chemicals. Surprisingly, starlings are predicted to be highly sensitive to the embryotoxic effects of DLCs based on their AHR1 genotype. The amino acids within the AHR1 LBD that predict sensitivity (sites 324 and 380) were identical to those in the highly-sensitive chicken. Furthermore, results from an *in vitro* luciferase reporter gene assay indicated that a full-length chicken AHR1 construct and a mutant construct containing the AHR1 LBD of starling were equally sensitive to AHR1 activation by TCDD, PeCDF, TCDF and non-*ortho* substituted PCBs (Farmahin *et al.*, 2013; Manning *et al.*, 2012).

Given that the sensitivity of avian hepatocytes to induction of EROD and CYP1A4/5 mRNA is predictive of the sensitivity of avian species to DLC toxicity (Head and Kennedy, 2010; Herve *et al.*, 2010b; Kennedy *et al.*, 1996; Manning *et al.*, 2013), we conducted a study with European starling hepatocytes to measure the expression levels of CYP1A4/5 mRNA after exposure to graded concentrations of TCDD, PeCDF and TCDF. The goals were (1) to determine starling hepatocyte sensitivity to CYP1A4/5 mRNA induction and (2) to compare the relative potencies of TCDD, PeCDF and TCDF. The results indicate that European starling and chicken hepatocytes have approximately the same sensitivity to CYP1A4/5 induction by TCDD. However, starling hepatocytes are

less sensitive than chicken hepatocytes to CYP1A4/5 induction by PeCDF and TCDF.

5.3. MATERIALS AND METHODS

5.3.1. Source of eggs and incubation conditions

European starling eggs (n=116) were collected from twenty-one nest boxes located in Delhi and St. George in southern Ontario. The boxes were monitored for forty days before the eggs were collected (in May 2010). The starlings readily re-nested or continued to lay eggs following egg removal, and no reduction in reproductive output was observed. All field and laboratory procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre.

Eggs were placed in portable incubators (Hova-Bator, G.Q.F. Manufacturing, Savannah, GA, USA) and temperature was maintained between 35-38°C. Humidity was provided by adding water to the water trays for the approximately eight hours it took to transport the eggs to the laboratory at the National Wildlife Research Center, after which they were incubated at 37.5°C and 58% relative humidity in Brinsea Contaq Z6 incubators (Sandford, England) until one to two days before the predicted hatch date (starlings hatch between days 12 and 13 days (Ricklefs and Smeraski, 1983)). The eggs were candled periodically and any dead or infertile embryos were discarded.

5.3.2. Preparation of TCDD, PeCDF and TCDF solutions

A detailed description of the analysis and purification of TCDD, PeCDF and TCDF solutions is provided elsewhere (Herve *et al.*, 2010b). In brief, serial dilutions of TCDD, PeCDF and TCDF were prepared from stock solutions in dimethyl sulfoxide (DMSO), and concentrations were determined by isotope dilution following EPA method 1613 (U.S.EPA, 1994), using high-resolution gas chromatography and high-resolution mass spectrometry.

5.3.3. Preparation and dosing of cultured hepatocytes

Primary cultures of hepatocytes were prepared from starling embryos using previous methods (Kennedy *et al.*, 1995), and included subsequent modifications (Head and Kennedy, 2007; Kennedy *et al.*, 2003). Briefly, starling embryos (n=82) were euthanized by decapitation and the livers were removed, pooled and digested with collagenase. Hepatocytes were separated from erythrocytes using Percoll (Amersham Bioscience, Uppsala, Sweden) density gradient centrifugation and cell clumping was prevented by use of DNAse (Roche, Laval, Quebec, Canada) treatment. The cells were plated in 48-well culture plates in cell culture medium 199 supplemented with insulin (1 µg/ml) and thyroxine (1 µg/ml), and incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. The cells were then exposed to DMSO (2.5 µl) or DMSO solutions of TCDD, PeCDF or TCDF for 24 h at concentrations ranging from 0.003 to 300 nM in triplicate, after which the plates were flash-frozen in powdered dry ice and stored at -80°C until analysis.

5.3.4. Cell viability

The Calcein-AM assay was used, as per the manufacturer's instructions (Invitrogen-Molecular Probes, Eugene, OR, USA), to determine the viability of the cells after 24 h exposure to the range of concentrations administered for each compound. Vehicle (DMSO) treated cells were used as a positive control and 99% ethanol-killed cells were the negative control. The culture medium was removed, 200 µl of the Calcein-AM working solution (3 µl of Calcein-AM in 10 mL of PBS-EDTA) was added to each well, and plates were then incubated in the dark for 45 min at room temperature. Fluorescence was measured using a Cytofluor 2350 (Millipore, Bedford, MA, USA) fluorescence plate reader with a 485 nm excitation wavelength and a 530 nm emission wavelength.

5.3.5. RNA isolation and complementary DNA (cDNA) synthesis

Total RNA was extracted from each well using RNeasy 96 kits (Qiagen, Mississauga, Ontario, Canada) as per the manufacturer's instructions and with the modifications described elsewhere (Herve *et al.*, 2010b). To eliminate residual DNA contamination, the on-column DNase treatment was performed during the RNA extraction. A second DNase treatment was performed using the Ambion TURBO DNA-free kit (Ambion, Austin, TX), as per the manufacturer's instructions, to ensure maximal removal of genomic DNA. Complementary DNA (cDNA) was synthesized by the reverse transcription of approximately 175 ng of DNase-treated total RNA from each well using Superscript II reverse transcriptase and random hexamers (Invitrogen, Burlington, Ontario, Canada) as

per the manufacturer's instructions. To verify the absence of genomic DNA in the RNA template, a control without the reverse transcriptase enzyme (no-reverse transcriptase control) was included for each plate.

5.3.6. Real -time reverse transcription polymerase chain reaction (real-time RT-PCR)

Multiplex real-time RT-PCR assays were employed to measure CYP1A4, CYP1A5 and β-actin (normalizer gene) mRNA abundance in starling embryo hepatocytes exposed to DMSO, TCDD, PeCDF or TCDF by use of dual-labeled fluorescent hydrolysis probes (Head and Kennedy, 2007). The primers and probes were designed as previously described (Herve *et al.*, 2010b) and obtained from Invitrogen and Biosearch Technologies (Novato, CA, USA), respectively. Primer and probe sets for European starling CYP1A4 and CYP1A5 mRNA were designed based on ClustalW alignments of available avian CYP1A4 and CYP1A5 sequences in GenBank (Table 5.1). Reactions were performed using Brilliant Multiplex QPCR Mastermix kits (Agilent Technologies, Santa Clara, CA, USA). Each 25 µL reaction mixture contained primers and probes for CYP1A4, CYP1A5 and β-actin, 1x Multiplex QPCR master mix, 30 nM reference dye (ROX) and 5 µl of cDNA. The thermal profile for all reactions was 10 min at 95°C, followed by 40 cycles of 95°C for 30 sec and 60°C for 1 min. All samples were assayed in duplicate. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008).

Table 5.1. European starling cytochrome P4501A4 (CYP1A4), CYP1A5 and β-actin primer and probe sequences, dye, quencher and amplicon size in base pairs (bp). Final primer and probe concentrations used in the QPCR master mix are shown in brackets.

European Starling	
CYP1A4	
Dye/Quencher	FAM/BHQ-3
Forward	5'-GCGGTCTTCAAGGACATCAA-3' (300 nM)
Reverse	5'-TCCCGAATGTGCTCCTTATC-3' (300 nM)
Probe[a]	5'-AAGGTCCTGTAATGCTCCTGCACAA-3' (200 nM)
Amplicon size	95
CYP1A5	
Dye/Quencher	Quasar 670/BHQ-2
Forward	5'-CGCAGCATGAAATTGTTTCT-3' (300 nM)
Reverse	5'-CGGATGTTGTTCTCGTCGTA-3' (300 nM)
Probe[b]	5'-CCTGCAGAAGAGGGTCAAGGAGCA-3' (200 nM)
Amplicon size	101
β-actin	
Dye/Quencher	JOE/BHQ-1
Forward primer	5'-AAATTGTGCGTGACATCAAGGA-3' (50 nM)
Reverse primer	5'-GAGGCAGCTGTGGCCATCT-3' (50 nM)
Probe[a]	5'-TGCTCCTTGACCCTCTTCTGCAGG-3' (200 nM)
Amplicon size	76

[a]Probe anneals to the same strand as the reverse primer.
[b]Probe anneals to the same strand as the forward primer.

5.3.7. Concentration-dependent effects of DLCs on CYP1A mRNA expression

The concentrations of TCDD, PeCDF and TCDF that elicited a response equal to 20, 50 or 80% of the TCDD maximal response ($TCDD_{20}$, $TCDD_{50}$ or $TCDD_{80}$), EC_{50}, and maximal response values are presented as the mean value detected for three replicate wells ± standard deviation (SD). To calculate $TCDD_{20}$, $TCDD_{50}$, and $TCDD_{80}$ for CYP1A4 and CYP1A5 induction, the fold change data were first converted to the percent maximum fold change elicited by TCDD. The normalized fold change response data were then plotted against log DLC concentration and concentration-response curves were fitted using a four parameter logistic equation (Head and Kennedy, 2007). $EC_{threshold}$ (EC_{thr}) values refer to the concentration of a compound that elicited a significantly higher level of CYP1A4/5 than that detected in DMSO-treated cells. Data from the average of three wells (each analyzed in duplicate by real-time RT-PCR) from the same cell culture plate were used to generate curve fits for induction of mRNA expression.

5.3.8. Statistical analysis

Statistical differences among maximal response, logEC_{50}, log $TCDD_{20}$, log $TCDD_{50}$, and log $TCDD_{80}$ values for TCDD, PeCDF and TCDF within each specific endpoint were determined by performing an unpaired t-test ($p < 0.05$) or a one-way ANOVA ($p < 0.05$) followed by Tukey's Multiple Comparison Test ($p < 0.05$). EC_{thr} was determined using a one-way ANOVA ($p < 0.05$) with a Dunnett's post-hoc test ($p < 0.05$). Only the concentrations that elicited a response less than 10% of the positive control were included in the ANOVA analysis for determining EC_{thr}.

5.3.9. Relative sensitivity (ReS) calculations

Because the chicken is believed to be the most sensitive avian species to the toxic and biochemical effects of DLCs, the sensitivity of European starling hepatocytes to CYP1A4 and 1A5 mRNA induction was expressed relative to that previously observed in chicken hepatocytes (Herve *et al.*, 2010b). Since the maximal response and slopes of concentration-response curves differed between chicken (Herve *et al.*, 2010b) and starling hepatocytes (current study), EC_{thr} values were used to calculate relative sensitivity ($ReS_{threshold}$, ReS_{thr}) for European starling. EC_{50}-based ReS values were also calculated for comparison to EC_{thr}-based ReS values. EC_{50} values for chicken were taken from a previously published study (Herve *et al.*, 2010b) and EC_{thr} values for chicken were calculated from raw data of the same study. EC_{thr}-based ReS or EC_{50}-based ReS values were calculated as follows: EC_{thr} or EC_{50} of compound *x* in chicken ÷ EC_{thr} or EC_{50} of compound *x* in European starling.

5.3.10. Relative potency (ReP) calculations

The potency of PeCDF and TCDF was measured relative to TCDD. The ReP is a comparison of the CYP1A-inducing potency of a compound relative to the CYP1A-inducing potency of TCDD in the same species (the European Starling in this case). Since the maximal responses and slopes of concentration-response curves differed among DLC treatments, the relative potency of PeCDF and TCDF were calculated based on the systematic approach proposed by Villeneuve and colleagues (Villeneuve *et al.*, 2000). ReP values (ReP_{TCDD20},

$ReP_{TCDD50,}$ ReP_{TCDD80} and $ReP_{TCDDmax}$) were calculated based on $TCDD_{20}$, $TCDD_{50}$, and $TCDD_{80}$ values obtained from the four parameter logistic models. When the maximal response of PeCDF or TCDF was less than 50% or 80% of the TCDD maximal response, a highest observed response for that chemical ($TCDD_{max}$) was calculated. ReP values were calculated as follows: $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ or EC_{thr} of TCDD ÷ $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ or EC_{thr} of PeCDF or TCDF. Average relative potency values (ReP_{avg}) were calculated from $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$ and EC_{thr} –based values. EC_{50}-based ReP values were not used for the ReP_{avg} calculation because these values overestimate the potency of chemicals with lower maximal responses than TCDD (Kennedy *et al.*, 1996). Finally, if the highest observed response was less than 20% of the TCDD maximal response, a ReP_{avg} was calculated based on EC_{thr} values only.

5.4. RESULTS

5.4.1. CYP1A4 and CYP1A5 mRNA induction

Results from the Calcein-AM cell viability assay showed no cytotoxic effect at any concentration of TCDD, PeCDF or TCDF (data not shown). TCDD, PeCDF and TCDF induced CYP1A4 mRNA and CYP1A5 mRNA in a concentration-dependent manner and mRNA expression of both isoforms reached a plateau at compound-specific concentrations (Figure 5.1). TCDD and PeCDF induced similar maximal levels of CYP1A4 mRNA expression (2547-fold and 2749-fold, respectively), while TCDF elicited a significantly lower maximal response (204-fold; Table 5.2; Figure 5.1). Maximal levels of CYP1A5 mRNA expression induced

by TCDD, PeCDF, and TCDF were 14-fold, 20-fold and 6.9-fold, respectively. Amplification was not detected in the no-reverse transcriptase and no-template controls for any of the assays, which confirmed the absence of contamination. β-actin (the normalizer gene) mRNA expression was consistent across all treatment groups and was therefore considered to be an appropriate control gene. The fold induction data were converted to the percent maximum fold induction elicited by TCDD (% TCDD) and concentration-dependent curves were generated to (a) provide a visual comparison among compounds and (b) calculate ReP values (see below) (Figure 5.2).

5.4.2. Relative sensitivity (ReS) and relative potency (ReP) values

The EC_{thr}-based ReS values of European starling hepatocytes exposed to TCDD, PeCDF and TCDF were 1, 0.1 and 0.03 (for CYP1A4) and 3, 0.1 and 0.01 (for CYP1A5), respectively (Table 5.2). The ReP_{avg} values were calculated based on EC_{thr}, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$ and $TCDD_{max}$ values for CYP1A4 and CYP1A5 mRNA expression. The ReP_{avg} values for CYP1A4 and CYP1A5 mRNA induction were 0.1 and 0.2 for PeCDF and 0.03 and 0.009 for TCDF, respectively (Table 5.3). Overall, the rank order of potency of the compounds was TCDD > PeCDF > TCDF (Figure 5.2, Table 5.3).

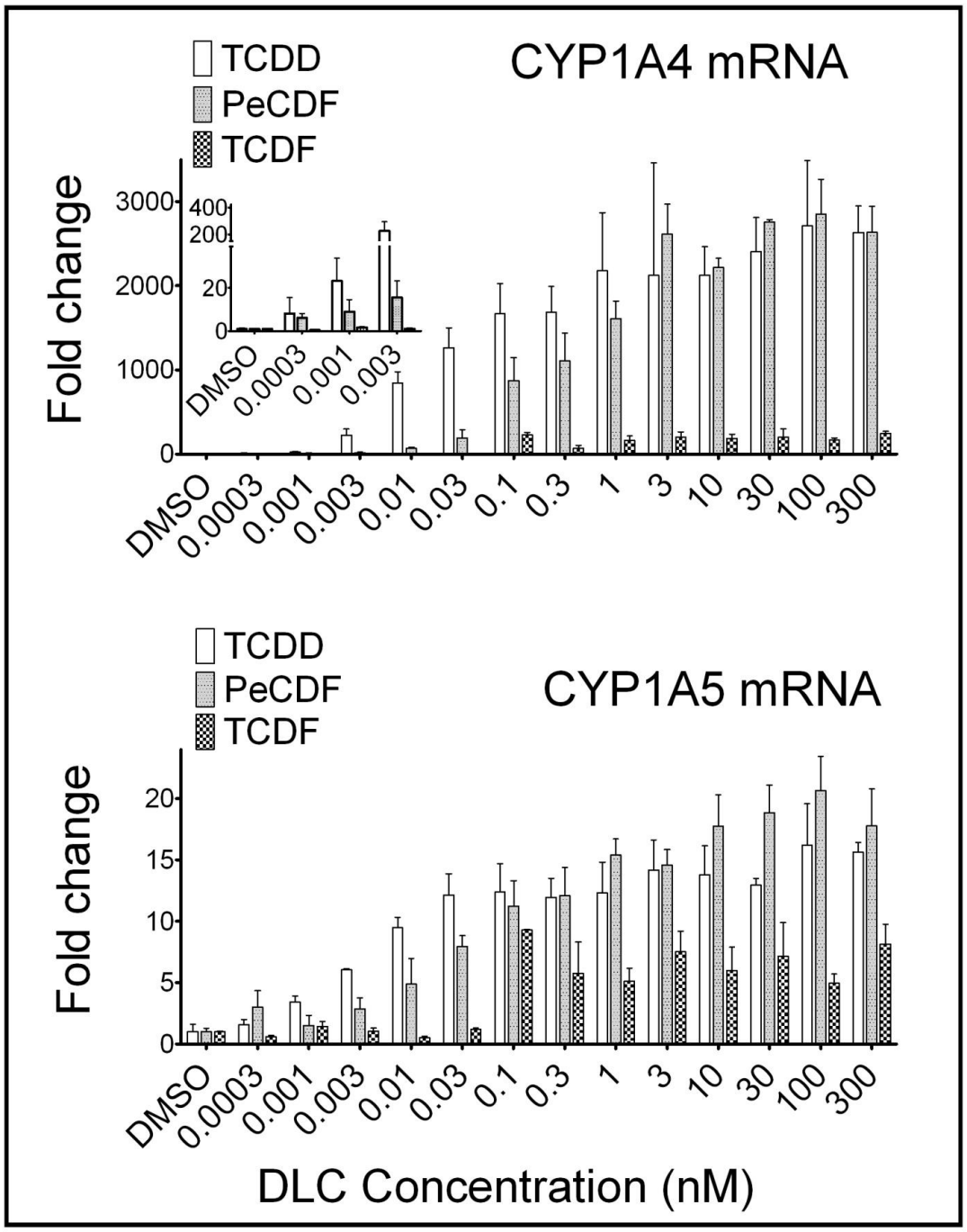


Figure 5.1. Concentration-dependent effects of TCDD, PeCDF and TCDF on CYP1A4 and CYP1A5 mRNA expression in European starling embryo hepatocyte cultures after exposure for 24 h. Bars represent the mean fold change ± standard deviation (SD) of three replicate cell culture wells assessed in duplicate by real-time RT-PCR.

Table 5.2. Threshold effective concentration (EC_{thr}), half maximal effective concentration (EC_{50}), maximal response, EC_{thr}–based relative sensitivity (ReS_{thr}) and EC_{50}–based relative sensitivity (ReS_{50}) for CYP1A4 and CYP1A5 mRNA data obtained from European starling and chicken embryo hepatocyte cultures exposed to TCDD, PeCDF and TCDF for 24 h. The chicken hepatocyte data are from an earlier study (Herve *et al.*, 2010b). ReS_{thr} and ReS_{50} for the European starling were calculated as follows: EC_{thr} or EC_{50} of compound *x* in chicken ÷ EC_{thr} or EC_{50} of compound *x* in European starling. EC_{thr} or EC_{50} ± standard deviation (SD) and maximal response ± SD values were calculated from the curve fit. EC_{thr} values were calculated using a one-way ANOVA ($p < 0.05$) followed by Dunnett's test ($p < 0.05$).

		Starling embryo hepatocytes			Chicken embryo hepatocytes*			Relative sensitivity	
Compound	**mRNA**	**EC_{thr} (nM)**	**EC_{50} ± SD (nM)**	**Maximal response ± SD (fold change)**	**EC_{thr} (nM)**	**EC_{50} ± SD (nM)**	**Maximal response ± SD (fold change)**	**EC_{thr}-based ReS**	**EC_{50}-based ReS**
TCDD	**CYP1A4**	0.003	0.045 ± 0.01[a]	2547 ± 210[a]	0.003	0.026 ± 0.01[a]	502 ± 81[a]	1	0.6
PeCDF	**CYP1A4**	0.03	0.47 ± 0.1[b]	2749 ± 61[a]	0.003	0.037 ± 0.01[a]	490 ± 40[a]	0.1	0.08
TCDF	**CYP1A4**	0.1	0.42 ± 0.1[b]	204 ± 25[b]	0.003	0.017 ± 0.02[a]	222 ± 38[b]	0.03	0.04
TCDD	**CYP1A5**	0.001	0.0062 ± 0.002[a]	14 ± 0.7[a]	0.003	0.021 ± 0.009[a]	78 ± 10[a]	3	3
PeCDF	**CYP1A5**	0.01	0.13 ± 0.06[b]	20 ± 1.2[b]	0.001	0.014 ± 0.005[a]	50 ± 3[b]	0.1	0.1
TCDF	**CYP1A5**	0.1	0.17 ± 0.1[b]	6.9 ± 0.8[c]	0.001	0.020 ± 0.01[a]	47 ± 3[b]	0.01	0.1

*Data from Herve et al. (2010a).
Mean values for starling and chicken were derived from the average of three different wells of the same cell culture plate, assessed in duplicate ± SD.
Superscript letters indicate significant differences ($p < 0.05$) in EC_{50} or maximal response of CYP1A4 or CYP1A5 after exposure to TCDD, PeCDF or TCDF for 24 hours.

Table 5.3. EC_{thr}, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ and relative potency (ReP) values in European starling embryo hepatocyte cultures exposed to TCDD, PeCDF and TCDF for 24 h. $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$ and $TCDD_{max}$ ± SD were calculated from the curve fit (Figure 5.2). EC_{thr} values were calculated using a one-way ANOVA ($p < 0.05$) followed by Dunnett's test ($p < 0.05$). Average relative potency values (ReP_{avg}) for PeCDF and TCDF relative to TCDD were calculated from the average of $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ and EC_{thr}-based ReP values. If the highest observed response was less than 20% of the TCDD maximal response, a ReP_{avg} was calculated only based on EC_{thr} values.

Compound	mRNA	EC_{thr} (nM)	$TCDD_{20}$ ± SD (nM)	$TCDD_{50}$ ± SD (nM)	$TCDD_{80}$ ± SD (nM)	$TCDD_{max}$ (nM)
TCDD	**CYP1A4**	0.003	0.0052 ± 0.001^{a}	0.045 ± 0.01^{a}	0.46 ± 0.1^{a}	NC
PeCDF	**CYP1A4**	0.03	0.074 ± 0.03^{b}	0.41 ± 0.18^{b}	2.2 ± 1.4^{a}	NC
TCDF	**CYP1A4**	0.1	N/A	N/A	N/A	NC
TCDD	**CYP1A5**	0.001	0.0011 ± 0.0006^{a}	0.0062 ± 0.002^{a}	0.049 ± 0.04^{a}	0.0024
PeCDF	**CYP1A5**	0.01	0.0030 ± 0.001^{a}	0.032 ± 0.004^{b}	0.21 ± 0.1^{b}	NC
TCDF	**CYP1A5**	0.1	0.16 ± 0.1^{b}	N/A	N/A	0.26*

Compound	mRNA	ReP_{ECthr}	ReP_{TCDD20}	ReP_{TCDD50}	ReP_{TCDD80}	$ReP_{TCDDmax}$	ReP_{avg}
TCDD	**CYP1A4**	1	1	1	1	NC	1
PeCDF	**CYP1A4**	0.1	0.07	0.1	0.2	NC	0.1
TCDF	**CYP1A4**	0.03	N/A	N/A	N/A	NC	0.03
TCDD	**CYP1A5**	1	1	1	1	1	1
PeCDF	**CYP1A5**	0.1	0.4	0.2	0.2	NC	0.2
TCDF	**CYP1A5**	0.01	0.007	N/A	N/A	0.01	0.009

N/A: not applicable since response was less than 20% of the TCDD maximal response.
NC: not calculated- $TCDD_{max}$ and $TCDD_{max}$ –based ReP values were calculated if the maximal response of PeCDF or TCDF did not reach 50% or 80% of the TCDD maximal response.
Superscript letters indicate significant differences ($p < 0.05$) in $TCDD_{20}$, $TCDD_{50}$ and $TCDD_{80}$ of CYP1A4 or CYP1A5 mRNA after exposure to TCDD, PeCDF or TCDF for 24 hours.
*maximal response of TCDF was 35% of the TCDD maximal response.

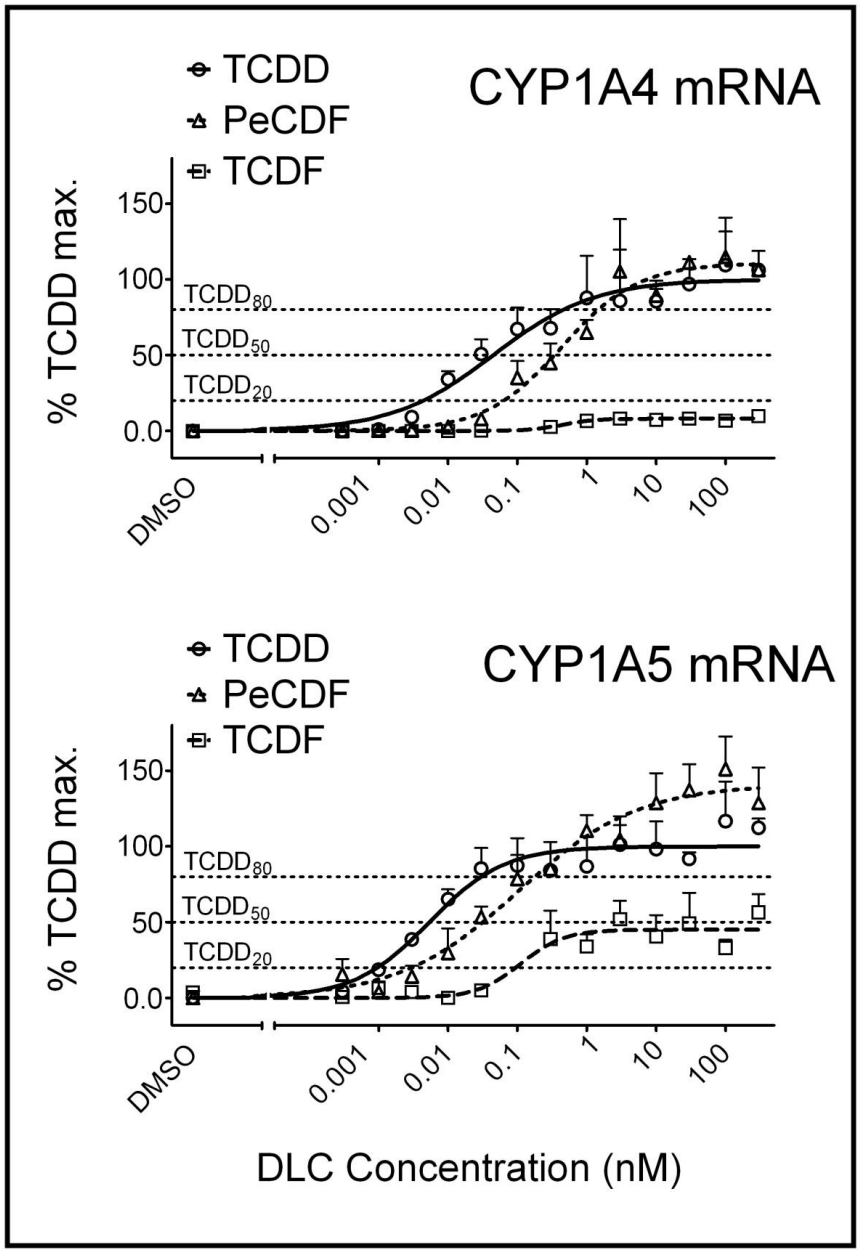


Figure 5.2. Concentration-dependent effects of TCDD (○), PeCDF (Δ) and TCDF (□) on CYP1A4 and CYP1A5 mRNA expression in European starling embryo hepatocyte cultures exposed for 24 h. Data are expressed as percent maximal response of TCDD. The DLC concentrations that elicited a response equal to 20, 50 or 80% of the TCDD maximal response ($TCDD_{20}$, $TCDD_{50}$ or $TCDD_{80}$) were calculated from the curve fit using a four parameter logistic equation. Each data point represents the mean fold change ± standard deviation (SD) of three replicate cell culture wells assessed in duplicate by real-time RT-PCR.

5.5. DISCUSSION

The goal of this study was to determine the concentration-dependent effects of TCDD, PeCDF and TCDF on CYP1A4 and CYP1A5 mRNA levels in primary hepatocyte cultures of embryonic European starlings. European starlings were of interest because (a) the sequencing results of the AHR1 LBD indicated that the amino acids at sites 324 and 380 were identical to those of the only avian species (the domestic chicken) known to be highly sensitive to the toxic effects of DLCs (Farmahin *et al.*, 2013) and (b) the North American population is very large and evidence of exposure to DLCs in the wild exists (Arenal *et al.*, 2004; Halbrook and Arenal, 2003; Van den Steen *et al.*, 2007). The results of the present study indicate that the relative sensitivity of European starlings to CYP1A mRNA induction by TCDD is similar to chicken (Table 5.2). This finding corroborates the results of the LRG assay presented in our previous studies (Farmahin *et al.*, 2013; Manning *et al.*, 2012) that suggested COS-7 cells expressing chicken mutant AHR1 containing the starling AHR1 LBD and cells expressing chicken AHR1 LBD were similar in sensitivity to the effects of DLCs.

Another study showed that among four species of wild birds, starlings were the most sensitive to Aroclor 1254 (a commercial mixture of PCBs) (Stickel *et al.*, 1984). In that study, European starlings (type 1), red-winged blackbirds (*Agelaius phoeniceus*; type 2), brown-headed cowbirds (*Molothrus ater*; type 2) and common grackles (*Quiscalus quiscula*; type 2) were exposed to dietary concentrations of 1,500 ppm Aroclor 1254 until 50% died. Fifty percent mortality for starlings, blackbirds, cowbirds and grackles was observed at 4, 6, 7 and 8 days, respectively. As such, the study by Stickel and colleagues supports the

hypothesis that the starling, a type 1 species, is more sensitive to certain DLCs than type 2 avian species given that the 50% mortality endpoint occurred earlier than the other species tested. Lower PCB residues were detected in dead starlings compared to those in the other species (Stickel *et al.*, 1984) suggesting that the clearance rate of PCBs (and perhaps other DLCs) was faster in starlings.

Starling hepatocytes had relatively low sensitivity to PeCDF and TCDF. Based on EC_{thr} values calculated from CYP1A4/5 mRNA expression for European starling hepatocytes and chicken hepatocytes (Herve *et al.*, 2010b), the European starling is 10-fold and 33- to 100-fold less sensitive than chicken to PeCDF and TCDF, respectively. The sensitivity of starling hepatocytes to dioxin and not furans might suggest differential metabolism of these chemicals in starling hepatocytes. A previous study in our laboratory demonstrated the effect of *in vitro* metabolism of DLCs on concentration-response curves (Bastien et al. 1997). The results of a study that determined the concentration of DLCs in 19 bald eagle *(Haliaeetus leucocephalus)* carcasses in British Colombia, Canada, showed birds with higher concentrations of TCDD tended to have unusually low concentrations of TCDF (Elliott *et al.*, 1996). Similarly, eagles, found dead in the Upper Peninsula of Lake Michigan during 2000, that had elevated TCDD concentrations tended to have greater TCDD:TCDF and PCB 126:PCB 77 ratios (Kumar *et al.*, 2002). These findings suggest differential metabolism of DLCs in certain avian species and may also indicate that hepatic CYP1A induction by TCDD results in metabolism of TCDF.

The results of the present study indicate that the potency of TCDD (ReP_{avg}) for CYP1A mRNA induction in European starling hepatocytes is

approximately 5- to 10-fold and 33- to 111-fold greater than the potencies of PeCDF and TCDF, respectively (Table 5.3). This finding is of interest because earlier studies that determined *in vitro* or *in vivo* potencies of these chemicals in several avian species found that PeCDF was equally potent or more potent than TCDD (Cohen-Barnhouse *et al.*, 2011; Farmahin *et al.*, 2012; Herve *et al.*, 2010b; Herve *et al.*, 2010c; Sanderson *et al.*, 1998). For example, the LRG assay showed that PeCDF was 20– and 26–fold more potent than TCDD in cells expressing Japanese quail and common tern (*Sterna hirundo*) AHR1 (Farmahin *et al.*, 2012).

The preparation of hepatocyte cultures from embryonic European starlings presented many challenges. Our laboratory has many years of experience preparing hepatocyte cultures from various species of domestic and wild species of birds, but this was our first attempt to prepare hepatocytes from a species with extremely small eggs. An average starling egg weighs approximately 7 g and hepatocytes extracted from each liver weigh approximately 2.7 mg. On the other hand, chicken and herring gull eggs weigh approximately 50 g and 92 g and hepatocytes extracted from chicken and herring gull livers weigh approximately 56 mg and 75 mg. For the work presented in this study we used eggs collected from the field that were in various stages of incubation. The small size of starling embryo livers required a relatively large number of starling embryos to seed one 48-well cell culture plate. Fourteen starling embryo livers were used to seed one 48-well cell culture plate while only one chicken or herring gull embryo is needed to seed 1.5 and approximately 2 plates, respectively. To overcome this limitation

of working with species that have small embryos, a study is currently underway in our laboratory to prepare hepatocyte cultures from adult European starlings.

In conclusion, the European starling, a type 1 species based on AHR1 LBD genotype, was as sensitive as the chicken to CYP1A4/5 mRNA induction after exposure to TCDD in primary hepatocyte cultures. Surprisingly, the starling was less sensitive than chicken to CYP1A4/5 mRNA induction by PeCDF and TCDF, and the reason for differences among these types 1 species is not known. It is possible that differential metabolism of DLCs causes such effects, and studies to test this hypothesis are warranted.

CHAPTER 6 : RESEARCH SUMMARY AND FUTURE DIRECTIONS

6.1. OVERALL OBJECTIVES

The main goal of my Ph.D. research was to develop *in vitro* methods and knowledge to explain and predict differences in avian sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and dioxin-like compounds (DLCs). To meet this goal, a luciferase reporter gene (LRG) assay and cell-based binding assays were developed to circumvent problems associated with previously established *in vitro* assays. The LRG assay measures aryl hydrocarbon receptor 1 (AHR1)-mediated gene expression *in vitro*, and the cell-based binding assay measures AHR1 binding affinity to ligands.

6.2. HYPOTHESIS TESTING AND SUMMARY OF STUDY OUTCOMES

6.2.1. The LRG assay can be used as an alternative to the ethoxyresorufin *O*-deethylase (EROD) assay to predict avian *in ovo* (egg injection) sensitivity to DLCs.

A strong correlation (r^2 = 0.77; $p < 0.005$) was found between LD_{50} data from egg injection studies (Cohen-Barnhouse *et al.*, 2011) and EC_{50} data from the LRG assay (data from Chapter 2) for chicken (*Gallus gallus domesticus*), ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*) exposed to TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). To compare LRG and EROD assays, the correlation between LD_{50} data (Cohen-Barnhouse *et al.*, 2011) and EROD EC_{50} values (Herve *et al.*, 2010b) for similar species and compounds was calculated (r^2

= 0.51, $p < 0.05$). Other studies have shown that the EROD and LRG assays predict LD_{50} values to the same extent (Farmahin *et al.*, 2012; Head and Kennedy, 2010). For example, in Chapter 3 I found a strong correlation (r^2=0.91, p=0.001; Figure 3.12 , panel A) between LD_{50} values (Cohen-Barnhouse *et al.*, 2011; Head *et al.*, 2008) and LRG EC_{50} values in wild-type AHR1 constructs (chicken, pheasant and quail) exposed to TCDD, PeCDF and PCB 126. A strong correlation (r^2 = 0.92, $p < 0.005$) was also reported between EROD EC_{50} values and embryonic LD_{50} values in different species of birds (chicken, ring-necked pheasant, turkey, double-crested cormorant and common tern) exposed to TCDD and PCB 126 (Head and Kennedy, 2010). Results from this thesis demonstrate that differences in sensitivity exist between the LRG and EROD assays. For example, I determined the EC_{50} of TCDD in COS-7 cells expressing chicken AHR1 to be 0.21 nM, but Herve *et al.* (2010) reported an EC_{50} for TCDD of 0.018 nM based on the EROD assay. The reduced sensitivity of the LRG assay limits its ability to predict avian sensitivity to weak AHR1-agonists. However, the LRG assay, unlike other *in vitro* methods such as induction of EROD activity and measurement of CYP1A mRNA expression, does not require the bird of interest to be euthanized because the full-length AHR1 sequence can be determined by using a drop of blood. Overall, the results support the hypothesis that the LRG assay is a useful alternative to the EROD assay for predicting species sensitivity to potent DLCs.

6.2.2. The major determinant of the sensitivity of avian AHR1 to DLCs resides within the LBD and not the DNA-binding domain (DBD) or transactivation domain (TAD).

The findings presented in Chapter 3 suggest that amino acids within the AHR1 DBD and TAD have no effect on the sensitivity of the birds studied to DLCs. This is because the linear regression equations between *in ovo* LD_{50}s and LRG EC_{50} data, in which wild-type avian AHR1 (r^2=0.91, p=0.001) or mutant AHR1 (r^2=0.93, p<0.0001) were used, had similar correlation coefficients, slopes and Y-intercepts. These results confirmed and extended the results of Karchner *et al.* (2006), which showed that differential sensitivity of chicken and common tern AHR1 to activation by TCDD resided in the ligand binding domain.

6.2.3. The sensitivity of birds to DLCs is determined by the amino acids at sites 324 and 380 in the AHR1 ligand binding domain (LBD).

In Chapter 2, site-directed mutagenesis was used to generate two mutated quail AHR1s: V324I (pheasant-like mutant quail AHR1 with substitution of a single amino acid at position 324), and V324I_A380S (chicken-like mutant quail AHR1 with substitution of two amino acids at sites 324 and 380). The relative sensitivity (ReS) of COS-7 cells expressing quail AHR1 and exposed to TCDD (ReS = 0.011) increased approximately 10-fold when valine at position 324 was changed to isoleucine (V324I; ReS = 0.13). The sensitivity of cells expressing the V324I mutant was identical to that of cells expressing pheasant AHR1 (ReS for pheasant = 0.13). Similarly, when the two amino acids at positions 324 and 380 were altered to obtain V324I_A380S (chicken-like mutant), the ReS increased to

0.7, which is similar to that of cells expressing chicken AHR1 (ReS = 1). ReS similarity between cells expressing pheasant and V324I, and chicken and V324I_A380S, was also observed when the cells were exposed to PeCDF or TCDF. In Chapter 3, chicken AHR1 was used to synthesize pheasant-like and quail-like AHR1 mutants and the results confirmed the findings reported using quail mutants in Chapter 2. The results of the homology modeling studies outlined in Chapter 3 showed that the side chains of amino acids 324 and 380 contribute to the binding cavity and ligand binding site locations for chicken, pheasant and quail are between these two amino acids side chains (Figure 3.5). This finding suggests there might be a strong interaction between a ligand and these two amino acid side chains. Together, these results support the hypothesis that avian sensitivity to DLCs is determined by the amino acids at positions 324 and 380 within the AHR1 ligand-binding domain.

6.2.4. The amino acid residues at sites 256, 257, 297 and 337 in avian AHR1 do not play a role in the determination of species sensitivity to DLCs.

The LRG assay results reported in Chapter 3 showed that the identities of four variable amino acid sites within the LBD (256, 257, 297 and 337) did not affect the ability of AHR1 to transactivate the aryl hydrocarbon responsive element (AHRE)-driven reporter gene when exposed to TCDD and PeCDF. As explained in Chapter 3, the identities of amino acids 256, 257, 297 and 337 vary among species and are used to define 13 sub-types (Table 3.1). Among avian AHR1 constructs, the rank order in sensitivity to induce AHRE-driven reporter gene activity was: type 1(1A, 1B and 1C) > type 2 (2A, 2B, 2C, 2D, 2E, 2F and

2G) > type 3 (3A, 3B and 3C). EC_{50} values obtained for all AHR1 types and sub-types exposed to TCDD indicated that there were no statistical differences among the sub-types within each type. Moreover, homology modeling in Chapter 3 showed that side chains of these residues do not contribute to the binding cavity surface (Figure 3.13) because they point toward the outside of the binding cavity. These results are consistent with a study that employed the LRG assay to determine avian species sensitivity to AHR1 activation by several PCB congeners (Manning *et al.*, 2012) and support the hypothesis that amino acids 256, 257, 297 and 337 do not play a role in determining species differences in sensitivity to DLCs.

6.2.5. TCDD and PeCDF are equipotent inducers of the reporter gene in cells expressing chicken, pheasant, Japanese quail and common tern (*Sterna hirundo*) AHR1.

The results in Chapter 2 show that PeCDF (ReP = 0.84) is as potent as TCDD (ReP = 1.0) at inducing AHR1-mediated reporter gene activity in COS-7 cells transfected with chicken AHR1, and more potent than TCDD for ring-necked pheasant (ReP = 3.7), Japanese quail (ReP = 20), and common tern (ReP = 26) AHR1. Thus, the results support the hypothesis that TCDD and PeCDF are equipotent in the chicken. However, the hypothesis is rejected for ring-necked pheasant, Japanese quail and common tern. In addition, results of a recent study showed that PeCDF was a more potent inducer of EROD, CYP1A4 mRNA and CYP1A5 mRNA than TCDD in ring-necked pheasant and Japanese quail embryo hepatocyte cultures, while PeCDF and TCDD were equipotent inducers in

chicken embryo hepatocytes (Herve *et al.*, 2010c). The results of these *in vitro* studies are in general agreement with the results from egg injection studies (Cohen-Barnhouse *et al.*, 2011). This finding is important because TCDD is commonly considered to be the most potent AHR agonist and is used as the reference DLC to derive the relative potency for all other DLCs.

6.2.6. The differences in PeCDF relative potency among avian species is due to differences in its binding affinity to AHR1 in each species.

The results of the cell-based binding assay and the hydroxyapatite (HAP) binding assay presented in Chapter 4 show that PeCDF binds to pheasant and Japanese quail AHR1 with greater affinity than TCDD, and that PeCDF and TCDD bind with equal affinity to chicken AHR1. These data support the hypothesis that the differences in PeCDF relative potency among these birds are due to differences in binding affinity between avian AHR1s and PeCDF/TCDD. The finding is consistent with previous studies that showed correlation between AHR binding affinities and EC_{50}s for CYP1A-catalyzed EROD induction (Hestermann *et al.*, 2000; Safe, 1990).

6.2.7. Two amino acids at positions 324 and 380 contribute to the differences in PeCDF potency among species.

Several *in ovo* and *in vitro* studies revealed that PeCDF was more potent than TCDD in certain avian species; however, the potency of PeCDF and TCDD in chicken is identical. Hepatocyte cell culture studies and LRG assays showed that the ReP of PeCDF was slightly higher than TCDD in ring-necked pheasant

(pheasant AHR1 has isoleucine at site 324 and alanine at site 380) and significantly higher than TCDD in avian species with a valine at site 324 and an alanine at site 380 of the AHR1; for example, double-crested cormorant (*Phalacrocorax auritus*; 10–13-fold), Forster's tern (*Sterna forsteri*; 10–15-fold), Japanese quail (13–27-fold) (Farmahin *et al.*, 2012; Herve *et al.*, 2010c; Sanderson *et al.*, 1998).

Site-directed mutagenesis, described in Chapters 2 and 3, showed that the amino acid identities at positions 324 and 380 affect the relative potency of PeCDF. For example, the PeCDF relative potencies presented in Chapter 2 in cells expressing quail, V324I (pheasant- like mutant), pheasant, V324I_A380S (chicken-like mutant) and chicken AHR1s are 20, 3.6, 3.7, 1.1 and 1, respectively. Similar findings were reported in Chapter 3 with chicken mutants and these results support the hypothesis that amino acids at positions 324 and 380 determine the relative potency of PeCDF.

6.2.8. The European Starling and chicken are equally sensitive to DLCs.

This hypothesis was supported for TCDD, but not for PeCDF or TCDF. The results in Chapter 5 of this thesis indicate that starling CYP1A4/5 mRNA expression EC_{50} values calculated from TCDD concentration-response curves were similar to chicken EC_{50} values (Herve *et al.*, 2010b). However, starling hepatocytes had relatively low sensitivity to PeCDF and TCDF; indeed, the EC_{50} values for PeCDF and TCDF were more similar to type 3 avian species, such as the herring gull *(Larus argentatus)* and Japanese quail. The sensitivity of starling

hepatocytes to dioxin but not furans could suggest variable metabolism of these chemicals in starling hepatocytes.

6.3. FUTURE RESEARCH AND SUGGESTIONS

6.3.1. Improve the sensitivity of the luciferase reporter gene assay

The studies in Chapter 2 and 3 were possible due to the development of a LRG assay that allows the determination of AHR1 activity in host cells. Unlike other reporter gene assays that use transiently transfected COS-7 cells (Karchner *et al.*, 2006; Lee *et al.*, 2009; Yasui *et al.*, 2007), all the steps in the modified LRG assay in this thesis (e.g. seeding, transfection, dosing and luciferase detection) were performed in 96-well plates. Using 96-well plates and employing a few other small modifications (Chapter 2, Discussion) allows the generation of approximately 32 concentration−response curves (1536 wells) per person-week. Despite the efficiency and high throughput nature of this assay, it is approximately 6-fold less sensitive than the assay used by Iwata and colleagues (Lee *et al.*, 2009; Mol *et al.*, 2012). This limits the ability of the LRG assay to measure less potent AHR-agonists and less sensitive AHR1 constructs. To improve this, the following studies are suggested:

6.3.1.1. Incubate the cells with DLCs at 33°C instead of 37°C for 24 h.

Zhao and colleagues reported a four- to five-fold increase in the overall induction of luciferase activity in the second generation (G2) of chemically activated luciferase expression (CALUX) in H1L6.1c2 cells (Zhao *et al.*, 2010). The authors speculated that the reason for such increased activity was due to a

simple change in incubation temperature, which led to more efficient protein folding at the lower temperature.

6.3.1.2. Increase the number of AHREs in CYP1A5 reporter constructs.

Increasing the number of AHREs from 7 to 20 in the CYP1A5 reporter constructs could increase the sensitivity of the LRG assay. He and colleagues modified the number of AHREs in order to improve the sensitivity of the CALUX system they were using (He *et al.*, 2011). In this system, the magnitude of induction and the sensitivity of luciferase activity responses increased with the number of AHREs in reporter constructs. The highest luciferase induction and sensitivity were observed in a cell line transfected with a reporter construct containing 20 AHREs (He *et al.*, 2011).

6.3.1.3. Add cycloheximide to the culture medium.

After adding cycloheximide to the culture medium, a five-fold increase in reporter gene assay sensitivity was reported in liver recombinant Hepa1c7 cells containing the firefly luciferase gene (Saito *et al.*, 2009). Cycloheximide has been shown to block AHR degradation and it also increases accumulation of the TCDD-activated AHR and functional AHR-ARNT (AHR nuclear translocator) complex (Ma *et al.*, 2000; Ma and Baldwin, 2002). Thus, adding cycloheximide to the culture medium could increase LRG assay sensitivity.

6.3.1.4. Use CYP1A4 AHRE upstream of the luciferase reporter gene

Maximal fold induction for mRNA expression of CYP1A4 is generally higher than CYP1A5. For example, results of a study showed the maximal fold induction of CYP1A4 (504-, 30- and 40-fold) was higher than CYP1A5 (78-, 21- and 20-fold) for chicken, pheasant and quail, respectively, when exposed to TCDD (Herve *et al.*, 2010b).

Results of studies that used the LRG assay indicated that the TCDD-EC_{50} estimated from this assay is not dependent on chicken or great cormorant (*Phalacrocorax carbo*) CYP1A5-reporter construct. However, maximal luciferase activities (efficacy) were approximately 2- to 3-times higher when a chicken AHRE-driven reporter gene (pGL4-ckCYP1A5-6XREs) was used instead of the cormorant AHRE-driven reporter gene (pGL4-ccCYP1A5-7XREs). As such, I suggest the use of chicken AHRE rather than cormorant AHRE and using CYP1A4 instead of CYP1A5 might increase the sensitivity of the LRG assay by increasing the fold-change in induction of the reporter gene and as a result increase the efficacy of the assay.

6.3.2. Perform all steps of the cell-based binding assay in one plate

The affinity of AHR-DLC binding is correlated with *in vivo* toxicity of DLCs (Okey *et al.*, 1989; Poland *et al.*, 1976; Poland and Glover, 1980) and the knowledge obtained from the AHR binding assay improves our understanding of the mechanism(s) underlying AHR-dependent toxicity. However, the AHR binding assays currently available are labour-intensive and have low sensitivity (Chapter 4). I have modified a cell-based assay to develop a batch assay with higher

sensitivity than the well-known HAP binding assay. Therefore, I suggest conducting studies to determine if all steps of this assay could be conducted in one cell culture plate. For example: (1) following exposure to DLCs, cells could be washed in the cell culture plate, the washing reagent could be aspirated and finally, scintillation cocktail could be added directly to the same plate or (2) cells could be grown on filter paper plates and all steps of binding, washing and measuring radioactivity could be done in one plate.

6.3.3. Determine the binding affinity between DLCs and AHR1 mutants by use of the cell-based binding assay

The findings from this thesis (Chapters 2 and 3) demonstrated that isoleucine and serine at positions 324 and 380 led to significant increases in AHR1 activity in the LRG assay. The mechanism(s) by which these two amino acids affect AHR1 activity remain unclear. In Chapter 3, the ligand binding site of three galliform species was shown to be located close to the centre of the side chains of amino acids 324 and 380, which suggests these two amino acids are involved in binding DLCs. Future studies could assess the binding affinity of DLCs to mutant AHR1s that have different amino acids at position 324 and 380 to determine whether these amino acids contribute to AHR1 binding or if they affect AHR1 activity through a different mechanism (e.g. changing the conformation of the protein).

6.3.4. Perform docking studies of DLCs with avian AHR1 LBDs to determine the reason for differential relative potency of PeCDF in various species.

Through site-directed mutagenesis in Chapters 2 and 3, I showed that the identity of amino acids 324 and 380 were responsible for the reduced potency of TCDD (≈100-fold) and PeCDF (≈ 5-fold) in the quail-like mutant AHR1. Potential reasons for the differential decreases in TCDD and PeCDF potencies to induce reporter gene activity in COS-7 cells include: (1) the position of PeCDF in the binding cavity of the AHR1 LBD compared to TCDD leading to a lower interaction of PeCDF with the amino acid side chains at positions 324 and 380, (2) the larger molecular size of PeCDF which could fit better in the bigger binding cavities of pheasant and quail AHR1 or (3) the additional electronegative Cl group of PeCDF compared to TCDD, which could contribute to augmented hydrogen binding with the side chains of amino acids in the binding cavity of pheasant and quail AHR1 LBD. Future molecular docking and binding energy studies are suggested to provide more information about the interaction between ligands and avian AHR1 LBDs.

6.3.5. Perform more studies to elucidate the role(s) of the multiple isoforms of AHR or ARNT (i.e., AHR2, ARNT2) in avian sensitivity to DLCs.

A limited number of studies that compare the properties of avian AHR1 and AHR2 have been conducted and the results from those studies indicate that AHR1 is the predominant AHR form responsible for regulating the response to DLCs. AHR2 was shown to be less active than AHR1 in the presence of TCDD and its functions are not well understood (Fujisawa *et al.*, 2012; Iwata *et al.*, 2010;

Yasui *et al.*, 2004; Yasui *et al.*, 2007). The role of ARNT2 in avian species has also not been elucidated. Lee and colleagues (2007) showed a significant positive correlation between ARNT1 and ARNT2 mRNA expression levels in the liver of wild cormorants. This suggests that their expression may be regulated by similar transcriptional regulation mechanisms (Lee *et al.*, 2007). Another study showed both chicken ARNT1 and ARNT2 can participate in the interaction with each AHR isoform and AHRE (Lee *et al.*, 2011). More studies are suggested to confirm previous findings and elucidate the role of AHR2 and ARNT2 in birds in the context of DLC toxicity.

6.3.6. Conduct egg injection studies and additional hepatocyte cell cultures with European starlings

European starlings were of particular interest because the sequencing results of the AHR1 ligand binding domain indicated that the amino acids at sites 324 and 380 were identical to those of the only avian species known to be highly sensitive to the toxic effects of DLCs, the chicken (Chapter 3, Table 3.1). The results from Chapter 5 showed that the starling was as sensitive as the chicken to TCDD but not to PeCDF and TCDF. This might suggest dioxin metabolism is different from furan in starling. Another study also showed that *in vitro* metabolism might be different for dioxin and furan (Elliott *et al.*, 1996b; Kumar *et al.*, 2002). Additional egg injection, hepatocyte culture and metabolism studies could be performed to confirm starling sensitivity to DLCs and elucidate the reason for starling sensitivity to dioxin but not furans.

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