Predicting the sensitivity of avian species to the embryotoxic effects of polychlorinated biphenyls (PCBs) using *in vitro* approaches

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Abstract

The goal of this thesis was to use a luciferase reporter gene (LRG) assay in order to predict avian species sensitivity to the toxic effects of polychlorinated biphenyl (PCB) congeners 126, 77, 105 and 118 based on their aryl hydrocarbon receptor 1 (AHR1) ligand binding domain (LBD) sequence. These results were then compared to ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A4/5 mRNA expression measured in three model species.

The amino acid residues at positions 324 and 380 in the AHR1 LBD are the major determinants of avian species sensitivity to PCBs. Both LRG and EROD activity were significantly correlated with embryolethality and were better predictors of PCB-induced embryolethality than CYP1A4/5 mRNA expression. These results support the hypothesis that the LRG assay in combination with a species' AHR1 LBD sequence can be used to predict PCB-induced embryolethality in potentially any avian species of interest without the use of lethal methods.

Résumé

L’objectif de cette thèse était d’utiliser un essai sur un gène rapporteur de la luciférase (GRL) afin de prévoir la sensibilité des espèces aviaires aux effets toxiques des biphényles polychlorés (BPCs) 126, 77, 105 et 118 basé sur la séquence du domaine de liaison aux ligands (DLL) du récepteur 1 aryl-hydrocarbone (RAH1). Ces résultats étaient ensuite comparés avec l'activité de l'éthoxyrésorufine *O*-déséthylase (EROD) et l’expression de l'ARNm du cytochrome P4501A4/5 (CYP1A4/5), dans les cultures d'hépatocytes de trois espèces modèles.

Les identités des acides aminés aux positions 324 et 380 dans le DLL RAH1 sont les facteurs principaux impliqués dans la sensibilité des espèces aviaires aux BPCs. Les activités de la luciférase et de l'enzyme EROD étaient tous les deux significativement corrélées avec les données sur l'embryolétalité, et ces activités ont été jugées comme étant de meilleurs indices de l'embryolétalité induite par les BPCs que l'expression de l'ARNm du CYP1A4/5. Ces résultats viennent appuyer l'hypothèse selon laquelle l'essai GRL et les connaissances sur la séquence du DLL RAH1 d'une espèce peuvent être combinés pour prévoir l'embryolétalité induite par les BPCs chez pratiquement n'importe quelle espèce aviaire d'intérêt sans avoir recours à des méthodes létales.

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AHR:	aryl hydrocarbon receptor
ANOVA:	analysis of variance
ARNT:	aryl hydrocarbon receptor nuclear translocator
bHLH-PAS:	basic helix-loop-helix Per-ARNT-Sim
BMF:	biomagnification factor
BNF:	β-naphthoflavone
CALUX:	chemically activated luciferase expression
CAR:	constitutive androstane receptor
cDNA:	complementary DNA
CEH:	chicken embryo hepatocytes
CMV:	cytomegalovirus
CYP:	cytochrome P450
DLC:	dioxin-like compound
DBD:	DNA binding domain
DCM:	dichloromethane
DMSO:	dimethyl sulfoxide
EC_{50}:	median effect concentration
$EC_{threshold}$:	threshold effect concentration
EDTA:	ethylenediaminetetraacetic acid
EI+:	electron impact positive
EROD:	ethoxyresorufin *O*-deethylase
GLEMEDS:	Great Lakes Embryo Mortality, Edema and Deformities Syndrome
GST:	glutathione S-transferase
HRGC:	high resolution gas chromatography
HRMS:	high resolution mass spectrometry
HSP90:	heat shock protein 90
JEH:	Japanese quail embryo hepatocytes
K_{ow}:	octanol-water partition coefficient
LBD:	ligand binding domain
LD_{50}:	median lethal dose
LD_{100}:	100% lethal dose
LOAEL:	lowest observable adverse effect level
LRG:	luciferase reporter gene
LRMS:	low resolution mass spectrometry
mRNA:	messenger RNA
Mrp:	multidrug resistance-associated protein
NADPH:	nicotinamide adenine dinucleotide phosphate
NOAEL:	no observable adverse effect level
No-RT:	no reverse transcriptase control
PB:	phenobarbital
PBS:	phosphate-buffered saline
PC_x:	x % positive control concentration
PCB:	polychlorinated biphenyl
PCB 77:	3,3’,4,4’-tetrachlorobiphenyl
PCB105:	2,3,3’,4,4’-pentachlorobiphenyl

PCB 105-P:	purified PCB 105
PCB 105-RG:	reagent-grade PCB 105
PCB 114:	2,3,4,4’,5-pentachlorobiphenyl
PCB118:	2,3’,4,4’,5-pentachlorobiphenyl
PCB 118-P:	purified PCB 118
PCB 118-RG:	reagent-grade PCB 118
PCB 123:	2’,3,4,4’,5-pentachlorobiphenyl
PCB 126:	3,3’,4,4’,5-pentachlorobiphenyl
PCB 138:	2,2’,3,4,4’,5’-hexachlorobiphenyl
PCB 156:	2,3,3’,4,4’,5-hexachlorobiphenyl
PCB 157:	2,3,3’,4,4’,5’-hexachlorobiphenyl
PCB 167:	2,3’,4,4’,5,5’-hexachlorobiphenyl
PCB 169:	3,3’,4,4’,5,5’-hexachlorobiphenyl
PCB 189:	2,3,3’,4,4’,5,5’-heptachlorobiphenyl
PCDD:	polychlorinated dibenzo-*p*-dioxin
PCDF:	polychlorinated dibenzofuran
PeCDF:	2,3,4,7,8-pentachlorodibenzofuran
PEH:	ring-necked pheasant embryo hepatocytes
QPCR:	real-time quantitative polymerase chain reaction
ReP:	relative potency
ReS:	relative sensitivity
ROS:	reactive oxygen species
SE:	standard error
SIR:	selective ion recording
SRC:	steroid receptor co-activator
T_4:	thyroxine
TAD:	transactivation domain
TCDD:	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
$TCDD_x$:	x % TCDD maximal response concentration
TEF:	toxic equivalency factor
TEQ:	toxic equivalent
TH:	thyroid hormone
TLC:	thin layer chromatography
TTR:	transthyretin
UDP-GT:	uridinediphosphate glucuronosyl transferase
WHO:	World Health Organization
XRE:	xenobiotic response element

Statement of contributions

Chapter 2	**Performed by:**
Experimental design, data analysis and manuscript preparation	Gillian Manning Reza Farmahin Doug Crump Sean Kennedy
PCB chemical analysis and purification	Jeffery Klein Alex Konstantinov David Potter
AHR1 sequencing and AHR1 expression constructs	Reza Farmahin Stephanie Jones Lukas Mundy
Luciferase reporter gene assays	Gillian Manning

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EROD assays	Lukas Mundy Gillian Manning
CYP1A4/5 mRNA expression	Lukas Mundy Gillian Manning

Publications

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction to polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) are a group of industrial chemicals that were manufactured in North America from 1929 until 1977 (De Voogt and Brinkman, 1989; Environment Canada, 2010). PCBs have several unique physical and chemical properties, including insulating and flame-retardant properties, thermal stability, resistance to oxidation and hydrolysis, low water solubility and low volatility (De Voogt and Brinkman, 1989; Peters *et al.*, 2006). These properties allowed for their use in several industrial applications, such as cooling and dielectric fluids for transformers and capacitors, heat-conducting fluids in heat-exchangers, flame retardants in lubricating oils, hydraulic fluids, ink solvents for carbonless copy paper, and plasticizers in synthetic resins, rubbers, paints, waxes and asphalt, but have also contributed to their environmental persistence (Blankenship *et al.*, 2005; Canadian Council of Resource and Environment Ministers, 1986; De Voogt and Brinkman, 1989; Environment Canada, 2010; Erickson, 2001; UNEP, 2008).

Since the time of their first production until 1989, 1.7 million tonnes of PCBs were manufactured globally (UNEP, 2008). Monsanto, which produced technical mixtures of PCBs under the name of Aroclor, was the main manufacturer of PCBs in North America with a total production of approximately 600,000 tonnes between 1930 and 1975 (De Voogt and Brinkman, 1989). Monsanto started reducing its production of PCBs in 1971 due to emerging concerns regarding the effects of PCBs on the environment and human health; by the end of 1977, Monsanto had completely phased out its production and sale of PCBs (De Voogt and Brinkman, 1989). A North American ban on the import and manufacture of PCBs started in 1977, however, global production only ended when Russia stopped its production of PCBs in 1993 (Carrie *et al.*,

2010; Environment Canada, 2010). Although a global ban on PCBs was enacted in 2004 (UNEP, 2009), PCB use is still allowed in existing closed electrical and hydraulic systems until the system reaches the end of its service life (Canadian Council of Resource and Environment Ministers, 1986; Environment Canada, 2010; Erickson, 2001). The Stockholm Convention states that the use of PCBs in these applications should be eliminated by 2025 (UNEP, 2009).

1.1.1 Sources of PCBs

Because of their numerous applications, PCBs were released to the environment from several sources. Processing and recycling of carbonless copy paper led to widespread releases of PCBs in areas such as the Kalamazoo River, MI, USA and the Fox River, WI, USA (Blankenship *et al.*, 2005; Imamoglu *et al.*, 2004), and PCBs discharged from manufacturing plants for electrical equipment led to contamination of the Hudson River, NY, USA and New Bedford Harbor, MA, USA (Erickson, 2001; U.S.EPA, 2012). Additional sources of PCBs include atmospheric transport following volatilization or combustion of PCBs, municipal waste discharges, land runoff, landfill disposal and accidental spills (Eisler and Belisle, 1996; Holoubek, 2001). Atmospheric deposition of PCBs in the water column and subsequent assimilation by fish throughout the food chain is a significant source of PCBs in Great Lakes biota (Erickson, 2001; Ward *et al.*, 2010). Less chlorinated, *ortho*-substituted PCBs tend to be more volatile and water soluble, whereas congeners with higher degrees of chlorination and no *ortho*-chlorines are more prominent in soils and sediments (Henry and DeVito, 2003).

While the import, manufacture and release of PCBs ended several decades ago, PCBs are still detected globally in the environment and in wildlife due to their high environmental persistence and ability to bioaccumulate and biomagnify in both aquatic and terrestrial food webs

(Blankenship *et al.*, 2005; Hong *et al.*, 2008; Kay *et al.*, 2005; Letcher *et al.*, 2010; Levengood and Schaeffer, 2010; Wang *et al.*, 2008). Processes such as atmospheric deposition, biovector transport and resuspension of PCBs from sediment contribute to continuous PCB exposure (Friedman *et al.*, 2011; Michelutti *et al.*, 2009; Ricca *et al.*, 2008). Although PCB levels in bird eggs from the Great Lakes and the arctic have declined since the 1970s (Braune, 2007; Norstrom and Hebert, 2006), increasing trends in PCB levels were recently observed in burbot (*Lota lota*) from the Mackenzie River and may be due to increased exposure as a result of warming temperatures (Carrie *et al.*, 2010).

1.1.2 Bioaccumulation, disposition and metabolism of PCBs in birds

Organisms accumulate PCBs through the water column, interstitial sediment waters and consumption of contaminated prey (Eisler and Belisle, 1996). PCB concentrations in common tern (*Sterna hirundo*), arctic tern (*Sterna paradisaea*) and great cormorant (*Phalacrocorax carbo sinensis*) from Germany were several times higher than the concentrations found in their fish and invertebrate prey items (Scharenberg, 1991a; Scharenberg, 1991b). Biomagnification factors (BMFs) from terrestrial invertebrates to house wren (*Troglodytes aedon*) and Eastern bluebird (*Sialia sialis*) eggs from the Kalamazoo River, Michigan, USA ranged from 3.1 to 35 (Blankenship *et al.*, 2005) and BMFs from aquatic emergent insects to tree swallow (*Tachycineta bicolor*) nestlings and adults ranged from 5 to 14 (Kay *et al.*, 2005). PCBs concentrate in several tissues, including liver, brain, muscle, heart and adipose tissue (Havera and Duzan, 1986; Kubota *et al.*, 2006; Naert *et al.*, 2007; Stickel *et al.*, 1984).

Hydrophobicity, often represented as octanol-water partition coefficients (K_{ow}), is an important property in determining PCB bioavailability (Henry and DeVito, 2003). The $\log K_{ow}$

and BMF factors from alewife (*Alosa pseudoharengus*) to herring gull (*Larus argentatus*) eggs for selected PCB congeners are presented in Table 1.1. $LogK_{ow}$ increases with the degree of PCB chlorination and is also generally associated with increased bioaccumulation and biomagnification of PCBs (Drouillard and Norstrom, 2003; Norstrom, 2002). Differences in metabolism and excretion among PCB congeners also affect their accumulation in birds (Drouillard and Norstrom, 2003). The elimination half-life for total PCBs in common grackles (*Quiscalus quiscula*) exposed to Aroclor 1254 was 89 days (Stickel *et al.*, 1984), but rates of elimination vary widely between PCB congeners. In general, highly chlorinated PCB congeners are metabolized to a lesser extent than less chlorinated congeners (Barron *et al.*, 1995). However, PCB congeners lacking vicinal chlorine substituents at *meta* and *para* sites (*m,p*-unsubstituted) on at least one phenyl ring are readily cleared and have lower BMFs than congeners with vicinal chlorine atoms at these sites (*m,p*-substituted), independent of their degree of chlorination or $logK_{ow}$ (Barron *et al.*, 1995; Drouillard *et al.*, 2007; Drouillard *et al.*, 2001; Maervoet *et al.*, 2004). For PCBs with similar $logK_{ow}$ values, *m,p*-unsubstituted congeners had elimination rates 3 to 4 times higher than *m,p*-substituted congeners in adult male American kestrels (*Falco sparverius*) (Drouillard *et al.*, 2001) and in juvenile American kestrels exposed to PCBs, retention factors ranged from 13.3 to 81.9% for *m,p*-substituted PCB congeners, but only 1 to 16.5% for *m,p*-unsubstituted congeners (Drouillard *et al.*, 2007). Accumulation, toxicokinetics and elimination of PCBs in birds also varies with life stage, sex and species (Barron *et al.*, 1995; Drouillard *et al.*, 2007; Kubota *et al.*, 2006; Naert *et al.*, 2007).

Table 1.1. Octanol-water partition coefficients ($logK_{ow}$) and biomagnification factors (BMFs) for selected dioxin-like PCB congeners.

Chemical	$logK_{ow}$[a]	BMF[b]
PCB 77	6.52	1.8
PCB 105	6.66	20
PCB 118	7.12	31
PCB 126	6.90	29
PCB 138	7.44	42
PCB 169	7.43	46

[a] $LogK_{ow}$ data from (Eisler and Belisle, 1996).
[b] Biomagnification factors from alewife to herring gull eggs from (Hoffman *et al.*, 1996)

1.2 Mechanisms of action of PCBs

1.2.1 Aryl hydrocarbon receptor (AHR) activation

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) superfamily and consists of three domains: the DNA-binding domain (DBD), ligand binding domain (LBD) and transactivation domain (TAD) (Okey, 2007). In its unliganded form, the AHR is part of a cytosolic complex containing heat shock protein 90 (HSP90), the HSP90 co-chaperone p23 and hepatitis B virus X-associated protein 2 (Fujii-Kuriyama and Kawajiri, 2010). Upon ligand binding, the AHR migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with the AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama, 2003). The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-activators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction of gene expression (Fujii-Kuriyama and Kawajiri, 2010). Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-

β, interleukin-1β), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism (Fujii-Kuriyama and Kawajiri, 2010; Giesy *et al.*, 2006; Mimura and Fujii-Kuriyama, 2003; Safe, 1994).

Two AHR isoforms (AHR1 and AHR2) have been identified in the black-footed albatross (*Phoebastria nigripes*), great cormorant and domestic chicken (*Gallus gallus domesticus*) (Yasui *et al.*, 2007). AHR1 mRNA levels were similar in the kidney, heart, lung, spleen, brain, gonad and intestine from the great cormorant but were lower in muscle and pancreas. AHR2 expression was mainly observed in the liver, but was also detected in gonad, brain and intestine. AHR1 levels represented a greater proportion (80%) of total AHR levels than AHR2 in the cormorant liver (Yasui *et al.*, 2007) and while both AHR isoforms bound to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which is considered to be the most potent AHR agonist, AHR2 was less effective at inducing TCDD-dependent transactivation compared to AHR1 in all three species of birds (Lee *et al.*, 2009; Yasui *et al.*, 2007).

The AHR can be activated by several structurally diverse chemicals, but binds preferentially to planar halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons. Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and certain PCB congeners, represent some of the most potent AHR ligands (Denison *et al.*, 2011). Only a subset of PCB congeners, including 4 non-*ortho* (PCB 77, 81, 126 and 169) and 8 mono-*ortho* substituted congeners (PCB 105, 114, 118, 123, 156, 157, 167 and 189), has been shown to bind to the AHR and cause toxic effects similar to those elicited by dioxins (van den Berg *et al.*, 1998). Of the dioxin-like PCBs, non-*ortho* congeners are the most toxicologically active, while mono-*ortho* PCBs are generally less potent (McFarland and Clarke, 1989; Safe, 1994). Chlorine substitution at *ortho* positions

increases the energetic costs of assuming the coplanar conformation required for binding to the AHR (McFarland and Clarke, 1989). Thus, a smaller proportion of mono-*ortho* PCB molecules are able to bind to the AHR and elicit toxic effects, resulting in reduced potency of these congeners. Other PCB congeners, such as di-*ortho* substituted PCBs, are very weak AHR agonists and do not likely contribute to dioxin-like effects (Safe, 1994). These non-coplanar PCBs may however exert toxic effects through other mechanisms.

The role of the AHR in mediating the toxic effects of planar hydrophobic contaminants has been well studied, however the endogenous role of the AHR is less clear (Okey, 2007). Some endogenous and natural substances, including prostaglandin PGG2 and the tryptophan derivatives indole-3-carbinol, 6-formylindolo[3,2-b]carbazole and kynurenic acid can bind to and activate the AHR (Fujii-Kuriyama and Kawajiri, 2010; Omiecinski *et al.*, 2011; Swedenborg and Pongratz, 2010). The AHR is thought to have important endogenous roles in reproduction, liver development, cardiovascular function, immune function and cell cycle regulation (Baba *et al.*, 2005; Denison *et al.*, 2011; Fernandez-Salguero *et al.*, 1995; Mimura *et al.*, 1997; Omiecinski *et al.*, 2011; Schmidt *et al.*, 1996), and activation of the AHR by DLCs may therefore adversely affect these processes.

1.2.2 Constitutive androstane receptor (CAR) activation

The constitutive androstane receptor (CAR) is a nuclear receptor involved in the regulation of genes responsible for phase I (CYP2A, CYP2B, CYP2C and CYP3A) and phase II metabolism (UDP-GT, GSTs and cytosolic sulfotransferase 2A1), lipid metabolism (insulin induced gene 2 and fatty acid synthase), cell cycle regulation (Mdm2) and xenobiotic transport (multidrug resistance-associated protein (Mrp) 2 and Mrp 4) (Graham and Lake, 2008; Huang *et*

al., 2005; Kopec *et al.*, 2010; Omiecinski *et al.*, 2011). Following ligand-dependent or independent activation, CAR forms a heterodimer with the retinoid X receptor α and associates with response elements in the promoter region of target genes to regulate their expression (Graham and Lake, 2008; Yoshinari *et al.*, 2010). Phenobarbital (PB) is the prototypical CAR activator, however it does so through indirect mechanisms and is not a direct CAR ligand (Omiecinski *et al.*, 2011). Several non-dioxin-like PCBs and some mono-*ortho* PCBs have also been shown to induce CAR-mediated transcriptional activity (Al-Salman and Plant, 2012; Martin and Klaassen, 2010; Safe, 1994; Sakai *et al.*, 2006). PB-activated CAR was shown to induce CY1A1 and CYP1A2 expression independently of the AHR (Yoshinari *et al.*, 2010) and although no studies have demonstrated CAR-mediated CYP1A induction by PCBs, it remains possible that mixed-type inducers, such as mono-*ortho* PCBs, could induce CYP1A expression through both AHR and CAR activation. PB- and PCB 153-induced CAR activation increased cell proliferation, inhibition of apoptosis and liver hypertrophy, and could lead to promotion of liver tumors in rodents (Dean *et al.*, 2002; Graham and Lake, 2008; Huang *et al.*, 2005; Kopec *et al.*, 2010). CAR was also shown to play a role in thyroid hormone (TH) metabolism, as thyroxine (T_4) levels were reduced in wild-type mice treated with the CAR ligand 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene, but not in CAR null mice (Omiecinski *et al.*, 2011). The reduction in T_4 levels was likely a result of CAR-mediated induction of metabolizing enzymes such as UDP-GT (Omiecinski *et al.*, 2011).

1.2.3 CYP1A activation and PCB toxicity

Of all AHR-regulated genes, the CYP1A subfamily of enzymes is the most studied and is often used as a biomarker of PCB, PCDD and PCDF exposure and toxicity (Harris and Elliott,

2011; Head and Kennedy, 2010; Rifkind, 2006; Safe, 1987). CYP1A4 and CYP1A5 are the isoforms expressed in birds and are orthologous to mammalian CYP1A1 and CYP1A2, respectively (Goldstone and Stegeman, 2006). Induction of CYP1A1 and CYP1A4 can be measured as ethoxyresorufin *O*-deethylase (EROD) or aryl hydrocarbon hydroxylase activity, whereas uroporphyrinogen oxidation activity and arachidonic acid metabolism are mediated mainly by CYP1A2 and CYP1A5 in mammals and birds, respectively (Rifkind *et al.*, 1994; Sinclair *et al.*, 1997).

Although CYP1A induction is a good predictor of DLC toxicity (Head and Kennedy, 2010; Safe, 1987), it is less clear whether CYP1A is in itself responsible for the toxic effects of DLCs. Production of reactive oxygen species (ROS) resulting from increased CYP1A expression has been suggested as a potential mechanism of DLC toxicity (Giesy *et al.*, 2006). This hypothesis was supported by a study demonstrating that co-treatment of chicken embryos with either TCDD and the CYP inhibitor piperonyl butoxide, or TCDD and vitamin E reduced the incidence of edema and hemorrhage compared to embryos exposed to TCDD alone (Blankenship *et al.*, 2003). CYP1A1-dependent ROS production was also implicated in the development of pericardial edema and vascular defects in TCDD-treated zebrafish (*Danio rerio*) (Rifkind, 2006).

CYP1A2 and CYP1A5 induction have been associated with the incidence of porphyria in mammals and birds. Porphyria is a disorder in which disturbance of heme biosynthesis results in accumulation and excretion of porphyrins (Kennedy and Fox, 1990). Through the normal heme biosynthesis pathway, uroporphyrinogen is converted to coproporphyrinogen by uroporphyrinogen decarboxylase (Smith *et al.*, 2001). However, CYP1A2 and CYP1A5 catalyze the oxidation of uroporphyrinogen to uroporphyrin, leading to uroporphyrin accumulation and liver damage (Rifkind, 2006; Smith *et al.*, 2001). PCB concentrations were significantly

correlated with levels of highly carboxylated porphyrins (uroporphyrin, hepta- and hexacarboxylic acid porphyrins) in herring gull livers (Kennedy *et al.,* 1998). Porphyrin accumulation was also observed in avian hepatocyte cultures exposed to dioxin-like and non-dioxin-like PCB congeners, indicating that heme biosynthesis may be affected by both groups of PCBs through different mechanisms of action (Henry and DeVito, 2003; Lorenzen *et al.,* 1997; Sanderson *et al.,* 1998).

Studies conducted with CYP1A1 and CYP1A2 null mice support a role for these enzymes in TCDD-induced porphyria, hepatotoxicity and lethality (Rifkind, 2006; Smith *et al.,* 2001; Uno *et al.,* 2004). However, TCDD-induced thymus atrophy and antibody suppression were still observed in CYP1A1 and/or CYP1A2 null mice (Rifkind, 2006; Smith *et al.,* 2001). The degree of protection against TCDD toxicity provided by loss of the CYP1A1 gene was also small in comparison to that provided by loss of the AHR gene (Uno *et al.*, 2004), indicating that CYP1A activation may play a role in some but not all of the toxic effects of DLCs.

1.2.4 AHR and avian species sensitivity

Species differ considerably in their sensitivity to DLCs. For example, PCB 126 and 77 LD_{50}s for chicken embryos were 2 to 3 orders of magnitude lower than the LD_{50}s measured for American kestrel and common tern embryos (Hoffman *et al.*, 1998). Differences in sensitivity to DLCs have been associated with differences in the AHR amino acid sequence in mammals, fish and birds (Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006; Mimura and Fujii-Kuriyama, 2003; Wirgin *et al.*, 2011).

Karchner *et al.,* (2006) used chimeric AHR1 constructs combining the DBD, LBD and TAD from the chicken (sensitive to DLC toxicity) and common tern (resistant to DLC toxicity)

to demonstrate that amino acid differences within the LBD were responsible for differences in avian species sensitivity to TCDD. More specifically, the amino acid residues found at positions 324 and 380 in the AHR1 LBD were associated with differences in TCDD binding affinity and transcriptional activation between the chicken (Ile324_Ser380) and common tern (Val324_Ala380) receptors (Karchner *et al.*, 2006).

The predicted AHR1 LBD amino acid sequences from 86 species of birds were compared and 6 amino acid residues differed among species (Table A.1.1) (Farmahin *et al.*, in press; Head *et al.*, 2008). However, only the amino acids at positions 324 and 380 in the AHR1 LBD were associated with differences in DLC toxicity *in ovo* and AHR1-mediated gene expression *in vitro* (Farmahin *et al.*, in press; Head *et al.*, 2008). These results indicate that species can be divided into one of three AHR1 types based on the amino acids found at positions 324 and 380 of the AHR1 LBD: type 1 (Ile324_Ser380), type 2 (Ile324_Ala380) and type 3 (Val324_Ala380) (Farmahin *et al.*, in press).

1.3 PCB toxicity in birds

Exposure to PCBs has been associated with embryolethality as well as changes in reproductive function and behaviour, development, immune function, endocrine homeostasis and biochemical responses in avian species (Cesh *et al.*, 2010; Fernie *et al.*, 2001; Hoffman *et al.*, 1998; Kennedy *et al.*, 1996; Lavoie and Grasman, 2007; McCarty and Secord, 1999a; Walker and Catron, 2000). The AHR, which has been shown to mediate most if not all the toxic effects of DLCs, is thought to play a role in several of these functions, based on studies conducted in AHR-null mice (Baba *et al.*, 2005; Fernandez-Salguero *et al.*, 1995; Mimura *et al.*, 1997; Schmidt *et al.*, 1996). Some of the effects observed in AHR-null mice included reduced growth

rates (Mimura *et al.*, 1997; Schmidt *et al.*, 1996), abnormal liver and immune system development (Fernandez-Salguero *et al.*, 1995; Omiecinski *et al.*, 2011), retinoid accumulation in the liver and reproductive defects (Baba *et al.*, 2005; Mimura and Fujii-Kuriyama, 2003). Disruption of these processes by DLCs may therefore result from dysregulation of endogenous AHR functions (Bock and Kohle, 2006).

1.3.1 PCB effects on signaling molecules

PCBs have been shown to cause changes in the levels of several hormones, such as TH, corticosterone and retinoids, across classes of vertebrates. Reduced T_4 levels have been observed in herring gulls, glaucous gulls (*Larus hyperboreus*), bald eagle (*Haliaeetus leucocephalus*) and American kestrels exposed to PCBs (Cesh *et al.*, 2010; McNabb and Fox, 2003; Smits *et al.*, 2002; Verreault *et al.*, 2004). In mammals, PCBs are known to disrupt thyroid status by interfering with thyroid hormone biotransformation enzymes and hormone transport by plasma proteins. UDP-GT conjugates with T_4 and facilitates its excretion in bile and induction of UDP-GT by PCBs has been shown to decrease circulating T_4 in mammals (McNabb and Fox, 2003; Webb and McNabb, 2008). UDP-GT activity was significantly induced in Japanese quail (*Coturnix japonica*) exposed to Aroclor 1254 and also resulted in decreased plasma T_4 levels in these birds, although this decrease was not statistically significant (Webb and McNabb, 2008). Decreased T_4 levels may be caused by both dioxin-like and non-coplanar PCB congeners (Martin and Klaassen, 2010) since expression of UDP-GT is induced through AHR and CAR activation (Mimura and Fujii-Kuriyama, 2003; Yoshinari *et al.*, 2010). PCBs may also decrease circulating levels of TH through competition with T_4 for binding to transthyretin (TTR), leading to the excretion of free T_4 in the urine or bile (Giesy *et al.*, 2006; McNabb and Fox, 2003; Webb and

McNabb, 2008). Hydroxylated PCBs were found to have greater binding affinity with gull TTR than T_4, indicating that PCB metabolites may displace T_4 from TTR leading to increased T_4 metabolism and excretion (Ucan-Marin *et al.*, 2009). Thus, several mechanisms of action may contribute to the effects of PCBs on decreased TH levels in birds.

Baseline and stress-induced corticosterone levels were reduced in American kestrels exposed to a mixture of Aroclor 1248, 1254 and 1260 compared to control birds (Love *et al.*, 2003). A significant, negative relationship was observed between circulating corticosterone levels and yolk sac concentrations of total PCBs in herring gull embryos from the Great Lakes (Lorenzen *et al.*, 1999). Long-term suppression of corticosterone levels could lead to increased susceptibility to environmental stressors, increased chick mortality and changes in immune responses through chronic activation of the hypothalamic-pituitary-adrenal axis (Letcher *et al.*, 2010; Love *et al.*, 2003).

Both positive and negative relationships have been observed between Vitamin A levels and PCBs in birds (Cesh *et al.*, 2010), however the general response pattern appears to be a reduction in Vitamin A levels with increased PCB exposure (Harris and Elliott, 2011). Increasing PCB exposure was associated with decreased levels of circulating retinol and/or liver retinyl palmitate in black guillemot (*Cepphus grille*), European shag (*Phalacrocorax aristotelis*) and great blue heron (*Ardea herodias*) nestlings (Champoux *et al.*, 2006; Kuzyk *et al.*, 2003; Murvoll *et al.*, 2006). The effects of PCBs on retinoids may be due to accelerated metabolism and clearance of vitamin A through interactions between PCBs and carrier proteins such as retinol binding protein and TTR and induction of phase I and phase II enzymes (Cesh *et al.*, 2010; Harris and Elliott, 2011; Murvoll *et al.*, 2006).

1.3.2 Reproductive success and behaviour

Exposure to PCBs has been associated with reductions in reproductive success, including decreased egg production, hatchability and fertility (Hoffman *et al.*, 1996). PCBs are maternally deposited in yolks of forming eggs and are present throughout embryonic development, when birds are most sensitive to the effects of contaminants (Barron *et al.*, 1995; McNabb and Fox, 2003). PCB-induced embryolethality has been demonstrated in several species of birds using egg injection studies, which mimic maternal deposition of chemicals to the egg. The embryotoxic effects of TCDD, PCB 77 and PCB 126 were demonstrated in egg injection studies conducted with chicken, American kestrel, common tern, double-crested cormorant (*Phalacrocorax auritus*), turkey (*Meleagris gallopavo*) and ring-necked pheasant (*Phasianus colchicus*) embryos, and in all cases the chicken was observed to be much more sensitive than other species (Brunström and Lund, 1988; Brunström and Reutergårdh, 1986; Hoffman *et al.*, 1998; Powell *et al.*, 1996; Powell *et al.*, 1998). Mono-*ortho* PCBs also increased the incidence of mortality in chicken embryos, however these compounds were approximately 3 orders of magnitude less potent than PCB 126 (Brunström, 1990).

PCB-induced effects other than overt embryotoxicity, including delayed egg laying, smaller clutch size, increased infertility and altered parental behaviour can also contribute to reductions in reproductive success (Fernie *et al.*, 2001; Harris and Elliott, 2011). PCB exposure was associated with reduced foraging trips, increased absence from the nest, increased nest abandonment and reduced nest quality in a number of bird species (Bustnes *et al.*, 2001; Halbrook and Arenal, 2003; McCarty and Secord, 1999a; McCarty and Secord, 1999b; Ward *et al.*, 2010). These effects were often accompanied by reductions in reproductive success. The specific mechanisms underlying changes in reproductive behaviour are unclear, however

endocrine disruption, neurological changes and stress have been identified as potential causes (Harris and Elliott, 2011).

1.3.3 Developmental abnormalities

Examination of avian embryos and chicks both in controlled laboratory settings and in the wild has linked dioxin-like PCB exposure with a number of developmental abnormalities including reduced growth, shortened limbs, skeletal abnormalities, beak defects, foot deformities, altered organ sizes, heart deformities, microphthalmia, liver necrosis, porphyria, hemorrhage and edema (Brunström, 1990; Brunström, 1988; Brunström and Andersson, 1988; Gilbertson and Fox, 1977; Gilbertson *et al.*, 1991; Harris and Elliott, 2011; Hoffman *et al.*, 1998; Hoffman *et al.*, 1996; Lavoie and Grasman, 2007; Letcher *et al.*, 2010; Powell *et al.*, 1996; Walker and Catron, 2000). As was observed with embryolethality, chicken embryos were highly sensitive to the teratogenic effects of PCBs in comparison to other species (Brunström, 1988; Brunström, 1989; Brunström *et al.*, 1990b; Brunström and Lund, 1988; Brunström and Reutergårdh, 1986; Hoffman *et al.*, 1998; Powell *et al.*, 1996; Powell *et al.*, 1998).

The suite of developmental effects caused by DLCs in egg injection studies are very similar to those observed in Great Lakes Embryo Mortality, Edema and Deformities Syndrome (GLEMEDS), a disease characterized by high embryonic and chick mortality, subcutaneous, pericardial and peritoneal edemas, growth retardation, liver necrosis, porphyria, bill deformities, club feet, eye deformities and skeletal abnormalities (Gilbertson *et al.*, 1991). GLEMEDS was reported in herring gull, common tern and double-crested cormorant colonies from the Great Lakes during the 1970s and 1980s (Gilbertson *et al.*, 1991), and reductions in the onset of

GLEMEDS and improvements in reproductive success of these bird populations coincided with declines in TCDD and PCB exposure (Gilbertson *et al.*, 1991).

1.3.4 Immune function

Immunological effects of PCBs have been observed in a number of species, including mammals, fish and birds (Giesy *et al.*, 2006). T-cell dependent inflammatory responses, antibody titres and white blood cell counts were affected by PCB exposure in American kestrels and/or chickens (Lavoie and Grasman, 2007; Smits and Bortolotti, 2001; Smits *et al.*, 2002). Changes in hormone levels due to PCB exposure may be responsible for the effects on immune responses, as triiodothyronine levels were positively correlated with T-cell dependent inflammatory responses in American kestrel nestlings exposed to PCBs *in ovo* (Smits *et al.*, 2002). TH plays an important role in the development, maintenance and function of antibody- and cell-mediated immune responses. In addition, depressed levels of circulating corticosterone were accompanied by higher levels of circulating lymphocytes in male kestrels exposed to PCBs (Smits *et al.*, 2002). The opposite effect was observed in mallards (*Anas platyrhynchos*) exposed to the synthetic glucocorticoid dexamethasone (Fowles *et al.*, 1993). These findings indicate that corticosteroid levels and immune responses interact with each other, and that the increase in white blood cell count in PCB-exposed kestrels could be caused by PCB-mediated suppression of corticosterone-related stress responses (Smits and Bortolotti, 2001; Smits *et al.*, 2002).

1.4 Toxic equivalents (TEQ) approach

DLCs represent a group of structurally-related chemicals that elicit similar toxic effects subsequent to binding and activation of the AHR (Denison *et al.*, 2011; Okey, 2007) and are

found as complex mixtures in environmental media. Since they are presumed to act through a common mechanism of action, DLCs are assessed as a group of chemicals using the toxic equivalents (TEQ) approach developed by the World Health Organization (WHO) (van den Berg *et al.*, 1998). In order to be included in the TEQ framework, compounds must (1) be structurally similar to PCDDs and PCDFs, (2) bind to the AHR, (3) elicit dioxin-like biochemical and toxic effects and (4) be persistent and accumulate in the food chain (van den Berg *et al.*, 1998). The TEQ approach assumes concentration additivity and uses toxic equivalency factors (TEFs) to estimate the toxic potency of each DLC relative to TCDD. TEFs specific to mammals, fish and birds are consensus values that were selected through expert examination of relative potency (ReP) values based on several endpoints in multiple species (Safe, 1997; van den Berg *et al.*, 1998). WHO-TEFs for TCDD and PCBs in birds are presented in Table 1.2. TEFs along with chemical residue data from animal tissues, soil, sediment or water samples can be used to calculate TEQ concentrations, as shown by equation 1 (van den Berg *et al.*, 1998). In order for the TEQ approach to remain up to date, TEFs should be reviewed and revised as new data becomes available (Safe, 1994). The most recent review of mammalian TEFs was conducted in 2005 (van den Berg *et al.*, 2006).

$$TEQ = \sum_{n1}\left[PCDD_i \times TEF_i\right] + \sum_{n2}\left[PCDF_i \times TEF_i\right] + \sum_{n3}\left[PCB_i \times TEF_i\right] \qquad (1)$$

Table 1.2. World Health Organization Toxic Equivalency Factors (WHO-TEFs) for birds.

Chemical	WHO-TEF[a]
Dioxins	
2,3,7,8-TCDD	1
Non-ortho PCBs	
PCB 77	0.05
PCB 81	0.1
PCB 126	0.1
PCB 169	0.001
Mono-ortho PCBs	
PCB 105	0.0001
PCB 114	0.0001
PCB 118	0.00001
PCB 123	0.00001
PCB 156	0.0001
PCB 157	0.0001
PCB 167	0.00001
PCB 189	0.00001

[a] From van den Berg *et al.* (1998)

1.4.1 Limitations of TEQ approach

Although the TEQ approach facilitates the risk assessment of DLCs, non-additive effects of DLCs and interspecies differences in ReP values may affect the predictive ability of this framework. A limited number of validation studies have found good correlations between the observed responses elicited by DLC mixtures and the predicted responses from the TEQ approach (Giesy and Kannan, 1998; van den Berg *et al.*, 1998). However, non-additive effects due to competition between full and partial agonists for AHR binding have also been observed for some DLC mixtures and have led to an overestimation of mixture toxicity (Howard *et al.*, 2010; Safe, 1997; van den Berg *et al.*, 1998).

ReP values for a given DLC can vary considerably among species and endpoints (Giesy and Kannan, 1998; Haws *et al.*, 2006; van den Berg *et al.*, 2006). The range of mammalian ReP values spread 2 to 3 orders of magnitude for PCDD and PCDF congeners, and 3 to 5 orders of magnitude for PCB congeners (Haws *et al.*, 2006). PCB 126 RePs based on EROD activity in human donor hepatocytes and HepG2 cells were 40 to 65 times lower than in rhesus monkey

(*Macaca mulatta*) and Sprague-Dawley rat hepatocyte cultures (Silkworth *et al.*, 2005). Significant differences in PeCDF and hexachlorobenzene (proposed for inclusion in TEQ concept) RePs were also observed between the chicken, ring-necked pheasant and Japanese quail (Cohen-Barnhouse *et al.*, 2011; Hervé *et al.*, 2010; Mundy *et al.*, 2012; Mundy *et al.*, 2010). These studies highlight the uncertainties associated with applying a single set of TEFs for all species within a particular class of vertebrates.

1.5 Thesis overview

1.5.1 Rationale

Avian-specific TEFs were developed by the WHO to simplify the environmental risk assessment of DLCs. However, TEFs do not account for differences in the relative potency of DLCs among species of birds that can result from differences in species sensitivity to individual DLCs. Birds differ widely in their sensitivity to the embryotoxic effects of PCBs (Cohen-Barnhouse *et al.*, 2011; Head *et al.*, 2008) and while PCB toxicity data are readily available for the chicken, fewer studies have measured the overt toxicity of PCBs in wild birds. Recently, the identities of amino acid residues 324 and 380 in the AHR1 LBD were shown to be associated with differences in avian species sensitivity to selected DLCs (Farmahin *et al.*, in press; Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006). In addition, *in vitro* CYP1A induction is significantly correlated with overt toxicity and sublethal effects induced by DLCs in birds and mammals (Head and Kennedy, 2010; Safe, 1987). Together, these studies support the use of AHR1 LBD sequence and *in vitro* CYP1A induction for predicting avian species sensitivity to DLCs.

The goal of this M.Sc. thesis was to use *in vitro* techniques to predict (1) the sensitivity of avian species to the embryotoxic effects of PCB 126, 77, 105 and 118 (Figure 1.1) and (2) the relative potency of these congeners in different species of birds. These congeners were selected because they display a wide range in their individual potencies. PCB 126 is the most toxic dioxin-like PCB congener (McNabb and Fox, 2003; van den Berg *et al.*, 1998), whereas PCB 77 was found to contribute the most to TEQ in tree swallows nesting along the Hudson River, NY, USA (Secord *et al.*, 1999). In the same study, PCB 105 and 118 were the dioxin-like PCBs found in the highest concentrations in tree swallow eggs and nestlings (Secord *et al.*, 1999). Previous studies indicated that contamination of mono-*ortho* PCB solutions with more potent AHR agonists could lead to overestimation of the ReP values for these PCBs (Peters *et al.*, 2006). Therefore, reagent-grade and purified solutions of PCB 105 and 118 were obtained in order to compare their effects and ReP values.

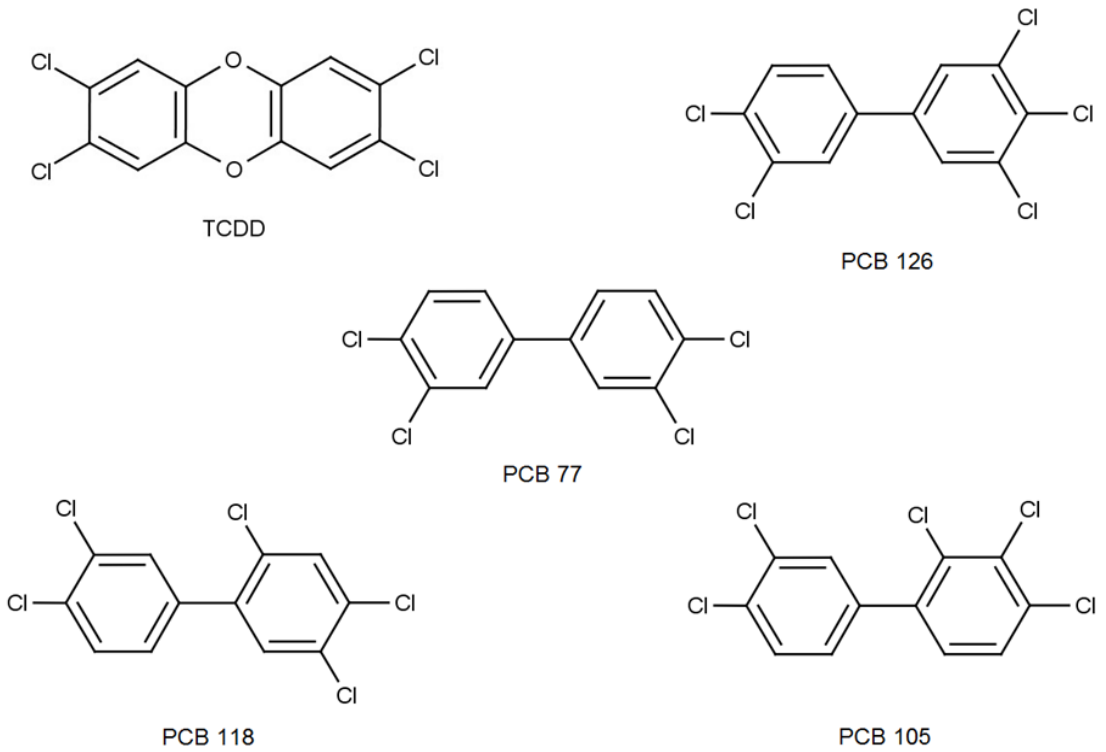

Figure 1.1. Molecular structures for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and PCB congeners 126, 77, 105 and 118.

1.5.2 Experimental objectives

1) Measure CYP1A reporter gene induction in COS-7 cells transfected with 15 AHR1 constructs (12 mutant and 3 wild-type constructs) representative of 86 avian species (Table A.1.1) and exposed to PCBs using a luciferase reporter gene (LRG) assay.
    a. Calculate relative sensitivity (ReS) values for each AHR1 subtype. ReS values for AHR1 constructs are calculated relative to chicken AHR1 (subtype 1A).
    b. Calculate ReP values of individual PCBs. ReP values for PCB congeners are calculated relative to TCDD.
2) Correlate CYP1A reporter gene induction data for different PCBs and different AHR1 subtypes with egg injection LD_{50} values from the literature.
3) Measure PCB-induced EROD activity and CYP1A4/5 mRNA expression in chicken, ring-necked pheasant and Japanese quail hepatocyte cultures and compare results to PCB-induced reporter gene activity and *in ovo* toxicity.
4) Compare relative potencies of purified and reagent grade PCB 105 and 118.

1.5.3 Hypotheses and predictions

The hypotheses for this thesis were as follows:

1) Amino acid residues at positions 324 and 380 in the avian AHR1 ligand binding domain are predictive of avian species sensitivity to PCBs.

Prediction: Species with type 1 AHR1 genotypes (Ile324_Ser380) will be most sensitive to CYP1A induction by PCBs, species with type 2 AHR1 genotypes (Ile324_Ala380) will be moderately sensitive and species with type 3 AHR1 genotypes (Val324_Ala380) will be the least sensitive.

2) The rank order of PCB potency and RePs for PCBs do not differ between species of birds.

Prediction: According to WHO-TEFs for birds (Table 1.2), the order of DLC potency will be as follows in all species and AHR1 subtypes tested:

TCDD > PCB 126 > PCB 77 > PCB 105 > PCB 118.

3) The LRG assay along with the knowledge of a species' AHR1 genotype can be used to predict the embryotoxic effects of PCBs in that species.

Prediction: LRG assay endpoints will be significantly correlated with *in ovo* LD_{50} values from the literature.

4) The LRG assay could be used instead of other *in vitro* techniques using primary hepatocyte cultures to predict species sensitivity to DLCs and the relative potency of DLCs in avian species.

Prediction: ReS and ReP values obtained from LRG assay results will be similar to those obtained for EROD activity and CYP1A4/5 mRNA expression.

5) The presence of AHR ligands found as impurities in reagent grade PCB 105 and PCB 118 preparations cause the potency of these congeners to be overestimated.

Prediction: The RePs for purified and reagent grade solutions of PCB 105 and 118 will be significantly different.

1.5.4 Species selected

EROD activity and/or CYP1A4/5 mRNA expression was measured in hepatocyte cultures from three species of galliformes: the domestic chicken, ring-necked pheasant and Japanese quail. Eggs for these species are easily obtainable and differences in their sensitivity to TCDD, PCB 126 and PCB 77 have been demonstrated both *in ovo* and *in vitro* (Cohen-Barnhouse *et al.*, 2011;

Head *et al.*, 2008; Hervé *et al.*, 2010). Each species is representative of one of the three major avian AHR1 LBD types (Table A.1.1), with the chicken having a type 1 genotype (Ile324_Ser380), the ring-necked pheasant having a type 2 genotype (Ile324_Ala380) and the Japanese quail having a type 3 genotype (Val324_Ala380).

CHAPTER 2: A LUCIFERASE REPORTER GENE ASSAY AND ARYL HYDROCARBON RECEPTOR 1 GENOTYPE PREDICT THE LD_{50} OF POLYCHLORINATED BIPHENYLS IN AVIAN SPECIES[1]

2.1 Abstract

Birds differ in their sensitivity to the embryotoxic effects of polychlorinated biphenyls (PCBs), which complicates environmental risk assessments for these chemicals. Recent research has shown that the identities of amino acid residues 324 and 380 in the avian aryl hydrocarbon receptor 1 (AHR1) ligand binding domain (LBD) are primarily responsible for differences in avian species sensitivity to selected dibenzo-*p*-dioxins and furans. A luciferase reporter gene (LRG) assay was developed in our laboratory to measure AHR1-mediated induction of a cytochrome P450 1A5 reporter gene in COS-7 cells transfected with different avian AHR1 constructs. In the present study, the LRG assay was used to measure the concentration-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and PCB 126, 77, 105 and 118 on luciferase activity in COS-7 cells transfected with AHR1 constructs representative of 86 avian species in order to predict their sensitivity to PCB-induced embryolethality and the relative potency of PCBs in these species. The results of the LRG assay indicate that the identity of amino acid residues 324 and 380 in the AHR1 LBD are the major determinants of avian species sensitivity to PCBs. The relative potency of PCBs did not differ greatly among AHR1 constructs. Luciferase activity was significantly correlated with embryolethality data obtained from the literature ($R^2 \geq 0.87$, $p < 0.0001$). Thus, the LRG assay in combination with the knowledge of a species' AHR1 LBD sequence can be used to predict PCB-induced embryolethality in potentially any avian species of interest without the use of lethal methods on a large number of individuals.

[1] Adapted from Manning G.E., Farmahin R., Crump D., Jones S.P., Klein J., Konstantinov A., Potter D., Kennedy S.W. (2012). A luciferase reporter gene assay and aryl hydrocarbon receptor 1 genotype predict the LD_{50} of polychlorinated biphenyls in avian species. *Toxicol. Appl. Pharmacol.* **263**(3): 390-401.

2.2 Introduction

Polychlorinated biphenyls (PCBs) were banned internationally in 2004 (UNEP, 2009) and their production and use was phased-out in several countries starting in the 1970s. However, PCBs are still detectable in the environment and biota, including birds, due to their high environmental persistence and bioaccumulation potential (Custer *et al.*, 2003; Kay *et al.*, 2005; Kubota *et al.*, 2006; Letcher *et al.*, 2010; Ward *et al.*, 2010). For example, levels of total PCBs ranging from 0.1 to 100 μg/g have been measured in house wren (*Troglodytes aedon*), herring gull (*Larus argentatus*) and tree swallow (*Tachycineta bicolor*) eggs from the Kalamazoo River, Great Lakes and Housatonic River, respectively (Custer *et al.*, 2003; Neigh *et al.*, 2007; Weseloh *et al.*, 2006). Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and certain PCBs, elicit a wide range of effects in birds including embryolethality, and changes in reproductive function, development, cardiovascular function, immune function, endocrine homeostasis and biochemical responses (Bortolotti *et al.*, 2003; Cesh *et al.*, 2010; Fernie *et al.*, 2003; Jenssen *et al.*, 2010; Kennedy *et al.*, 1996; Lavoie *et al.*, 2007; Letcher *et al.*, 2010; Smits *et al.*, 2002; Walker and Catron, 2000). The toxic effects of DLCs have been studied extensively and most, if not all, effects are mediated through binding to the aryl hydrocarbon receptor (AHR) followed by transactivation of AHR-regulated genes, such as cytochrome P450 1A (CYP1A) enzymes (Denison *et al.*, 2011; Okey, 2007).

The wide range in avian species sensitivity to DLCs can lead to complications in conducting risk assessments for these chemicals (Cohen-Barnhouse *et al.*, 2011; Hervé *et al.*, 2010). However, recent research has shown that differences in sensitivity are associated with the

identity of amino acid residues 324 and 380 in the avian AHR1 ligand binding domain (LBD) (Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006). The AHR1 LBD amino acid sequences of 86 avian species were studied and species were grouped into one of three main AHR1 types: high sensitivity, type 1 [chicken-like species (Ile324_Ser380)]; moderate sensitivity, type 2 [ring-necked pheasant-like species (Ile324_Ala380)]; or low sensitivity, type 3 [Japanese quail-like species (Val324_Ala380)]. Species were further divided into one of 13 subtypes based on the predicted amino acid at positions 256, 257, 297 and 337 in the AHR1 LBD (Table 2.1) (Farmahin *et al.*, in press; Head *et al.*, 2008). Employing our knowledge of the AHR1 LBD sequences in various species of birds, a high-throughput luciferase reporter gene (LRG) assay that measures induction of a CYP1A5 reporter gene was developed in our laboratory with the goal of predicting the toxic effects of DLCs in potentially any species of bird (Farmahin *et al.*, in press; Farmahin *et al.*, 2012). CYP1A induction by PCBs in primary avian hepatocyte cultures is strongly correlated with embryolethality, supporting the use of CYP1A induction for predicting PCB toxicity (Head *et al.*, 2010). However, measuring CYP1A induction from hepatocyte cultures is not feasible for rare or endangered species. The LRG assay circumvents the need for using large numbers of individuals because only the AHR1 genotype of a species of interest is needed to determine its sensitivity to AHR1-mediated reporter gene activity by DLCs (Farmahin *et al.*, in press; Farmahin *et al.*, 2012).

The goals of the present study were: (1) to use the LRG assay to determine the concentration-dependent effects of PCB congeners 126, 77, 105 and 118 on reporter gene activity in cells transfected with AHR1 expression constructs representative of several avian species, and (2) to use the data generated from the LRG assay to predict species sensitivity to the toxic effects of PCBs and differences in the relative potency of PCBs among species. PCB 105

and 118 were of special interest due to recent concerns that the presence of low levels of potent AHR agonists found as impurities in mono-*ortho* PCB preparations could affect the relative potency (ReP) values obtained for mono-*ortho* PCBs (Peters *et al.*, 2006; van den Berg *et al.*, 2006). Therefore, another goal of this study was to compare the effects of purified and reagent-grade solutions of PCB 105 and 118 on AHR1-mediated reporter gene activity using the LRG assay.

2.3 Materials and methods

2.3.1 Analysis of mono-ortho PCBs

Crystalline, reagent-grade samples of PCB 105 and PCB 118, referred to hereafter as PCB 105-RG and PCB 118-RG, were obtained by Wellington Laboratories Inc. (Guelph, ON, Canada) and were subsequently tested to confirm their identity and purity using high resolution gas chromatography/low resolution mass spectrometry (HRGC/LRMS). For this purpose, an Agilent (Santa Clara, CA, USA) 7890A/5975C HRGC/LRMS system was used. The LRMS was operated using electron impact positive (EI+) ionization with full scan (50-1000 amu) acquisition. The HRGC column used was a 30 m DB5 (J&W) column (0.25 mm ID, 0.25 µm film thickness) with the following oven temperature program: 100ºC (5 minutes), 10ºC/minute to 325ºC, 325ºC (20 minutes). Helium carrier gas was used and the injector (splitless) and detector temperatures kept at 250ºC. Both PCBs were found to be > 99% pure by HRGC/LRMS and their identities confirmed by retention time comparison to standards from another source.

2.3.2 Purification of mono-ortho PCBs

Assuming that other PCB congeners could be present as contaminants, PCB 105-RG and 118-RG were initially purified using preparative thin layer chromatography (TLC). This was performed using glass plates coated with Silica Gel 60 GF254 (EMD Chemicals, Gibbstown, NJ, USA). PCB 105-RG and 118-RG were dissolved in hexane and applied to the origin of the TLC plates. The TLC plates were developed using hexane and then dried. The major bands observed under UV, corresponding to the PCBs, were collected and eluted from the silica with dichloromethane (DCM). The DCM eluates were then evaporated to dryness on a rotary evaporator leaving the recovered, crystalline PCBs.

The PCBs were additionally purified using carbon columns consisting of PX21 carbon (Amoco Corp., Chicago, IL, USA) dispersed on Celite™ (Fisher Scientific Ltd., Ottawa, ON, Canada). The PCBs were dissolved in hexane and applied to the tops of columns containing the PX21/Celite. The PCBs were then eluted with hexane, which was subsequently removed on a rotary evaporator. The purified PCBs are hereafter referred to as PCB 105-P and PCB 118-P.

2.3.3 Preparation and quantification of PCB and TCDD solutions

Dimethyl sulfoxide (DMSO) stock solutions of PCB 105-P, 118-P (>99% pure; lot number 060611), 105-RG, 118-RG and 126 (>99% pure; lot number 031711) prepared at 196 μg/ml (6.0 X 10^{-4} M) were obtained from Wellington Laboratories and used to make serial dilutions. Actual concentrations were 202.8 μg/ml, 207.2 μg/ml, 196.9 μg/ml, 198.9 μg/ml and 205.3 μg/ml for PCB 105-P, 105-RG, 118-P, 118-RG and 126, respectively. To prepare the DMSO stock solutions, accurate weights (9.80 mg) of the crystalline PCBs were first dissolved in pentane (ca. 10 ml) and transferred to volumetric flasks (Class A; 50 ml). DMSO (ca. 3 ml) was then added to

the pentane solutions and the pentane removed by evaporation. Once the pentane had been removed, the solutions were made to volume by the addition of DMSO and sonicated in an ultra-sonic bath. All solutions were visually inspected to ensure that no crystalline material was present.

To ensure that the crystalline PCBs had completely dissolved in the DMSO, 1 ml aliquots were taken and spiked with the appropriate 13C-labeled PCB analogues (1 ml; 50 µg/ml; nonane). Water (3 ml) was added to the spiked aliquots, and the mixtures were shaken vigorously and then centrifuged. The upper layer (nonane) was removed and the lower aqueous layer was extracted again with 3 ml of toluene. The nonane and toluene extracts were combined and analysed by HRGC/LRMS as described in Section 2.3.1. They were compared to a mixed standard containing accurate amounts of the PCBs and their 13C analogues. All of the DMSO stock solutions were found to be within ± 5% of the 196 µg/ml target concentration.

Serial dilutions of PCB 77 were prepared from a DMSO stock solution with a nominal concentration of 120 µg/ml. The PCB 77 stock solution was prepared by dissolving 0.48 mg of PCB 77 (ULTRA Scientific, Kingstown, RI, USA; >99% pure; lot number A-0094) in 4 ml of DMSO (4.0×10^{-4} M). A detailed description of the preparation and analysis of TCDD stock solutions is provided elsewhere (Hervé *et al.*, 2010). Serial dilutions of TCDD were prepared from a DMSO stock solution with a nominal concentration of 80 µg/ml. Identification and quantification of TCDD in the stock solution were determined by isotope dilution following EPA Method 1613 by use of high-resolution gas chromatography/high resolution mass spectrometry (HRGC/HRMS). The measured concentration of the stock solution was 72.9 µg/ml (2.3×10^{-4} M).

2.3.4 Testing of mono-ortho PCB solutions for impurities

The DMSO solutions of PCB 105-P, 105-RG, 118-P and 118-RG were tested for PCDDs, PCDFs and non-*ortho* PCBs. This involved taking 0.5 ml aliquots of the DMSO solutions and spiking them with 13C-labeled PCDD, PCDF and PCB surrogates (in nonane). Water (2.5 ml) was added to the spiked aliquots, and the mixture was shaken vigorously and then centrifuged. The upper organic layer (nonane) was removed and the aqueous/DMSO mixture was further extracted with an additional 3 ml of toluene. The extracts (nonane and toluene) were combined and analyzed by HRGC/HRMS using isotope dilution.

HRGC/HRMS analyses were performed using an Agilent 6890 series HRGC with direct capillary interface to a Waters (Milford, MA, USA) Autospec Ultima HRMS. Chromatographic separations were carried out on a 60 m J&W DB5 (0.25 mm ID, 0.25 µm film thickness) column in constant flow mode (Helium, 1 ml /minute) with the following oven temperature program: 150°C (1 minute), 12°C/minute to 200°C, 3 °C/minute to 235°C, 8°C/minute to 310°C, 310°C (10 minute). All injections were 1 µl using splitless injection at 280°C. The mass spectrometer was operated in EI+ selective ion recording mode (SIR) at a mass resolving power of 10,000 or greater. Where present, the PCDDs, PCDFs and PCBs were quantitated by internal standard methods.

2.3.5 Expression constructs

Details concerning the preparation of full length chicken, ring-necked pheasant and Japanese quail AHR1 constructs are provided elsewhere (Farmahin *et al.*, 2012). In addition, twelve mutant AHR1 constructs were previously prepared based on AHR1 LBD sequences obtained for 86 avian species (Table 2.1) (Farmahin *et al.*, in press). In brief, the twelve mutant

AHR1 constructs, representative of each AHR1 subtype identified in Table 2.1, were prepared by site-directed mutagenesis of the following amino acid residue sites in the chicken AHR1 LBD: 256, 257, 297, 324, 337 and 380. All mutant constructs were sequenced following site-directed mutagenesis to ensure the correct sequence was obtained. *In vitro* transcription and translation assays and western blot analyses were also conducted to ensure consistent expression levels among AHR1 constructs (Farmahin *et al.*, in press; Farmahin *et al.*, 2012).

A cormorant ARNT1 vector (pcDNA-ccArnt1) and a firefly luciferase reporter vector containing the cormorant CYP1A5 promoter region (pGL4-ccCYP1A5) were generously provided by Dr. Hisato Iwata (Ehime University, Japan) (Lee *et al.*, 2009; Yasui *et al.*, 2007). A *Renilla* luciferase vector carrying the cytomegalovirus promoter (phRL-CMV) was obtained from Promega (Madison, WI, USA).

Table 2.1. AHR1 subtypes identified based on predicted amino acid sequences of the avian AHR1 ligand binding domain (LBD). Avian AHR1 LBD sequences from 86 species were obtained from GenBank or were determined from liver or blood samples obtained from the National Wildlife Research Centre, Ottawa, ON, commercial suppliers near Ottawa, ON, or the Tittabawassee River Basin, Michigan, USA (Farmahin *et al.*, in press).

	LBD amino acid residues						
AHR1 subtype	256	257	297	**324**[a]	337	**380**[a]	Examples[b]
1A	A	A	T	**I**	V	**S**	domestic chicken (*Gallus gallus domesticus*)
1B	A	A	T	**I**	I	**S**	European starling (*Sturnus vulgaris*)
1C	A	T	T	**I**	I	**S**	gray catbird (*Dumetella carolinensis*)
2A	A	A	I	**I**	V	**A**	wild turkey (*Meleagris gallopavo*)
2B	A	A	T	**I**	I	**A**	indigo bunting (*Passerina cyanea*)
2C	A	A	V	**I**	V	**A**	rock ptarmigan (*Lagopus muta*)
2D	A	P	T	**I**	V	**A**	spotted sandpiper (*Actitis macularius*)
2E	A	T	T	**I**	I	**A**	tree swallow (*Tachycineta bicolor*)
2F	A	T	T	**I**	V	**A**	black-footed albatross (*Phoebastria nigripes*)
2G	T	A	I	**I**	V	**A**	ring-necked pheasant (*Phasianus colchicus*)
3A	A	A	T	**V**	V	**A**	Japanese quail (*Coturnix japonica*)
3B	A	T	T	**V**	V	**A**	herring gull (*Larus argentatus*)
3C	T	T	T	**V**	V	**A**	wood duck (*Aix sponsa)*

[a] Amino acid residues at positions 324 and 380 were used to classify species into three major AHR1 types, which are indicated by white (type 1), light grey (type 2) and dark grey (type 3) shading.
[b] The full list of 86 species is presented in Table A.1.1. (Farmahin *et al.*, in press).

2.3.6 Cell culture, transfection and luciferase reporter gene (LRG) assay

Minor modifications were made to the LRG assay procedure previously employed by our group (Farmahin *et al.*, in press; Farmahin *et al.*, 2012). In brief, COS-7 cells, provided by Dr. R. Haché (University of Ottawa, Ottawa, ON), were seeded in 96-well plates at a concentration of 10,000 cells per well and transfected after 18 hours with an AHR1 expression construct, cormorant ARNT, pGL4-ccCYP1A5, phRL-CMV and salmon sperm DNA (Invitrogen). Approximately 50 ng of DNA was complexed with 0.2 µl, rather than 0.15 µl, of Fugene 6 transfection reagent (Roche, Laval, QC, Canada) and 6 µl of the mixture was added to each well. The amounts of transfected expression vectors were 8 ng of chicken, ring-necked pheasant, Japanese quail or mutant avian AHR1, 1.55 ng of cormorant ARNT, 5 ng of pGL4-ccCYP1A5 and 0.75 ng of phRL-CMV. The total amount of transfected DNA was kept constant at 50 ng through the addition of salmon sperm DNA (Invitrogen). Cells were dosed 5 hours after transfection with a vehicle control (DMSO) or DMSO solutions of TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P or PCB 118-RG at 0.5% instead of 0.05% final DMSO concentration. The final concentration of DMSO was modified in order to increase the range of concentrations that could be tested. Final nominal concentrations of TCDD and PCBs ranged from 0.03 to 300 nM and 0.1 to 3000 nM, respectively. A positive control, consisting of a nominal concentration of 300 nM TCDD, was also included for each AHR1 construct that was tested.

Reporter gene activity was measured as luminescence according to the manufacturer's instructions 20 hours after dosing using Dual-Glo luciferase assay kits (Promega) in a LuminoSkan Ascent luminometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Final

luminescence values were expressed as a ratio of firefly luciferase units to *Renilla* luciferase units (luciferase ratio).

2.3.7 Analysis of concentration-response curves

Triplicate concentration-response curves were obtained from three independent experiments for each AHR1 construct and DLC treatment, each with four technical replicates per concentration of DMSO, TCDD or PCB. Luciferase ratios for each concentration-response curve were normalized according to the protocol described in OECD guideline 455 (OECD, 2009), where data were expressed as the relative luciferase activity compared to the positive control response. For each 96-well plate transfected with a particular AHR1 construct, the mean luciferase ratio, obtained from four replicate DMSO-treated wells, was subtracted from the luciferase ratios of DLC-treated wells. Using data normalized to the DMSO response, the mean luciferase ratio from four replicate positive control-treated wells was calculated and luciferase ratios of all wells were divided by the mean luciferase ratio for the 300 nM TCDD positive control. Luminescence data, represented as a percent of the positive control response, were imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) and fitted to a four parameter logistic model (Head and Kennedy, 2007b). The equation integrates the EC_{50}, slope, baseline response and maximal response as parameters. In cases where concentration-response curves did not reach a plateau (maximal response), the highest observed response is reported (Table A.2.1). Since the maximal response of a concentration-response curve must be known in order to calculate a reliable EC_{50}, EC_{50} values for these curves are not presented. The TCDD and PCB concentrations that elicited a response equal to 20, 50 and 80% that of the positive control (PC), referred to as PC_{20}, PC_{50} and PC_{80}, were determined for each replicate

concentration-response curve using the logistic model curve fit. An $EC_{threshold}$ value (Mundy *et al.*, 2010), representing the concentration of a compound that elicits a significantly higher response than DMSO-treated cells, was also calculated to allow for the calculation of relative sensitivity (ReS) or ReP values in cases where a treatment elicited a response less than 20% of the positive control at the highest concentration tested. $EC_{threshold}$ values were determined from concentration-response curves averaged from 3 replicate curves. EC_{50}, PC_{20}, PC_{50}, PC_{80} and maximal response values represent the mean obtained from three concentration-response curves ± standard error (SE).

2.3.8 Calculation of ReS and ReP values

Since the chicken is considered to be the most sensitive avian species to the toxic and biochemical effects of DLCs (Head *et al.*, 2008; Hervé *et al.*, 2010), the sensitivity of all wild-type and mutant AHR1 constructs was expressed relative to luciferase activity data from cells transfected with the chicken AHR1 construct. Full concentration-response curves were not always obtained, and in these cases an EC_{50} value could not be calculated. Therefore, $EC_{threshold}$ values were used to calculate ReS values for each AHR1 construct. For concentration-response curves that did achieve a maximal response, EC_{50}-based ReS values were also calculated for comparison to $EC_{threshold}$-based ReS values (Table A.2.2). ReS values were calculated as follows: $EC_{threshold}$ or EC_{50} of compound *x* in chicken AHR1 ÷ $EC_{threshold}$ or EC_{50} of compound *x* in the AHR1 construct of interest.

The maximal responses and slopes of concentration-response curves differed between DLC treatments. Therefore, the potency of PCBs was measured relative to TCDD using the systematic framework proposed by Villeneuve *et al.* (2000) with some modifications. ReP values based on

PC_{20}, PC_{50} and PC_{80} values (ReP_{PC20}, ReP_{PC50} and ReP_{PC80}) obtained from the four parameter logistic models were calculated. If the maximal response or highest observed response of the PCB congener was less than 80% of the positive control, a ReP estimate corresponding to the highest observed response for that PCB congener (ReP_{PCmax}) was also calculated. If the highest observed response was less than 20% of the positive control, a ReP was calculated based on $EC_{threshold}$ values (ReP_{thr}). ReP values were calculated as follows: PC_{20}, PC_{50}, PC_{80}, PC_{max} or $EC_{threshold}$ of TCDD in AHR1 *x* ÷ PC_{20}, PC_{50}, PC_{80}, PC_{max} or $EC_{threshold}$ of the PCB congener of interest in AHR1 *x*.

2.3.9 Statistical analysis

$EC_{threshold}$ values were determined using a one-way analysis of variance (ANOVA) ($p \leq 0.05$) followed by Dunnett's test ($p \leq 0.05$). Only the concentrations that elicited a response less than 20% of the positive control were included in the statistical analysis. Significant differences between maximal response, $logEC_{50}$, $logPC_{20}$, $logPC_{50}$ and $logPC_{80}$ values for different DLC solutions or different AHR1 constructs were determined by performing a t-test ($p \leq 0.05$) or a one-way ANOVA ($p \leq 0.05$), followed by Tukey's Multiple Comparison Test ($p \leq 0.05$). Linear regression analyses were conducted between log-transformed LD_{50} values and log-transformed EC_{50}, $EC_{threshold}$ and PC_{20} data obtained from the LRG assay. LD_{50} values for the DLCs used in these analyses were obtained from the literature.

2.4 Results and discussion

2.4.1 Levels of impurities in mono-ortho PCB preparations

Purification of PCB 105 and 118 was conducted to determine whether AHR1-mediated effects induced by these congeners could be partly due to contamination of the solutions with more potent AHR1 agonists. The absolute and TEQ concentrations of PCDDs, PCDFs and non-*ortho* PCBs found in PCB 105-P, 105-RG, 118-P and 118-RG are presented in Table 2.2. Most PCDD and PCDF congeners were not detected in PCB 105 and 118 solutions before or after purification, but 1.2 ppm of 1,2,3,4,6,7,8-heptachlorodibenzo-*p*-dioxin was detected in PCB 105-RG. Purification successfully removed this PCDD (Table 2.2). PCB 77 was the major contaminant detected in the PCB 105 and 118 solutions, with the highest concentration in PCB 118-RG (2470 ppm). Most of the PCB 77 was removed using a combination of TLC and carbon column cleanups, but approximately 20 ppm of PCB 77 could not be removed from either PCB 105-P or PCB 118-P. The inability to completely remove PCB 77 may have been caused by competition between PCB 77 and the major PCB components (i.e. PCB 105 or 118) for active sites on the carbon columns, and the ability of PCB 105 or PCB 118 to act as a solvent, which would carry some PCB 77 through the carbon column. Although cells treated with purified PCB solutions were exposed to some residual PCB 77 (a maximum of 0.1 nM PCB 77 in the highest dose group), this level of exposure is well below the PCB 77 $EC_{threshold}$ value in all AHR1 constructs (Table A.2.1). Thus, PCB 77 contamination in PCB 105-P and 118-P is not expected to affect induction of luciferase activity in cells exposed to these solutions.

Table 2.2. Concentrations of PCDDs, PCDFs and non-*ortho* PCBs (µg/g; ppm) in purified (P) and reagent-grade (RG) solutions of PCB 105 and PCB 118. Avian WHO-TEF values are from van den Berg *et al.* (1998). TEQ concentrations (µg/g; ppm) are the product of each compound concentration and its associated avian WHO-TEF. Total TEQ concentrations are the sum of TEQ concentrations for each compound and represent the maximum possible TEQ concentration because detection limit concentrations for all non-detected compounds were used in total TEQ calculations.

	Avian	PCB 105-P (µg/g)		PCB 105-RG (µg/g)		PCB 118-P (µg/g)		PCB 118-RG (µg/g)	
	WHO-TEF	concentration	TEQ	concentration	TEQ	concentration	TEQ	concentration	TEQ
DIOXINS									
2,3,7,8-TCDD	1	ND[a] (0.3)	<0.3	ND (0.4)	<0.4	ND (0.4)	<0.4	ND (0.4)	<0.4
1,2,3,7,8-PeCDD	1	ND (0.3)	<0.3	ND (0.3)	<0.3	ND (0.2)	<0.2	ND (0.2)	<0.2
1,2,3,4,7,8-HxCDD	0.05	ND (0.2)	<0.01	ND (0.6)	<0.03	ND (0.8)	<0.04	ND (0.3)	<0.015
1,2,3,6,7,8-HxCDD	0.01	ND (0.2)	<0.002	ND (0.5)	<0.005	ND (0.7)	<0.005	ND (0.2)	<0.002
1,2,3,7,8,9-HxCDD	0.1	ND (0.2)	<0.02	ND (0.6)	<0.06	ND (0.8)	<0.08	ND (0.3)	<0.03
1,2,3,4,6,7,8-HpCDD	0.001	ND (0.2)	<0.0002	1.2	0.0012	ND (0.4)	<0.0004	ND (0.5)	<0.0005
OCDD	0.0001	ND (0.7)	<0.00007	ND (0.5)	<0.00005	ND (0.4)	<0.00004	ND (0.3)	<0.00004
FURANS									
2,3,7,8-TCDF	1	ND (0.2)	<0.2	ND (0.2)	<0.2	ND (0.3)	<0.3	ND (0.3)	<0.3
1,2,3,7,8-PeCDF	0.1	ND (0.2)	<0.02	ND (0.2)	<0.02	ND (0.7)	<0.07	ND (0.9)	<0.09
2,3,4,7,8-PeCDF	1	ND (0.2)	<0.2	ND (0.2)	<0.2	ND (0.2)	<0.2	ND (0.2)	<0.2
1,2,3,4,7,8-HxCDF	0.1	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.1)	<0.01
1,2,3,6,7,8-HxCDF	0.1	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.1)	<0.01
1,2,3,7,8,9-HxCDF	0.1	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.1)	<0.01
2,3,4,6,7,8-HxCDF	0.1	ND (0.2)	<0.02	ND (0.1)	<0.01	ND (0.1)	<0.01	ND (0.2)	<0.02
1,2,3,4,6,7,8-HpCDF	0.01	ND (0.1)	<0.001	ND (0.1)	<0.001	ND (0.1)	<0.001	ND (0.1)	<0.001
1,2,3,4,7,8,9-HpCDF	0.01	ND (0.2)	<0.002	ND (0.2)	<0.002	ND (0.2)	<0.002	ND (0.1)	<0.001
OCDF	0.0001	ND (0.2)	<0.00002	ND (0.3)	<0.00003	ND (0.3)	<0.00003	ND (0.2)	<0.00002
Non-ortho PCBs									
PCB 77	0.05	24	1.2	17	0.85	21	1.05	2470	123.5
PCB 81	0.1	ND (0.3)	<0.03	ND (0.6)	<0.06	ND (0.2)	<0.02	ND (0.3)	<0.03
PCB 126	0.1	ND (5.4)	<0.54	ND (7.9)	<0.79	ND (6.6)	<0.66	ND (5.9)	<0.59
PCB 169	0.001	ND (0.1)	<0.0001	2.3	0.0023	ND (0.1)	<0.0001	ND (0.1)	<0.0001
TOTAL TEQ			2.9		3.0		3.1		125

[a] ND: Not detected (detection limits in brackets)

2.4.2 Induction of luciferase reporter gene activity in COS-7 cells

The concentration-dependent effects of TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG on luciferase reporter gene activity in cells transfected with wild-type and mutant AHR1 constructs are presented in Figures 2.1 and 2.2. The EC_{50}, $EC_{threshold}$, PC values and maximal responses associated with each concentration-response curve are provided in the supplementary information (Table A.2.1). TCDD and PCB 126 induced luciferase activity in all AHR1-transfected cells. The maximal responses for TCDD were approximately 100% in all AHR1-transfected cells and therefore the 300 nM concentration was an appropriate positive control for normalizing luciferase activity data. There were no significant differences between TCDD and PCB 126 maximal responses in cells transfected with type 1, ring-necked pheasant (subtype 2G), 2B and type 3 AHR1 constructs. However, PCB 126 induced significantly lower maximal responses (62-75% positive control) relative to TCDD in cells transfected with constructs 2A, 2C, 2D, 2E, 2F and 2G (Table A.2.1). PCB 77 induced luciferase activity in cells transfected with all type 1 and type 2 constructs. No significant induction was observed in cells transfected with 3A and 3B constructs, but 2065 nM PCB 77 significantly induced luciferase activity in cells transfected with the Japanese quail (subtype 3A) and 3C constructs. Maximal responses induced by PCB 77 were significantly lower than those induced by TCDD and PCB 126 in all AHR1-transfected cells (13-64%), except in cells transfected with the chicken (subtype 1A) and 1C constructs (72-76%) (Table A.2.1). The maximal responses induced by PCB 105-P, 105-RG, 118-P and 118-RG were significantly lower (3-38%) than responses elicited by TCDD and PCB 126 in all AHR1-transfected cells (Table A.2.1).

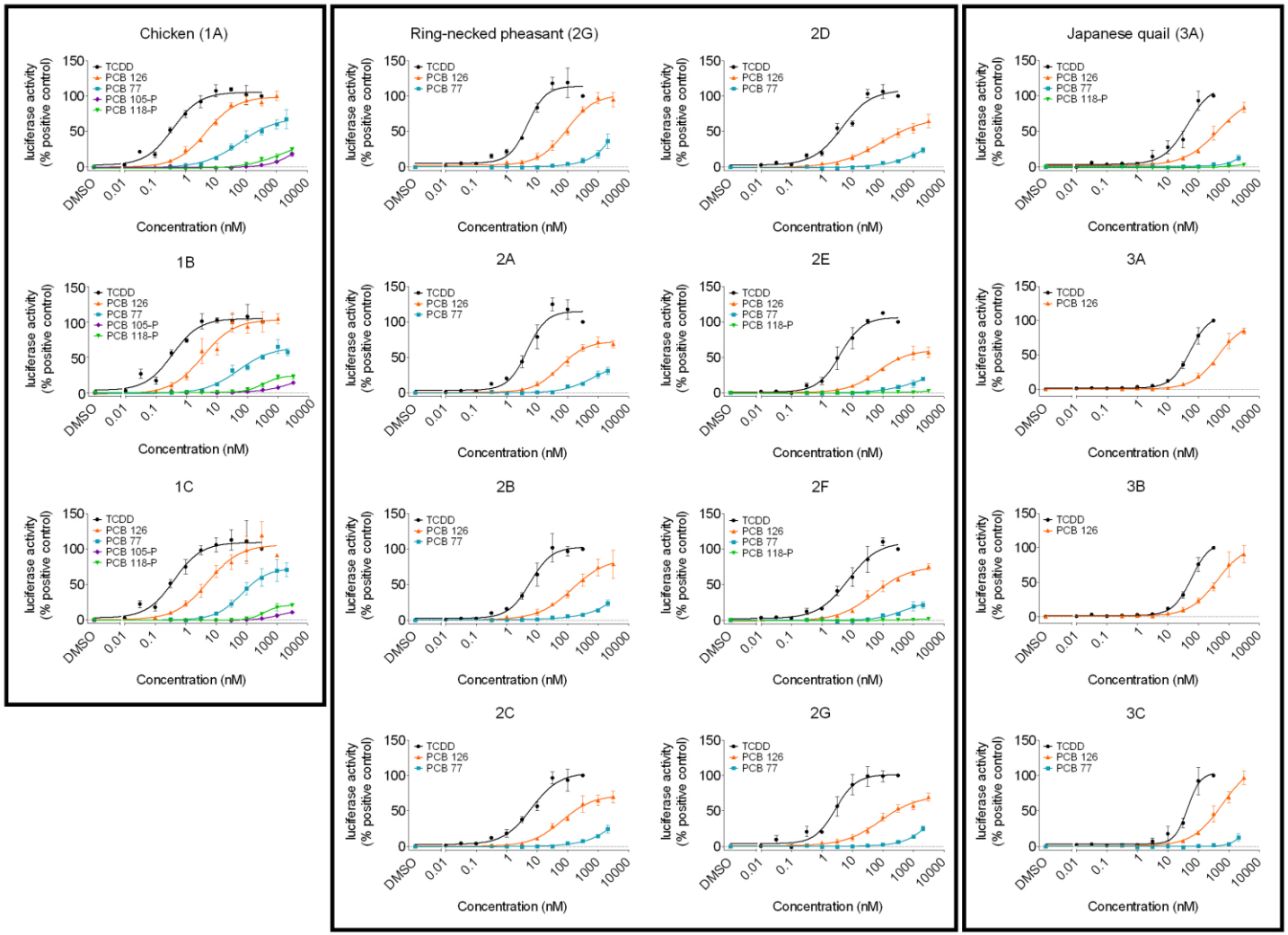

Figure 2.1. Effects of TCDD, PCB 126, PCB 77, PCB 105-P and PCB 118-P on AHR1-mediated luciferase reporter gene activity in COS-7 cells transfected with wild-type (chicken, ring-necked pheasant and Japanese quail) and mutant (1B-C, 2A-G and 3A-C) AHR1 constructs. Data are presented as the percent response relative to a 300 nM TCDD positive control. Concentration-response curves are included for constructs that showed a significant ($p < 0.05$), concentration-dependent increase in reporter gene activity, relative to the DMSO response, following exposure to TCDD or PCBs. Points represent mean positive control-normalized luciferase ratios obtained from 3 independent concentration-response curves. Each concentration-response curve had quadruplicate doses for each compound concentration. Bars represent standard error. Boxes contain concentration-response curves from the three main AHR1 types.

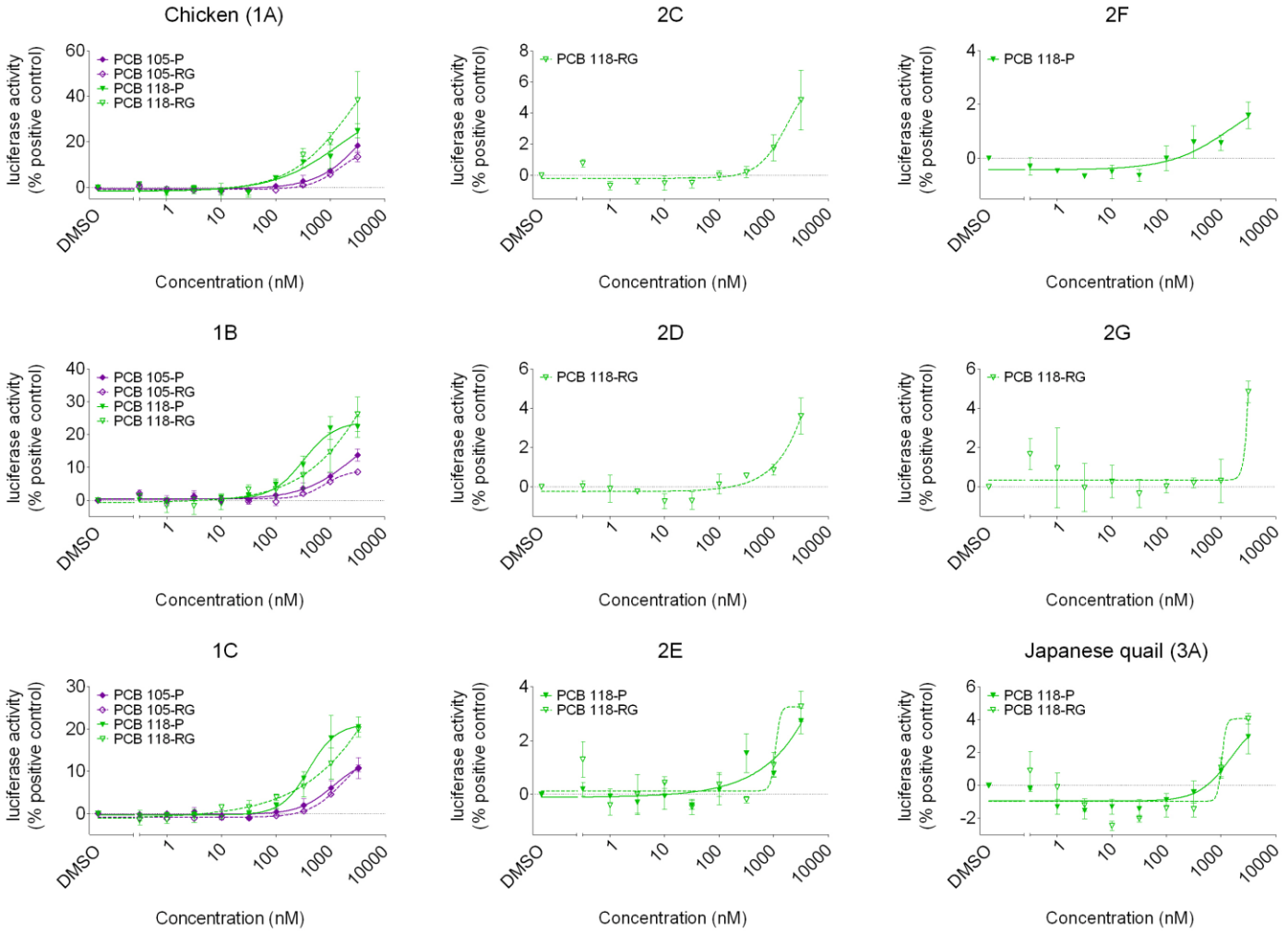

Figure 2.2. Effects of purified (P) and reagent grade (RG) solutions of PCB 105 and PCB 118 on AHR1-mediated luciferase reporter gene activity in COS-7 cells transfected with wild-type (chicken and Japanese quail) and mutant (1B-C and 2C-G) AHR1 constructs. Data are presented as the percent response relative to a 300 nM TCDD positive control. Concentration-response curves are presented for constructs that showed a significant ($p < 0.05$), concentration-dependent increase in reporter gene activity relative to the DMSO response. Points represent mean positive control-normalized luciferase ratios for results obtained from 3 independent concentration-response curves. Each concentration-response curve had quadruplicate doses for each compound concentration. Bars represent standard error.

The potency of a ligand depends on its binding affinity with the receptor and the ability of the ligand-receptor complex to induce a response (Hestermann *et al.*, 2000). AHR ligands can induce diverse changes in receptor conformation, leading to modifications in the interaction of the AHR/ARNT complex with co-activators and xenobiotic response elements (XREs) and ultimately, differences in transcriptional activation (Denison *et al.*, 2011; Hestermann *et al.*, 2000; Zhou *et al.*, 2003). Similarly, species-specific variations in the avian AHR1 amino acid sequence may also lead to differences in AHR-mediated transcriptional activity. This could account for the lower responses induced by PCB 126 relative to TCDD in several mutant type 2 constructs.

One drawback of the LRG assay is its inability to detect induction of luciferase activity by PCB 77, PCB 105 and PCB 118 in cells transfected with some type 2 and type 3 constructs. A ten-fold difference in sensitivity between our assay and a similar LRG assay developed by another laboratory (Lee *et al.*, 2009; Mol *et al.*, 2012) was previously reported (Farmahin *et al.*, 2012) and could be due to differences in the choice of luciferase assay systems. We selected the Dual-Glo Luciferase Assay system (Promega) because of its suitability for high-throughput screening applications (Hawkins *et al.*, 2002). This system includes more stable luciferase reagents with longer half-lives than the luciferase reagents included in the Dual Luciferase Reporter Assay (Promega) employed by the other laboratory (Lee *et al.*, 2009; Mol *et al.*, 2012). However, the improved stability of the luciferase used in the Dual-Glo assay is achieved by reducing catalytic turnover of the enzymes, which leads to a reduction in assay sensitivity (Hawkins *et al.*, 2002). Our assay is useful for high-throughput screening of DLC effects on AHR1-mediated transactivation in several avian species and inferences on the potency of DLCs or the sensitivity of avian species can still be made when no induction of luciferase activity is observed. However, if one was specifically

interested in observing induction of AHR1-mediated transactivation by weak AHR1 agonists in cells transfected with insensitive AHR1 constructs, the Dual Luciferase Reporter Assay might be more appropriate.

2.4.2.1 ReS of AHR1 constructs exposed to DLCs

$EC_{threshold}$-based ReS values (Table A.2.1) are presented in Table 2.3. When available, EC_{50} values were also used to calculate ReS values for comparison with $EC_{threshold}$-based ReS values (Table A.2.2). EC_{50}- and $EC_{threshold}$-based ReS values were generally similar. Type 1 AHR1 constructs were the most sensitive to induction of luciferase activity by all DLC treatments and no significant differences were observed among their EC_{50} values for a given DLC treatment (Table A.2.2). Type 2 and type 3 constructs exposed to TCDD were 10 times and 33 to 333 times less sensitive than the chicken construct, respectively. EC_{50} values for type 1 constructs exposed to TCDD were significantly different from all other type 2 and type 3 constructs, while EC_{50} values for almost all type 2 constructs exposed to TCDD were significantly different from type 1 and type 3 constructs (Table A.2.2).

Relative to the chicken construct, type 2 and type 3 constructs were 10 to 33 and 33 to 100 times less sensitive to induction of luciferase activity by PCB 126, respectively. EC_{50} values for the chicken and 1B constructs exposed to PCB 126 were significantly lower than type 2 and type 3 EC_{50} values (Table A.2.2). Although few statistically significant differences were observed between EC_{50} values for type 2 and type 3 constructs, type 3 constructs were generally less sensitive to PCB 126 than type 2 constructs. However, the differences in sensitivity between type 2 and type 3 constructs exposed to PCB 126 were not as distinct as for TCDD.

Type 2 constructs exposed to PCB 77 were 10 to 30 times less sensitive than the chicken construct. Luciferase activity was significantly induced by 2065 nM PCB 77 in cells transfected with the Japanese quail and 3C constructs, but not in cells transfected with constructs 3A or 3B. Therefore, type 3 constructs were determined to be at least 210 times less sensitive than the chicken construct.

Because no significant induction of luciferase activity was observed in cells transfected with type 2 or type 3 constructs following exposure to PCB 105-P and 105-RG, these constructs were at least 3 times less sensitive than the chicken construct based on the highest concentration of PCB 105-P and 105-RG tested (3000 nM). These data cannot elucidate whether type 2 constructs are more sensitive to PCB 105 than type 3 constructs, or vice versa.

Since exposure to 3000 nM PCB 118-P and 118-RG caused a significant increase in luciferase activity in cells transfected with some type 2 (2C, 2D, 2E, 2F, 2G) and type 3 (Japanese quail) constructs, all type 2 and type 3 AHR1 constructs are at least 10 times less sensitive to PCB 118-P and 118-RG than the chicken construct. Type 2 constructs may be more sensitive to PCB 118 than type 3 constructs given that luciferase activity was induced by either PCB 118-P or PCB 118-RG in cells transfected with five of eight type 2 constructs, but only 1 of 4 type 3 constructs.

Table 2.3. Sensitivity of wild-type (chicken, ring-necked pheasant and Japanese quail) and mutant (1B-C, 2A-G and 3A-C) AHR1 constructs to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG relative to chicken AHR1. Relative sensitivity (ReS) values were calculated based on $EC_{threshold}$ values obtained from the luciferase reporter gene (LRG) assay. If no induction of luciferase activity was observed, ReS values were estimated by dividing the chicken value by the highest concentration tested (2065 nM for PCB 77 and 3000 nM for all other compounds).

AHR1	**ReS_{thr}[a]**						
	TCDD	**PCB126**	**PCB 77**	**PCB 105-P**	**PCB 105-RG**	**PCB 118-P**	**PCB 118-RG**
chicken (1A)[b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1B	1.0	1.0	1.0	1.0	1.0	1.0	0.30
1C	1.0	1.0	3.3	1.0	1.0	1.0	0.30
ring-necked pheasant (2G)[b]	0.10	0.030	0.033	<0.33	<0.33	<0.10	<0.10
2A	0.10	0.030	0.10	<0.33	<0.33	<0.10	<0.10
2B	0.10	0.030	0.10	<0.33	<0.33	<0.10	<0.10
2C	0.10	0.10	0.10	<0.33	<0.33	<0.10	0.10
2D	0.10	0.030	0.10	<0.33	<0.33	<0.10	0.10
2E	0.10	0.10	0.10	<0.33	<0.33	0.10	0.10
2F	0.10	0.10	0.10	<0.33	<0.33	0.10	<0.10
2G	0.10	0.10	0.033	<0.33	<0.33	<0.10	0.10
Japanese quail (3A)[b]	0.0030	0.030	0.0048	<0.33	<0.33	0.10	0.10
3A	0.030	0.010	<0.0048	<0.33	<0.33	<0.10	<0.10
3B	0.0030	0.010	<0.0048	<0.33	<0.33	<0.10	<0.10
3C	0.0030	0.010	0.0048	<0.33	<0.33	<0.10	<0.10

[a] ReS_{thr}, $ReS_{ECthreshold}$

[b] Wild-type AHR1 constructs containing the full length sequence for the chicken, ring-necked pheasant or Japanese quail AHR1.

Type 1 constructs were the most sensitive to induction of AHR1-mediated reporter gene activity by all PCB congeners tested, followed by type 2 and type 3 constructs. No significant differences were observed among EC_{50} values for constructs within each main AHR1 type (Table A.2.2), and $EC_{threshold}$ values for constructs within each main AHR1 type were typically within one dose of each other. Therefore, amino acids at positions 256, 257, 297 and 337 within the AHR1 LBD do not appear to play a significant role in determining avian species sensitivity to TCDD and PCBs. However, the significant differences in EC_{50} values observed between type 1, type 2 and type 3 AHR1 constructs exposed to TCDD, PCB 126 and PCB 77 support previous findings (Farmahin *et al.*, in press; Farmahin *et al.*, 2012; Head *et al.*, 2008; Karchner *et al.*, 2006) that amino acids at positions 324 and 380 in the AHR1 LBD are important in determining species sensitivity to DLCs.

2.4.2.2 ReP of DLCs in different AHR1 constructs

Based on average ReP values (ReP_{avg}) (Table 2.4), visual inspection of the concentration-response curves (Figures 2.1 and 2.2) and the results of statistical analyses performed between LRG assay endpoints for different DLC treatments in a given AHR1 construct (Table A.2.1), the rank order of potency for most AHR1 constructs was: TCDD > PCB 126 > PCB 77 ≥ PCB 118-P ≈ PCB 118-RG ≥ PCB 105-P ≈ PCB 105-RG. ReP_{avg} values were calculated from ReP_{thr}, ReP_{PC20}, ReP_{PC50}, ReP_{PC80} and ReP_{PCmax} values (Table A.2.3). ReP_{EC50} values (Table A.2.3) were excluded from the ReP_{avg} calculation since these values can overestimate potency, especially when significant differences in maximal responses exist among the compounds being compared (Kennedy *et al.*, 1996) as observed in the present study.

PCB 77 significantly induced luciferase activity in cells transfected with the Japanese quail and 3C constructs at a concentration of 2065 nM, but not in constructs 3A and 3B, making it at least 210 times less potent than TCDD in type 3 AHR1 constructs. Both PCB 77 and 118-P have similar ReP_{thr} values for the Japanese quail construct and although PCB 77 induced a greater response (13%) than PCB 118-P (3%), it is difficult to say whether PCB 77 is significantly more potent than PCB 118-P in this construct. PCB 105-P, 105-RG, 118-P and 118-RG failed to significantly induce luciferase activity in cells transfected with several type 2 and type 3 constructs. In these cases, both purified and reagent grade solutions of PCB 105 and 118 were determined to be at least 10,000 times or 300 to 3000 times less potent than TCDD in type 2 and type 3 constructs, respectively and no distinction could be made between the potency of PCB 105 and 118 in these constructs. However, either PCB 118-P or PCB 118-RG significantly induced luciferase activity in cells transfected with the 2C, 2D, 2E, 2F, 2G and Japanese quail constructs. Therefore, PCB 118 may be a more potent inducer of *in vitro* AHR1-mediated transcriptional activation than PCB 105 in these constructs.

No considerable differences were noted in ReP values among AHR1 constructs for a given PCB congener. Average ReP values for PCB 126 in type 1, type 2 and type 3 constructs were 0.069, 0.040 and 0.18, respectively. Although ReP values for PCB 126 in type 3 constructs were 3 to 4 times higher relative to those for type 1 and type 2 constructs (Table 2.4), ReP_{avg} values for most constructs are comparable to the World Health Organization toxic equivalency factor (WHO-TEF) value of 0.1 for PCB 126 in birds (van den Berg *et al.*, 1998). All constructs had similar ReP values for PCB 77, however ReP_{avg} values for PCB 77 were at least an order of magnitude lower than the WHO-TEF value of 0.05 for PCB 77 in birds. These differences between LRG assay ReP values and WHO-TEFs are not surprising since TEFs are selected based on RePs for many endpoints and can span

several orders of magnitude (Haws *et al.*, 2006). The most notable difference between LRG assay results and the WHO-TEFs for birds is the finding that PCB 118 is equally potent or more potent than PCB 105 in the LRG assay, while the current WHO-TEFs for birds assume that PCB 118 is 10 times less potent than PCB 105. RePs obtained for PCB 105-P and 105-RG in the LRG assay were slightly lower than its WHO-TEF of 0.0001, or could not be precisely determined. However, ReP values for PCB 118-P and PCB 118-RG were greater or equal to the PCB 105 RePs (Table 2.4, Table A.2.3). These findings are in contrast with chicken egg injection studies where PCB 105 was found to cause embryolethality at much lower concentrations than PCB 118 (Brunström, 1990; Brunström *et al.*, 1990a). This could be due to reduced absorption of PCB 118 relative to PCB 105, as only 41.9% of PCB 118, compared to 81.9% of PCB 105, was retained in juvenile American kestrels (*Falco sparverius*) exposed to a 1:1:1 mixture of Aroclors 1248:1254:1260 from day 19 of incubation until 29 days post-hatch (Drouillard *et al.*, 2007). Increased metabolism and excretion of PCB 118 relative to 105 by embryos could also explain its decreased potency *in ovo*, however, PCB 105 and 118 had similar clearance rates in adult male American kestrels exposed to a 1:1:1 mixture of Aroclors 1248:1254:1260 for 120 days (Drouillard *et al.*, 2001).

Table 2.4. Potency of PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG relative to TCDD in wild-type (chicken, ring-necked pheasant and Japanese quail) and mutant (1B-C, 2A-G and 3A-C) AHR1 constructs. The average relative potency (ReP_{avg}), calculated from $EC_{threshold}$, PC_{20}, PC_{50}, PC_{80} and PC_{max}-based ReP values, is presented. If no induction of luciferase activity was observed, ReP values were estimated by dividing the TCDD value by the highest concentration tested (2065 nM for PCB 77 and 3000 nM for all other compounds).

AHR1	**ReP_{avg}**						
	TCDD	**PCB 126**	**PCB 77**	**PCB 105-P**	**PCB 105-RG**	**PCB 118-P**	**PCB 118-RG**
chicken (1A)[a]	1.0	0.074	0.0021	0.000030	0.000030	0.000062	0.000069
1B	1.0	0.066	0.0019	0.000030	0.000030	0.00011	0.000038
1C	1.0	0.067	0.0035	0.000030	0.000030	0.000047	0.000027
ring-necked pheasant (2G)[a]	1.0	0.037	0.0010	<0.00010	<0.00010	<0.00010	<0.00010
2A	1.0	0.038	0.0024	<0.00010	<0.00010	<0.00010	<0.00010
2B	1.0	0.041	0.0016	<0.00010	<0.00010	<0.00010	<0.00010
2C	1.0	0.045	0.0013	<0.00010	<0.00010	<0.00010	0.00010
2D	1.0	0.017	0.0013	<0.00010	<0.00010	<0.00010	0.00010
2E	1.0	0.039	0.0019	<0.00010	<0.00010	0.00010	0.00010
2F	1.0	0.062	0.0015	<0.00010	<0.00010	0.00010	<0.00010
2G	1.0	0.038	0.00073	<0.00010	<0.00010	<0.00010	0.00010
Japanese quail (3A)[a]	1.0	0.32	0.0048	<0.0033	<0.0033	0.0033	0.0033
3A	1.0	0.072	<0.00048	<0.00033	<0.00033	<0.00033	<0.00033
3B	1.0	0.18	<0.0048	<0.0033	<0.0033	<0.0033	<0.0033
3C	1.0	0.16	0.0048	<0.0033	<0.0033	<0.0033	<0.0033

[a] Wild-type AHR1 constructs containing the full length sequence for the chicken, ring-necked pheasant or Japanese quail AHR1.

ReP values for PCB 105-P, 105-RG, 118-P and 118-RG from the LRG assay were similar to those measured from EROD activity in avian embryo hepatocyte cultures (Kennedy *et al.*, 1996). PCB 118 induced EROD activity in 7 of the 9 species tested, whereas PCB 105 induced EROD activity in only 3 of these species. Furthermore, maximal responses for EROD activity were higher for PCB 118 than for PCB 105, as was seen with the LRG assay, and PC_{10}[2]-based ReP values for PCB 105 and 118 were equal (Kennedy *et al.*, 1996). The effects of PCB 118 and 105 on EROD activity in avian hepatocyte cultures are consistent with results obtained from the LRG assay and support the finding that PCB 118 is either equally potent or more potent than PCB 105 at inducing AHR1-mediated transactivation *in vitro*.

2.4.2.3 ReP of purified vs. reagent-grade mono-ortho PCB preparations

Contaminant levels were essentially equal in PCB 105-P and PCB 105-RG (Table 2.2). Concentration-response curves for both PCB 105 preparations were similar (Figure 2.2) and no differences were observed between their ReP values and maximal responses (Table 2.4, Table A.2.1, Table A.2.3) for type 1 AHR1 constructs. Although PCB 118-RG induced luciferase activity in cells transfected with twice as many type 2 constructs as PCB 118-P, the concentration-response curves did not differ significantly in cases where both preparations elicited significant increases in luciferase activity. Furthermore, PCB 118-P, but not PCB 118-RG, significantly induced luciferase activity in cells transfected with construct 2F. High levels of PCB 77 were detected in PCB 118-RG and at the highest concentration of PCB 118-RG (3000 nM), cells would have been exposed to approximately 8 nM PCB 77.

[2] Referred to as $EC_{TCDD10\%}$ in Kennedy *et al.* (1996).

This concentration of PCB 77 is within the observed range of $EC_{threshold}$ values for PCB 77 in type 1 constructs (Table A.2.1). However, the $EC_{threshold}$, PC_{20} and maximal responses (Table A.2.1) did not differ significantly between PCB 118-P and PCB 118-RG in type 1 constructs, indicating that the levels of PCB 77 found in PCB 118-RG did not cause an overestimation of PCB 118 potency in the LRG assay.

2.4.3 Relationship between AHR1-mediated luciferase reporter gene activity and in ovo LD_{50} values

Linear regression analyses comparing log-transformed LD_{50} values and log-transformed EC_{50}, $EC_{threshold}$ and PC_{20} values were performed to assess the ability of the LRG assay to predict the sensitivity of different avian species to DLC-induced embryolethality. LD_{50} values used in regression analyses were obtained from the literature and are presented in Table 2.5. As shown in Figure 2.3, $logLD_{50}$ values were associated with $logEC_{50}$, $logEC_{threshold}$ and $logPC_{20}$ values and significant relationships ($p < 0.0001$) with R^2 values of 0.90, 0.87 and 0.93 were observed. In Figure 2.3b, the TCDD $EC_{threshold}$ values for the wild-type Japanese quail construct (10 nM) and mutant 3A construct (1 nM), differed by an order of magnitude. However, the lower 3A $EC_{threshold}$ was due to lower variability among replicates relative to the Japanese quail and other type 3 constructs and does not indicate that the 3A construct is more sensitive to TCDD. This is supported by the observation that EC_{50} and PC_{20} values were similar among type 3 constructs (Figure 2.3, Table A.2.1).

Table 2.5. *In ovo* LD_{50} values for TCDD and PCBs used in linear regression analyses.

Species (AHR1 subtype)	Compound	LD_{50} (ng/g egg)	LD_{50} (pmol/g egg)	Reference
chicken (1A)	TCDD	0.21	0.65	(Cohen-Barnhouse *et al.*, 2011)
	PCB 126	1.1[a]	3.4	(Head *et al.*, 2008)
	PCB 77	8.6	29	(Brunström and Andersson, 1988)
	PCB 105	2200	6739	(Brunström, 1990)
	PCB 118	8000[b]	24507	(Brunström, 1990)
wild turkey (2A)	PCB 126	29[c]	88	(Brunström, 1989)
	PCB 77	800[b]	2740	(Brunström and Lund, 1988)
eastern bluebird (2E)	TCDD	4.8[d]	15	(Thiel *et al.*, 1988)
bobwhite quail (2G)	PCB 126	24	74	(Hoffman *et al.*, 1996)
ring-necked pheasant (2G)	TCDD	1.2	3.5	(Cohen-Barnhouse *et al.*, 2011)
Japanese quail (3A)	TCDD	9.7	30	(Cohen-Barnhouse *et al.*, 2011)
American kestrel (3B)	PCB 126	65	199	(Hoffman *et al.*, 1998)
common tern (3B)	PCB 126	104	351	(Hoffman *et al.*, 1998)
double-crested cormorant (3B)	TCDD	4	12	(Powell *et al.*, 1998)
	PCB 126	177	542	(Powell *et al.*, 1998)

[a] LD_{50} calculated based on a review of several egg injection studies (Head *et al.*, 2008).
[b] LD_{50} taken from Giesy *et al.* (2006).
[c] LD_{50} derived by linear interpolation between the LOAEL (20 ng/g; 36% mortality) and the LD_{100} (60 ng/g; 100% mortality) (Head and Kennedy, 2010).
[d] LD_{50} derived by linear interpolation between the LOAEL (1 ng/g, 14.3% mortality) and the LD_{100} (10 ng/g; 100% mortality).

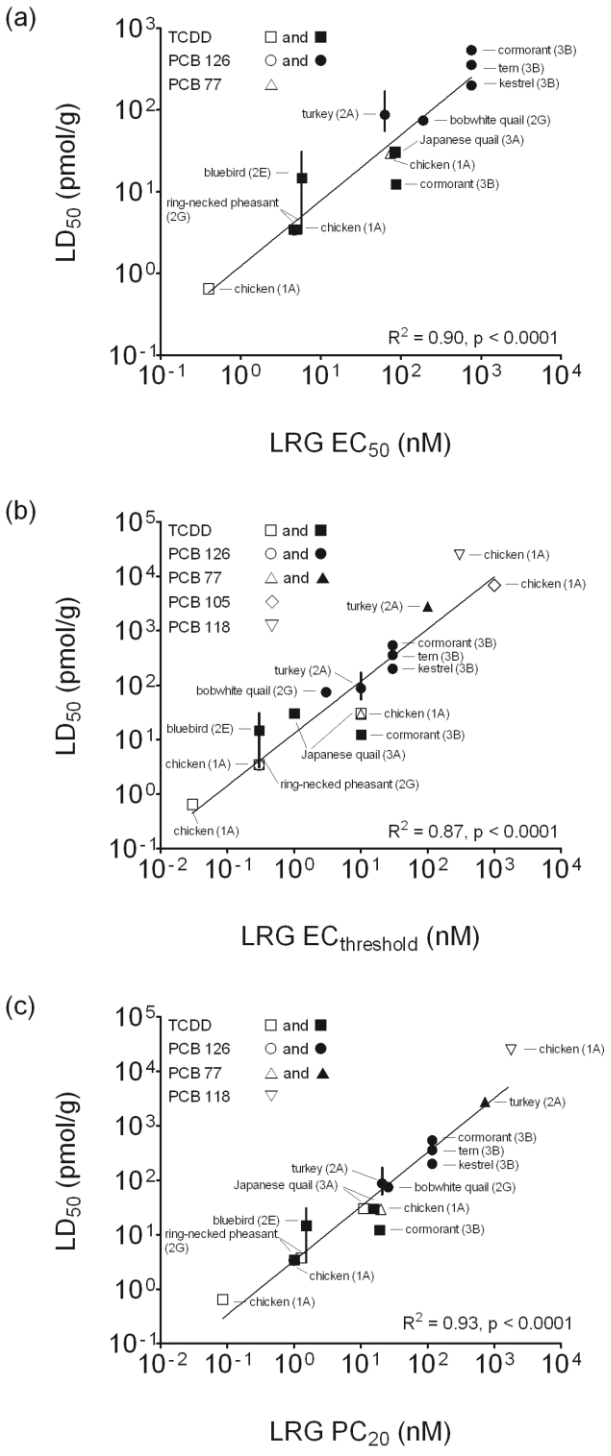

Figure 2.3. Linear regression analyses comparing LD_{50} values with (a) EC_{50} ($logLD_{50} = 0.80logEC_{50} + 0.09$), (b) $EC_{threshold}$ ($logLD_{50} = 0.96logEC_{threshold} + 1.11$) and (c) PC_{20} ($logLD_{50} = 0.99logPC_{20} + 0.53$) values derived from luciferase reporter gene (LRG) assay concentration-response curves. Open symbols represent LRG data from wild-type chicken, ring-necked pheasant or Japanese quail AHR1 constructs. Closed symbols represent LRG data from mutant AHR1 constructs. LRG data from PCB 105-P and PCB 118-P were included in regression analyses. LD_{50} values for TCDD in the bluebird and PCB 126 in the turkey were derived by linear interpolation between LOEL and LD_{100} values (Table 2.5), which is shown using error bars.

The development of *in vitro* techniques that can be used to predict the toxic effects of DLCs in wild birds is an important research goal in our laboratory. Since the results of the LRG assay indicate that birds fall into one of three main classes of sensitivity to PCBs, average EC_{50}, $EC_{threshold}$ and PC_{20} values were calculated for each main AHR1 type and DLC treatment and inputted into the corresponding linear regression model. The range of LD_{50} values predicted by the three regression models are provided in Table 2.6. Predicted LD_{50} values were similar among the three models and were generally consistent with the results of other egg injection studies investigating DLC-induced embryolethality that were not used to generate the regression models. For instance, the wood duck (*Aix sponsa*) was classified as type 3C based on its AHR1 LBD (Table 2.1) (Farmahin *et al.*, in press) and thus, the predicted TCDD LD_{50} is between 14 and 28 ng/g (Table 2.6). This predicted LD_{50} is in accordance with a no observable adverse effect level (NOAEL) of 4.6 ng/g reported for wood duck embryos exposed to TCDD (Augspurger *et al.*, 2008). Predicted LD_{50} values for PCB 77 in type 3 species (> 161 to 6310 ng/g) were also consistent with a reported LD_{50} of 316 ng/g obtained from an American kestrel egg injection study (Hoffman *et al.*, 1998). Although the predicted LD_{50} for PCB 126 in type 3 species was between 91 and 125 ng/g, no effects on mortality were observed in Japanese quail embryos exposed to 240 ng/g PCB 126 (Brunström and Halldin, 1998). This could indicate that other factors in addition to the AHR1 genotype, such as interspecies differences in the accumulation, distribution and metabolism of PCBs *in vivo* (Kubota *et al.*, 2006), are responsible for differences in avian species sensitivity to DLCs. Variability in experimental factors during egg injection studies, including the vehicle used to administer chemicals to the egg, the developmental stage at which embryos are exposed, the site of chemical injection and the orientation of eggs during incubation, could have also affected the LD_{50} value obtained (Heinz *et al.*, 2006). Overall,

the regression models give reasonable estimates of PCB LD_{50} values and could be used to predict PCB embryolethality in wild avian species as part of risk assessments at PCB-contaminated sites.

Table 2.6. *In ovo* LD_{50} values predicted from linear regression models between $logLD_{50}$ values and $logEC_{50}$ ($logLD_{50} = 0.80logEC_{50} + 0.09$), $logEC_{threshold}$ ($logLD_{50} = 0.96logEC_{threshold} + 1.11$) and $logPC_{20}$ values ($logLD_{50} = 0.99logPC_{20} + 0.53$) from the luciferase reporter gene (LRG) assay. Average EC_{50}, $EC_{threshold}$ and PC_{20} for type 1, 2 and 3 AHR1 constructs were calculated and used to estimate LD_{50} values for each DLC tested. The range of LD_{50} values predicted from the 3 linear regression models is presented.

Compound	AHR1 type	Average EC_{50} (nM)	Average EC_{thr}[a] (nM)	Average PC_{20} (nM)	LD_{50} range (ng/g)
TCDD	type 1	0.39	0.030	0.082	0.092-0.19
	type 2	7.3	0.30	1.3	1.3-1.9
	type 3	84	7.8	16	14-28
PCB 126	type 1	4.9	0.30	1.0	1.1-1.4
	type 2	103	6.5	24	16-26
	type 3	1306	25	114	91-125
PCB 77	type 1	75	7.7	25	11-29
	type 2	NC[b]	150	1621	509-1490
	type 3	>2065	2065	N/A[c]	>161-6310
PCB 105	type 1	788	1000	N/A	83-3150
	type 2	>3000	>3000	N/A	>243->9030
	type 3	>3000	>3000	N/A	>243->9030
PCB 118	type 1	550	300	1973	63-3060
	type 2	>3000	3000	N/A	>243-9030
	type 3	>3000	3000	N/A	>243-9030

[a] EC_{thr}, $EC_{threshold}$
[b] NC: EC_{50} not calculated since maximal response was not reached, but significant induction of luciferase activity was observed.
[c] N/A: not applicable since response less than 20% of positive control response.

2.4.4 General conclusions

Based on the LRG assay results, the amino acids at positions 324 and 380 in the AHR1 LBD are the major determinants of avian species sensitivity to PCBs (this study), PCDDs and PCDFs (Farmahin *et al.*, in press; Farmahin *et al.*, 2012), whereas amino acids at positions 256, 257, 297 and 337 do not appear to play any role in determining species sensitivity to DLCs. This indicates that the AHR1 LBD sequence alone could be used to predict DLC-induced embryolethality in a given species of bird. Egg injection studies in additional type 1, type 2 and type 3 species should be conducted to fully test this hypothesis.

The relative potency of PCBs did not differ significantly among AHR1 constructs and the LRG assay ReP values were generally similar to WHO-TEFs, with the notable exception that PCB 118 was equipotent or possibly more potent than PCB 105 *in vitro*. However, PCB 105 may still be more toxic than PCB 118 *in vivo* due to increased absorption or retention of PCB 105 (Drouillard *et al.*, 2007). Finally, regression analyses between LD_{50} values and LRG assay endpoints support the use of the LRG assay as a tool for predicting DLC-induced embryolethality in several avian species. Using the LRG assay along with the knowledge of a species' AHR1 genotype, the LD_{50} values of PCBs can be predicted in any avian species of interest without requiring large numbers of individuals.

CHAPTER 3: CYTOCHROME P4501A INDUCTION IN AVIAN HEPATOCYTE CULTURES EXPOSED TO POLYCHLORINATED BIPHENYLS: COMPARISONS WITH AHR1-MEDIATED REPORTER GENE ACTIVITY AND *IN OVO* TOXICITY[3]

3.1 Abstract

Avian-specific toxic equivalency factors (TEFs) were developed by the World Health Organization to simplify environmental risk assessments of dioxin-like compounds (DLCs), but TEFs do not account for differences in the toxic and biochemical potencies of DLCs among species of birds. Such variability may be due to differences in species sensitivity to individual DLCs. The sensitivity of avian species to DLCs was recently associated with the identity of amino acids 324 and 380 in the aryl hydrocarbon receptor 1 ligand binding domain (AHR1 LBD). A luciferase reporter gene (LRG) assay, measuring AHR1-mediated induction of a cytochrome P450 1A5 (CYP1A5) reporter gene, in combination with a species' AHR1 LBD sequence, were also shown to predict avian species sensitivity to polychlorinated biphenyls (PCBs) and PCB relative potency in a given species. The goals of the present study were to (1) measure induction of ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A4/5 mRNA by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and PCBs 126, 77, 105 and 118 in chicken, ring-necked pheasant and Japanese quail embryo hepatocytes and (2) compare these *in vitro* results to those previously generated by the LRG assay and *in ovo* toxicity studies. EROD activity and CYP1A4/5 mRNA expression data support and complement the findings of the LRG assay. CYP1A enzyme activity and mRNA expression were significantly correlated both with luciferase activity and *in ovo* toxicity

[3] Adapted from Manning G.E., Mundy L.J., Crump D, Jones S.P., Chiu S., Klein J., Konstantinov A., Potter D., Kennedy S.W. (2013). Cytochrome P4501A induction in avian hepatocyte cultures exposed to polychlorinated biphenyls: Comparisons with AHR1-mediated reporter gene activity and *in ovo* toxicity. *Toxicol. Appl. Pharmacol.* **266**(1): 38-47.

induced by PCBs. Relative potency values were generally similar between the LRG and EROD assays and indicate that the relative potency of some PCBs may differ among species.

3.2 Introduction

Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and certain polychlorinated biphenyl (PCB) congeners, are a group of structurally-related chemicals that cause toxicity subsequent to binding and activation of the aryl hydrocarbon receptor (AHR) (Denison *et al.*, 2011; Okey, 2007). Because PCDDs, PCDFs and PCBs exist as complex mixtures of congeners within environmental and biological samples, the toxic equivalents (TEQ) approach was developed by the World Health Organization (WHO) to simplify risk assessment of DLC mixtures (van den Berg *et al.*, 1998). Under this framework, toxic equivalency factors (TEFs) are used to represent the toxic potency of a DLC relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Relative potency (ReP) values from the literature were evaluated by an expert panel to derive TEFs specific to mammals, fish and birds for each DLC (van den Berg *et al.*, 1998); however, these TEFs do not consider differences in DLC potency among species within these classes of animals. For example, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is equipotent to TCDD in the chicken (*Gallus gallus domesticus*) but it is up to 6 and 30 times more potent than TCDD in the ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*), respectively (Cohen-Barnhouse *et al.*, 2011; Farmahin *et al.*, 2012; Hervé *et al.*, 2010).

Differences in the relative potency of PeCDF observed among species are likely related to differences in species sensitivity to individual DLCs, as Japanese quail embryos were 45 times less sensitive to TCDD but only 7 times less sensitive to PeCDF when compared to

chicken embryos (Cohen-Barnhouse *et al.*, 2011). Avian species sensitivity to DLCs has been associated with the identity of amino acids at sites 324 and 380 within the AHR1 ligand binding domain (LBD) (Farmahin *et al.*, 2012; Karchner *et al.*, 2006), and the results of AHR1 LBD sequencing of 86 species of birds indicates that species can be divided into three main groups based on their AHR1 genotype: type 1 (Ile324_Ser380), type 2 (Ile324_Ala380) and type 3 (Val324_Ala380) (Farmahin *et al.*, in press; Head *et al.*, 2008).

In vitro measures of AHR1 activation, such as cytochrome P4501A (CYP1A) induction, are good predictors of overt DLC toxicity (Head and Kennedy, 2010). Relative to egg injection studies, the use of primary avian hepatocyte cultures for measuring CYP1A enzyme activity or mRNA expression significantly reduces the number of animals required for testing the effects of DLCs. A luciferase reporter gene (LRG) assay, which measures AHR1-mediated induction of a CYP1A5 reporter gene, in combination with the knowledge of a species' AHR1 genotype has recently been shown to predict DLC toxicity in potentially any avian species and requires the lethal sampling of even fewer individuals than *in vitro* hepatocyte screening studies (Farmahin *et al.*, in press).

The goals of the present study were (1) to measure ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A4/5 mRNA expression induced by TCDD, PCB 126, PCB 77, PCB 105 and PCB 118 in primary hepatocyte cultures from three domestic species representative of each major AHR1 type, namely chicken (type 1), ring-necked pheasant (type 2) and Japanese quail (type 3), and (2) to calculate relative species sensitivity and relative PCB potency among the three species for comparison with results from the LRG assay presented in Chapter 2 and *in ovo* toxicity studies from the literature. In addition, it was previously suggested that contamination of mono-*ortho* PCB solutions with more potent AHR agonists results in overestimation of their ReP values (Peters *et al.*, 2006; van den Berg

et al., 2006). Therefore, the effects of reagent-grade and purified solutions of PCB 105 and 118 on EROD activity and CYP1A4/5 mRNA expression were also compared.

3.3 Materials and methods

3.3.1 Purification of mono-ortho PCBs

A complete description of the purification of PCB 105 and 118 is provided in Chapter 2. In brief, purified crystalline, reagent-grade samples of PCB 105 and 118, hereafter referred to as PCB 105-RG and 118-RG, were obtained by Wellington Laboratories (Guelph, ON, Canada) and analyzed by high resolution gas chromatography/low resolution mass spectrometry (HRGC/LRMS) to confirm their identity and purity. PCB 105-RG and 118-RG were purified by thin layer chromatography (TLC) and carbon column chromatography to obtain purified crystalline samples of PCB 105 and 118, hereafter referred to as PCB 105-P and PCB 118-P.

3.3.2 Preparation and analysis of TCDD and PCB solutions

3.3.2.1 Chicken and Japanese quail studies

Serial dilutions of TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG were prepared from dimethyl sulfoxide (DMSO) stock solutions as described in Chapter 2.

3.3.2.2 Ring-necked pheasant study

Serial dilutions were prepared from TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG stock solutions. The TCDD and PCB 77 stock solutions used in the chicken and Japanese quail studies were also used in the ring-necked pheasant study. PCB 105-RG

(AccuStandard, New Haven, CT, USA; lot # 101905AG-AC; purity stated to be 100%), PCB 118-RG (AccuStandard; lot # 032494; purity stated to be 100%) and PCB 126 (AccuStandard, lot #061204MS-AC; 99.7% pure) were weighed (0.5-1.0 mg) and stock solutions were prepared in DMSO. The nominal concentrations of the stock solutions were 83, 160 and 153 μg/ml for PCB 105-RG, 118-RG and 126, respectively.

3.3.3 Preparation and dosing of cultured hepatocytes

Primary cultures of hepatocytes were prepared from avian embryos using methods described elsewhere (Kennedy *et al.*, 1995) and included subsequent modifications (Head and Kennedy, 2007b; Kennedy *et al.*, 2003). All procedures were conducted according to protocols approved by the Animal Care Committee at the National Wildlife Research Centre and followed the guidelines of the Canadian Council on Animal Care. Reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Fertilized, unincubated chicken, ring-necked pheasant and Japanese quail eggs were obtained from the Canadian Food Inspection Agency (Ottawa, Ontario, Canada), Couvoir Simetin (Mirabel, QC, Canada) and Crazy Quails (Oshawa, Ontario, Canada), respectively. Eggs were incubated at 37.5°C and 60% relative humidity until 1-3 days prehatch (19, 23 and 16 days for chicken, ring-necked pheasant and Japanese quail, respectively). Eggs were candled periodically and infertile eggs or eggs containing dead embryos were discarded. In brief, 61 chicken, 51 ring-necked pheasant and 125 Japanese quail embryos were euthanized by decapitation, and livers were removed, pooled and digested with collagenase (Head *et al.*, 2006). Percoll (Amersham Bioscience, Uppsala, Sweden) density gradient centrifugation was used to separate erythrocytes from hepatocytes, and DNAse (Roche, Laval, Quebec, Canada) treatment was carried out to prevent cell clumping. Cells were plated in 48-well culture

plates by adding 25 µl of the cell suspension to 500 µl of cell culture medium 199, supplemented with insulin (1 µg/ml) and thyroxine (1 µg/ml), and incubated for 24 hours at 37°C in a humidified incubator with 5% CO_2. After 24 hours, cells were treated in triplicate with in-well concentrations of TCDD ranging from 0.0003 to 10 nM, and concentrations of PCB 126, 77, 105 and 118 ranging from 0.03 to 3000 nM. After another 24 hour incubation period, plates to be used for real-time quantitative PCR (QPCR) analysis were flash-frozen in powdered dry ice and stored at -80°C until the time of analysis. Plates used for EROD assays were rinsed with approximately 200 µl/well of phosphate-buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA) prior to being flash-frozen.

3.3.4 Cell viability

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

3.3.5 Ethoxyresorufin O-deethylase (EROD) assay

A detailed description of the EROD assay is provided elsewhere (Head and Kennedy, 2007b; Kennedy *et al.*, 1995). In brief, 3 replicate plates of hepatocytes per chemical were incubated at 37.5°C in the presence of nicotinamide adenine dinucleotide phosphate (NADPH, reduced) and 7-ethoxyresorufin for 7 minutes. Reactions were stopped by the addition of cold acetonitrile containing fluorescamine. Standard curves of resorufin and protein were prepared on each 48-well plate for each run. Plates were analyzed for both EROD activity (excitation wavelength: 530 nm, emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm, emission wavelength 460 nm) using a Cytofluor 2350 fluorescence plate reader.

3.3.6 RNA isolation and complementary DNA synthesis

Total RNA was extracted from 48-well plates using RNeasy 96 kits (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions with modifications described elsewhere (Head and Kennedy, 2007a). An on-column DNAse treatment was performed and, to ensure the maximal removal of genomic DNA, total RNA was treated a second time with DNAse from the Ambion TURBO DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. Total RNA was reverse transcribed to complementary DNA (cDNA) with Superscript II Reverse Transcriptase and random hexamers (Invitrogen, Burlington, Ontario, Canada) as per manufacturer's instructions. From each plate, a control without reverse transcriptase enzyme (No-RT control) was included to verify the absence of genomic DNA in the RNA template.

3.3.7 Quantitative Polymerase Chain Reaction (QPCR)

Multiplex QPCR assays using dual-labelled fluorescent hydrolysis probes (Hervé *et al.*, 2010) were used to quantify CYP1A4, CYP1A5 and β-actin (normalizer gene) mRNA abundance in chicken and Japanese quail embryo hepatocytes exposed to TCDD and PCBs. With the exception of primers and probes, all QPCR reactions were carried out using reagents from Stratagene and either the Stratagene Mx3000P or Mx3005 instrument (Stratagene, La Jolla, CA, USA). All primers (Invitrogen) and probes (Biosearch, Novato, CA, USA) were designed and validated as previously described (Hervé *et al.*, 2010). Taqman reactions were performed using Brilliant Multiplex QPCR Mastermix kits (Agilent Technologies, Santa Clara, CA, USA). Each 25 µl reaction contained forward and reverse primers and probes for the genes of interest (CYP1A4, CYP1A5) and the normalizer gene (β-actin), 1x Multiplex QPCR master mix, 30 nM reference dye (ROX) and 5 µl of cDNA. The thermal profile for all reactions was as follows: 10 minutes at 95°C, followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. Data were collected at the end of the 60°C phase. Each assay was performed once with samples in duplicate. Fold changes of CYP1A4/5 mRNA expression in TCDD- and PCB-treated hepatocytes were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

3.3.8 EROD and CYP1A4/5 mRNA data analysis

Fluorescence data were imported into GraphPad (GraphPad Prism 5.0 software, San Diego, CA, USA) for curve fitting. EROD activity data measured in chicken embryo hepatocytes (CEH), ring-necked pheasant embryo hepatocytes (PEH) and Japanese quail embryo hepatocytes (JEH) were fit to a modified Gaussian curve as explained elsewhere (Kennedy *et al.*, 1993). For each chemical, three EROD curves were generated from three

separate cell culture plates. The TCDD and PCB concentrations that elicited a response equal to 20, 50 and 80% of the TCDD maximal response in a given species ($TCDD_{20}$, $TCDD_{50}$ and $TCDD_{80}$), EC_{50} and maximal response values are presented as the mean value of replicates obtained from three 48-well plates ± standard error (SE). $EC_{threshold}$ values, which represent the concentration of a compound that elicited a significantly greater EROD response than DMSO-treated cells, were determined from concentration-response curves averaged from 3 replicate curves using a one-way analysis of variance (ANOVA) ($p < 0.05$) with a Dunnett's post-hoc test ($p < 0.05$). Only the concentrations that elicited a response less than 20% of the TCDD maximal response were included in the statistical analysis.

CYP1A4/5 mRNA expression data measured in CEH and JEH were fitted to a four-parameter logistic model as described previously (Head and Kennedy, 2007b). The equation integrates the hillslope, EC_{50}, baseline response and maximal response as parameters. A single curve fit was generated for induction of CYP1A4/5 mRNA expression using data from the average of three wells from the same cell culture plate, assessed in duplicate. EC_{50} ± SE, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$ and maximal responses ± SE were calculated from the curve fit. $EC_{threshold}$ values for CYP1A4/5 mRNA expression were determined from a single concentration-response curve using a one-way analysis of variance (ANOVA) ($p < 0.05$) with a Dunnett's post-hoc test ($p < 0.05$). Only the concentrations that elicited a response less than 20% of the TCDD maximal response were included in the statistical analysis.

Statistical differences among EC_{50}, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$ and maximal response values for EROD activity or CYP1A4/5 mRNA expression were determined by performing a one-way ANOVA ($p < 0.05$) followed by Tukey's post-hoc test ($p < 0.05$).

3.3.9 Calculation of relative sensitivity (ReS) and relative potency (ReP)

Since the chicken is considered to be the most sensitive avian species to the toxic and biochemical effects of DLCs (Head *et al.*, 2008; Hervé *et al.*, 2010), the sensitivity of the ring-necked pheasant and Japanese quail were expressed relative to chicken for EROD activity and/or CYP1A4/5 mRNA expression. Full concentration-response curves were not always obtained, and in these cases an EC_{50} value could not be calculated. Therefore, $EC_{threshold}$ values were used to calculate ReS values for each species and endpoint measured. For concentration-response curves that did achieve a maximal response, EC_{50}-based ReS values were also calculated for comparison to $EC_{threshold}$-based ReS values (Table A.3.1). ReS values were calculated as follows: EC_{50} or $EC_{threshold}$ of compound *x* in chicken ÷ EC_{50} or $EC_{threshold}$ of compound *x* in species of interest.

The maximal responses and slopes of concentration-response curves differed among DLC treatments. Therefore, the potency of PCBs was measured relative to TCDD using the systematic framework proposed by Villeneuve *et al.* (2000) with some modifications. ReP values based on $TCDD_{20}$, $TCDD_{50}$ and $TCDD_{80}$ values obtained from the Gaussian curves or four parameter logistic models (ReP_{TCDD20}, ReP_{TCDD50} and ReP_{TCDD80}) were calculated. If the maximal response or highest observed response of the PCB congener was less than 80% of the TCDD maximal response, a ReP estimate corresponding to the highest observed response for that PCB congener ($ReP_{TCDDmax}$) was also calculated. If the highest observed response was less than 20% of the TCDD maximal response, a ReP was calculated based on $EC_{threshold}$ values. ReP values were calculated as follows: EC_{50}, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ or $EC_{threshold}$ of TCDD in species *x* ÷ EC_{50}, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ or $EC_{threshold}$ of the DLC congener of interest in species *x*.

3.4 Results and discussion

3.4.1 Concentration-dependent effects of TCDD and PCBs on CYP1A induction

All DLCs significantly induced EROD activity in a concentration-dependent manner in CEH, PEH and JEH (Figures 3.1 and 3.2). Maximal EROD activity was followed by a decrease in activity at higher DLC concentrations, but this decrease was not due to cytotoxicity, as measured by the Calcein-AM assay (data not shown). EC_{50}, $EC_{threshold}$, TCDD values and maximal responses associated with each EROD curve are presented in Table 3.1. Significant differences in EC_{50}, TCDD values and maximal EROD activity among DLC treatments are also indicated.

CYP1A4 and CYP1A5 mRNA were induced in a concentration-dependent manner by all DLCs tested in CEH and JEH (Figures 3.3 and 3.4). There were no significant differences in β-actin mRNA expression among hepatocytes treated with graded concentrations of TCDD or PCBs, indicating that changes in CYP1A4/5 mRNA expression were not a result of altered β-actin expression. EC_{50}, $EC_{threshold}$, TCDD values and maximal responses associated with each curve are presented in Table 3.2.

Significant differences in maximal EROD activity and CYP1A4/5 mRNA expression were observed both among DLCs in a given species and among species exposed to a given DLC (Tables 3.1 and 3.2, Figures 3.1 and 3.3). DLCs may induce different CYP1A responses due to ligand-specific differences in AHR conformation, resulting in altered co-activator recruitment and interactions with xenobiotic response elements (XREs) (Denison *et al.*, 2011; Hestermann *et al.*, 2000; Zhou *et al.*, 2003). However, differences in cell uptake or metabolism could also contribute to differences in maximal CYP1A responses among DLCs (Hestermann *et al.*, 2000). Interspecies variation in CYP1A expression may be due to differences in the AHR amino acid sequence, leading to differences in ligand-receptor

conformation (Abnet *et al.*, 1999; Zhou *et al.*, 2003). Alternatively, interspecies differences in CYP1A catalytic activity (Darwish *et al.*, 2010; Martignoni *et al.*, 2006) could explain differences in maximal EROD activity among species.

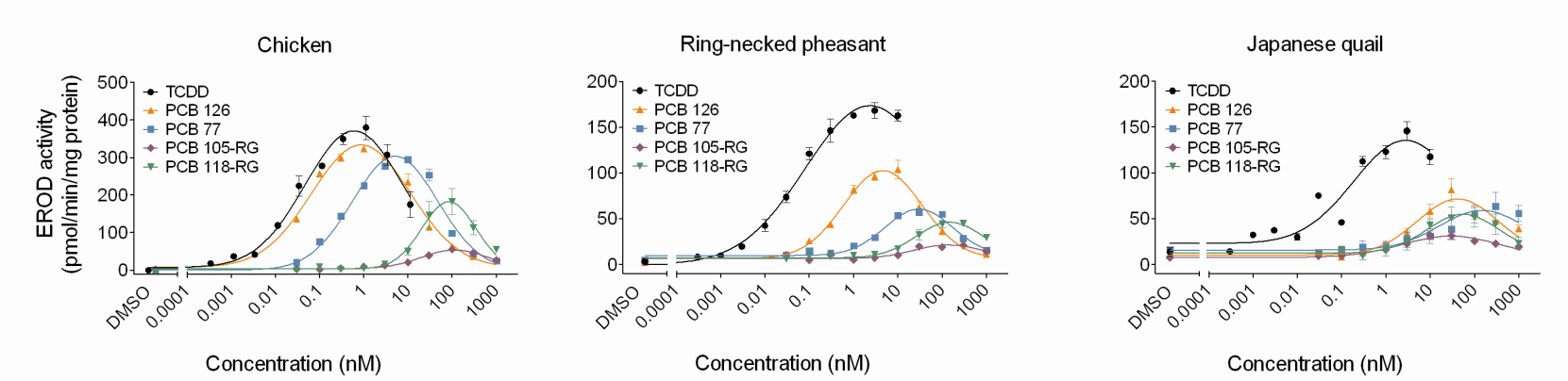

Figure 3.1. Concentration-dependent effects of TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG on EROD activity in chicken, ring-necked pheasant and Japanese quail embryo hepatocyte cultures exposed for 24 hours. Points represent mean EROD activity obtained from 3 replicate cell culture plates ± standard error. Each plate received triplicate doses of each concentration of TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG.

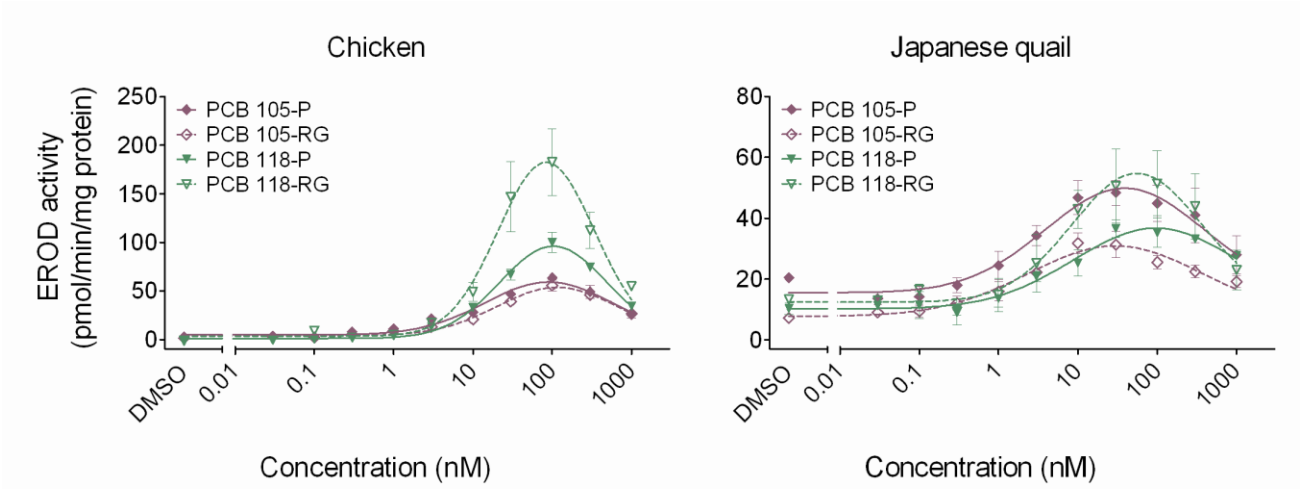

Figure 3.2. Concentration-dependent effects of reagent-grade (RG) versus purified (P) solutions of PCB 105 and PCB 118 on EROD activity in chicken and Japanese quail embryo hepatocyte cultures exposed for 24 hours. Points represent mean EROD activity obtained from 3 replicate cell culture plates ± standard error. Each plate received triplicate doses of each PCB concentration.

Table 3.1. EROD activity in chicken, ring-necked pheasant and Japanese quail embryo hepatocyte cultures exposed to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG for 24 h. EC_{50}, $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ and maximal responses represent the average of three replicates ± standard error (SE). $EC_{threshold}$ (EC_{thr}) values were determined from a single concentration-response curve averaged from 3 replicate curves using a one-way ANOVA ($p < 0.05$) followed by Dunnett's test ($p < 0.05$).

Species	Compound	EC_{50} ± SE (nM)	EC_{thr} (nM)	$TCDD_{20}$ ± SE (nM)	$TCDD_{50}$ ± SE (nM)	$TCDD_{80}$ ± SE (nM)	$TCDD_{max}$ ± SE (nM)	Maximal response ± SE (pmol/min/mg protein)
Chicken	TCDD	0.027±0.004[a]	0.00030	0.0058±0.0008[a]	0.028±0.004[a]	0.10±0.01[a]	N/A	373±14[a]
	PCB 126	0.043±0.001[a]	0.030	0.010±0.0004[a]	0.055±0.001[b]	0.26±0.01[b]	N/A	335±4[a]
	PCB 77	0.38±0.04[b]	0.030	0.12±0.01[b]	0.56±0.04[c]	2.85±0.12[c]	N/A	304±2[a]
	PCB 105-P	10.7±2.3[c]	3.00				N/A	59.8±4.4[b]
	PCB 105-RG	15.2±0.4[c]	10.0				N/A	53.8±4.3[b]
	PCB 118-P	16.5±1.1[c]	3.00	37.7±9.0[c]			55.1±14	96.4±9.3[b]
	PCB 118-RG	16.6±1.1[c]	10.0	16.7±6.0[c]	44.6±16[d]		40.8±13	183±39[c]
Ring-necked pheasant	TCDD	0.047±0.007[a]	0.0030	0.0063±0.002[a]	0.050±0.02[a]	0.31±0.1	N/A	174±7[a]
	PCB 126	0.43±0.06[b]	0.10	0.26±0.02[b]	1.73±0.18[b]		2.77±0.60	104±5[b]
	PCB 77	3.77±0.20[c]	1.00	6.88±1.0[c]			16.6±4.2	60.6±3.0[c]
	PCB 105-RG	17.4±2.2[d]	30.0				N/A	21.4±2.4[d]
	PCB 118-RG	20.5±4.1[d]	10.0	70.3±16[d]			88.0±32.8	46.6±2.5[c]
Japanese quail	TCDD	0.13±0.04[a]	0.030	0.028±0.01[a]	0.13±0.03[a]	0.60±0.2	N/A	137±10[a]
	PCB 126	3.79±0.73[b,c]	1.00	2.69±0.62[b]	10.5±0.7[b]		13.4±0.2	73.2±11[b]
	PCB 77	11.2±1.2[b]	100	15.6±5.0[b,c]			63.3±26	59.0±12[b,c]
	PCB 105-P	4.13±1.5[b,c]	3.00	5.95±1.8[b,c]			24.9±14	53.6±3.2[b,c]
	PCB 105-RG	1.66±0.52[c]	1.00	14.8±7.6[b,c]			24.2±15	31.2±1.7[c]
	PCB 118-P	6.57±1.4[b]	3.00	36.0±16[c]			37.2±8.7	37.3±3.0[b,c]
	PCB 118-RG	6.38±0.86[b]	10.0	9.01±3.2[b,c]			18.3±12	54.9±11[b,c]

Superscript letters indicate significant differences among treatments ($p < 0.05$) within each species.
N/A: not applicable since response was either above 80% or below 20% of TCDD maximal response
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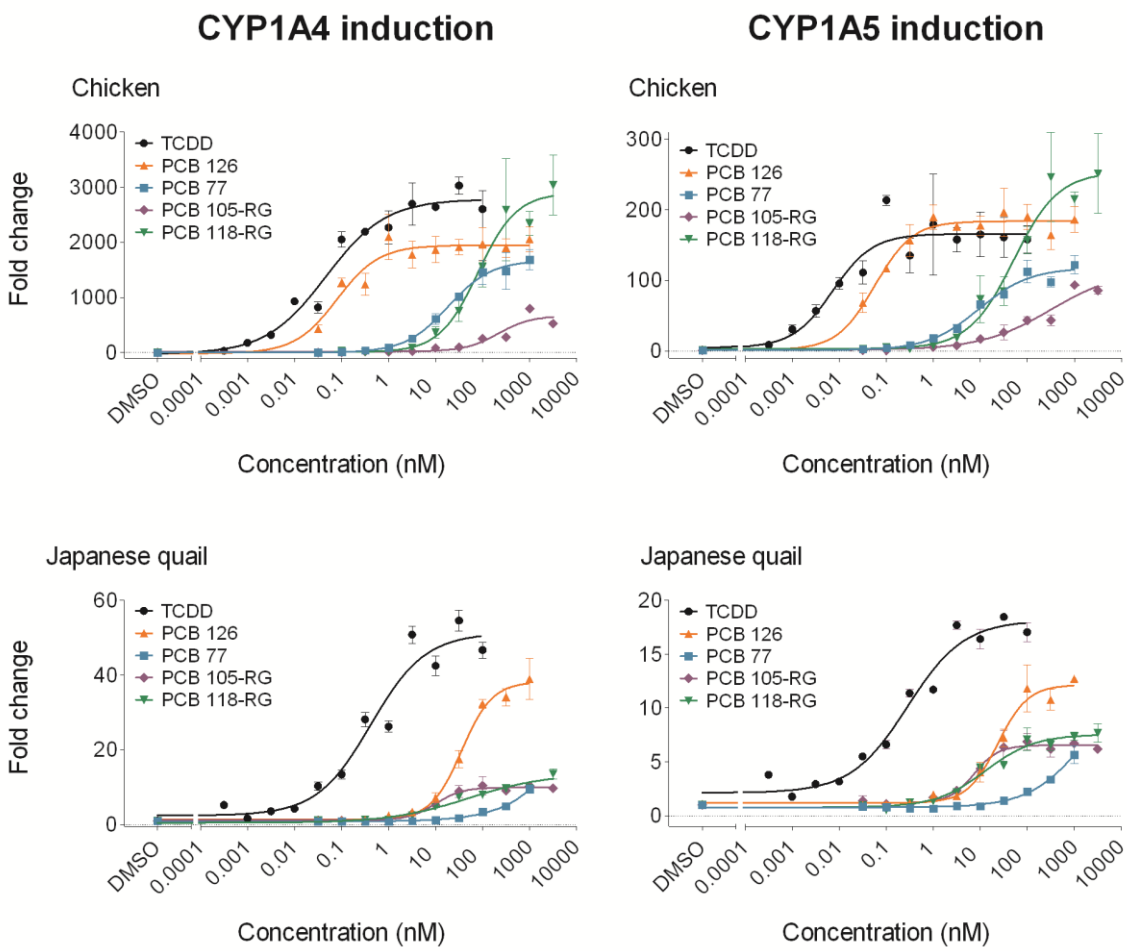

Figure 3.3. Concentration-dependent effects of TCDD, PCB 126, PCB 77, PCB 105-RG and PCB 118-RG on CYP1A4/5 mRNA expression in chicken and Japanese quail hepatocyte cultures exposed for 24 h. Points represent mean fold change of three replicate wells from the same cell culture plate, assessed in duplicate, ± standard error.

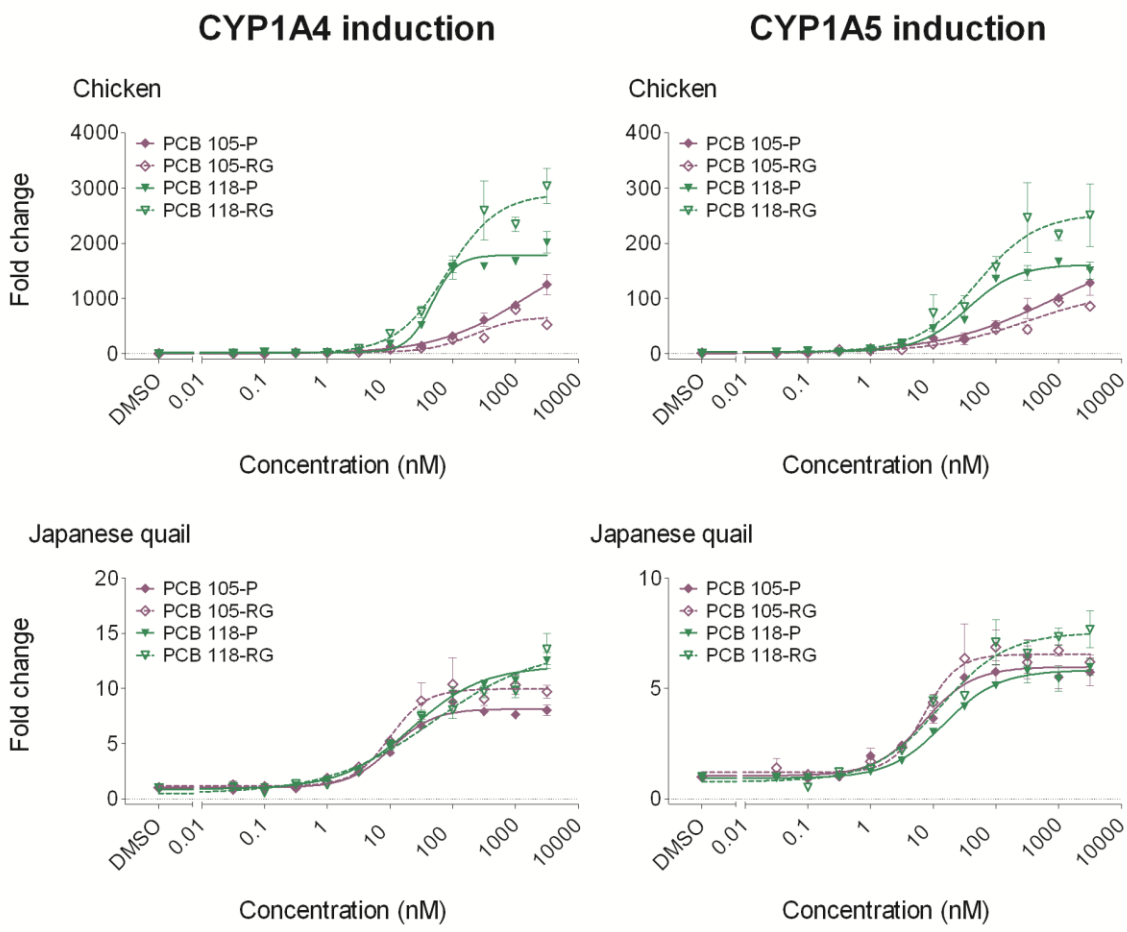

Figure 3.4. Concentration-dependent effects of reagent-grade (RG) versus purified (P) solutions of PCB 105 and PCB 118 on CYP1A4/5 mRNA expression in chicken and Japanese quail embryo hepatocyte cultures exposed for 24 hours. Points represent mean fold change of three replicate wells from the same cell culture plate, assessed in duplicate, ± standard error.

Table 3.2. CYP1A4/5 mRNA expression in chicken and Japanese quail embryo hepatocyte cultures exposed to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG for 24 h. EC_{50} ± standard error (SE), $TCDD_{20}$, $TCDD_{50}$, $TCDD_{80}$, $TCDD_{max}$ and maximal responses ± SE were calculated from the curve fit. $EC_{threshold}$ (EC_{thr}) values were calculated using a one-way ANOVA ($p < 0.05$) followed by Dunnett's test ($p < 0.05$).

Species	Compound	Endpoint	EC_{50} ± SE (nM)	EC_{thr} (nM)	$TCDD_{20}$ (nM)	$TCDD_{50}$ (nM)	$TCDD_{80}$ (nM)	$TCDD_{max}$ (nM)	Maximal response ± SE (fold change)
Chicken	TCDD	CYP1A4	0.046±1.4[a]	0.0010	0.0064	0.047	0.36	N/A	2780±130[a]
	PCB 126	CYP1A4	0.080±1.4[a]	0.030	0.032	0.21		6.43	1950±66[a,b]
	PCB 77	CYP1A4	18.6±1.2[b]	0.30	9.0	99.4		10000	1670±76[a,b]
	PCB 105-P	CYP1A4	1490±3[c]	3.00	295	5301		1200000	2040±710[a,b]
	PCB 105-RG	CYP1A4	215±1[b,c]	10.0	766			64500	669±72[b]
	PCB 118-P	CYP1A4	46.1±1.1[b]	10.0	31.7	80.8		949	1780±52[a,b]
	PCB 118-RG	CYP1A4	80.1±1.3[b]	10.0	20.4	73.8	246	N/A	2900±200[a]
Japanese quail	TCDD	CYP1A4	0.42±1.3[a]	0.0030	0.055	0.39	2.54	N/A	51.3±2.6[a]
	PCB 126	CYP1A4	35.8±1.2[b,c]	3.00	16.6	61.1		18200	38.2±1.8[b]
	PCB 77	CYP1A4	NC	100				N/A	9.47±1.9*[c]
	PCB 105-P	CYP1A4	10.5±1.1[b]	3.00				N/A	8.15±0.18[c]
	PCB 105-RG	CYP1A4	9.84±1.3[b]	10.0				N/A	9.95±0.45[c]
	PCB 118-P	CYP1A4	22.3±1.2[b,c]	10.0	477			1340	12.0±0.4[c]
	PCB 118-RG	CYP1A4	49.8±1.7[c]	10.0	871			119000	13.6±1.5[c]
Chicken	TCDD	CYP1A5	0.0072±1.8[a]	0.0010	0.0017	0.0068	0.025	N/A	166±11[a]
	PCB 126	CYP1A5	0.057±1.4[a]	0.030	0.014	0.047	0.14	N/A	184±7[a]
	PCB 77	CYP1A5	9.31±1.3[b]	1.00	3.0	28.6		2000	118±7[a]
	PCB 105-P	CYP1A5	842±6[c]	1.00	29.5	432	3911	N/A	197±79[a]
	PCB 105-RG	CYP1A5	309±2[b,c]	3.00	64.3	1559		327000	115±25[a]
	PCB 118-P	CYP1A5	35.7±1.2[b,c]	1.00	9.82	36.5	139	N/A	162±6[a]
	PCB 118-RG	CYP1A5	50.4±1.6[b,c]	1.00	5.81	22.0	54.4	N/A	254±27[a]
Japanese quail	TCDD	CYP1A5	0.28±1.3[a]	0.0030	0.024	0.23	1.71	N/A	18.2±0.6[a]
	PCB 126	CYP1A5	23.5±1.3[b]	10.0	12.3	56.3		4350	12.2±0.7[b]
	PCB 77	CYP1A5	NC	100	553			995	5.66±0.83*[c]
	PCB 105-P	CYP1A5	7.12±1.3[c]	1.00	14.4			28400	5.97±0.22[c]
	PCB 105-RG	CYP1A5	7.32±1.3[c]	3.00	9.43			114	6.54±0.27[c]
	PCB 118-P	CYP1A5	14.7±1.2[b,c]	3.00	37.3			4180	5.82±0.17[c]
	PCB 118-RG	CYP1A5	12.8±1.3[b,c]	10.0	24.9			23300	7.54±0.36[c]

Superscript letters indicate significant differences among treatments ($p < 0.05$) within each species.
* No maximal response was reached. Values represent highest observed response; NC: EC_{50} not calculated since maximal response was not reached
N/A: not applicable since response was either above 80% or below 20% of TCDD maximal response

3.4.2 Relative species sensitivity to CYP1A induction by DLCs

$EC_{threshold}$-based ReS values (ReS_{thr}) indicated that the chicken was the most sensitive species, the ring-necked pheasant was moderately sensitive and the Japanese quail was the least sensitive to induction of EROD activity by TCDD, PCB 126 and PCB 77 (Table 3.3). Similar trends were observed from EC_{50}-based ReS values. EC_{50} values for PCB 126 and 77 were significantly different among the three species (Table A.3.1). The EC_{50} value for TCDD in CEH was significantly lower than that in JEH; however the CEH and PEH EC_{50}s for TCDD were not significantly different. ReS_{thr} values for CYP1A4/5 mRNA also indicated that JEH were less sensitive than CEH to TCDD, PCB 126 and PCB 77 (Table 3.3, Table A.3.1). Similar results were obtained from EC_{50}-based ReS values; however, an EC_{50} could not be determined in JEH exposed to PCB 77 since no maximal response for CYP1A4/5 mRNA expression was achieved.

PEH and JEH were equally sensitive or more sensitive to PCB 105-P, 105-RG, 118-P or 118-RG than CEH based on ReS_{thr} values for EROD activity (Table 3.3). No differences were observed between EROD EC_{50} values for CEH and PEH dosed with PCB 105-RG or 118-RG, while EC_{50} values for PCB 105-RG, 118-P and 118-RG in JEH were significantly lower than those measured in CEH and PEH (Table A.3.1). CEH and JEH had similar sensitivities to induction of CYP1A4/5 mRNA expression by PCB 105-P, 105-RG, 118-P and 118-RG based on ReS_{thr} values and EC_{50}-based ReS values (Table 3.3, Table A.3.1).

Table 3.3. Relative sensitivity (ReS) and relative potency (ReP) values obtained from EROD activity, CYP1A4/5 mRNA expression and luciferase reporter gene (LRG) activity. $EC_{threshold}$-based ReS values (ReS_{thr}) for the ring-necked pheasant and Japanese quail were calculated relative to the chicken. Average relative potency values (ReP_{avg}) for PCB 126, 77, 105 and 118 relative to TCDD were calculated from $EC_{threshold}$-, $TCDD_{20}$-, $TCDD_{50}$-, $TCDD_{80}$- and $TCDD_{max}$-based ReP values.

Species	**Compound**	**ReS_{thr}**				**ReP_{avg}**			
		EROD	**CYP1A4**	**CYP1A5**	**LRG[a]**	**EROD**	**CYP1A4**	**CYP1A5**	**LRG[a]**
Chicken	TCDD	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	PCB 126	1.00	1.00	1.00	1.00	0.36	0.12	0.12	0.074
	PCB 77	1.00	1.00	1.00	1.00	0.035	0.0011	0.00046	0.0021
	PCB 105-P	1.00	1.00	1.00	1.00	0.00010	0.000091	0.00027	0.000030
	PCB 105-RG	1.00	1.00	1.00	1.00	0.000030	0.000036	0.000091	0.000030
	PCB 118-P	1.00	1.00	1.00	1.00	0.0010	0.00025	0.00039	0.000062
	PCB 118-RG	1.00	1.00	1.00	1.00	0.00041	0.00063	0.00052	0.000069
Ring-necked pheasant	TCDD	0.10			0.10	1.00			1.00
	PCB 126	0.30			0.030	0.022			0.037
	PCB 77	0.030			0.033	0.00085			0.0010
	PCB 105-RG	0.33			<0.33	0.000033			<0.00010
	PCB 118-RG	1.00			<0.10	0.000092			<0.00010
Japanese quail	TCDD	0.010	0.33	0.33	0.0030	1.00	1.00	1.00	1.00
	PCB 126	0.010	0.010	0.0030	0.030	0.016	0.0027	0.0016	0.32
	PCB 77	0.0010	0.0030	0.010	0.0048	0.0010	0.000030	0.000041	0.0048
	PCB 105-P	0.30	1.00	1.00	<0.33	0.0057	0.0010	0.0016	<0.0033
	PCB 105-RG	10.00	1.00	1.00	<0.33	0.011	0.00030	0.0014	<0.0033
	PCB 118-P	1.00	1.00	0.33	0.10	0.0039	0.00015	0.00055	0.0033
	PCB 118-RG	1.00	1.00	0.10	0.10	0.0030	0.00012	0.00043	0.0033

[a] LRG assay ReS and ReP values from Chapter 2.

Avian species sensitivity to dioxins, furans and PCBs has been related to the identity of amino acids at positions 324 and 380 within the AHR1 LBD (Cohen-Barnhouse *et al.*, 2011; Farmahin *et al.*, 2012; Head *et al.*, 2008; Hervé *et al.*, 2010; Karchner *et al.*, 2006). The results of AHR1 LBD sequencing for 86 avian species indicate that birds can be divided into one of 3 main groups based on the amino acids at these sites – type 1: Ile324_Ser380, type 2: Ile324_Ala380 and type 3: Val324_Ala380 (Farmahin *et al.*, in press; Head *et al.*, 2008). ReS values for PEH and JEH exposed to TCDD, PCB 126 and PCB 77 resembled ReS values obtained for *in ovo* toxicity in other type 2 and type 3 species, respectively (Head *et al.*, 2008). Thus, the results presented herein support previous findings that type 1 species (e.g. chicken) are the most sensitive to CYP1A induction by TCDD, PCB 126 and PCB 77, type 2 species (e.g. ring-necked pheasant) are moderately sensitive and type 3 species (e.g. Japanese quail) are the least sensitive (Head *et al.*, 2008). However, as was previously observed in avian embryo hepatocytes dosed with PeCDF and hexachlorobenzene (Hervé *et al.*, 2010; Mundy *et al.*, 2012; Mundy *et al.*, 2010), the ring-necked pheasant and Japanese quail were equally sensitive or more sensitive than the chicken to CYP1A induction by PCB 105 and 118. These results are in agreement with those of a previous study where EROD-based ReS values for PCB 105 and 118 in ring-necked pheasant, turkey (*Meleagris gallopavo*) (type 2 species) and duck (*Anas platyrhynchos*) (type 3 species) hepatocyte cultures were similar to those calculated for chicken hepatocyte cultures (Kennedy *et al.*, 1996). Ligand-dependent differences in binding with the different AHR1 types could explain such reversals in relative species sensitivity and could be determined by AHR1 binding assays or homology modeling studies.

3.4.3 Relative PCB potency in chicken, ring-necked pheasant and Japanese quail hepatocyte cultures

Based on average ReP values (ReP_{avg}) (Table 3.3), visual inspection of concentration-response curves (Figures 3.1 and 3.2) and the results of statistical analyses performed among EROD endpoints for different DLC treatments in a given species (Table 3.1), the rank order of DLC potency was: TCDD ≥ PCB 126 > PCB 77 > PCB 118-P ≈ PCB 118-RG ≥ PCB 105-P ≈ PCB 105-RG in CEH, TCDD > PCB 126 > PCB 77 > PCB 118-RG ≥ PCB 105-RG in PEH and TCDD > PCB 126 ≥ PCB 105-RG ≈ PCB 105-P ≈ PCB 118-RG ≈ PCB 118-P ≈ PCB 77 in JEH. The rank order of DLC potency for CYP1A4/5 mRNA expression based on ReP_{avg} values (Table 3.3), visual inspection of concentration-response curves (Figures 3.3 and 3.4) and the results of statistical analyses performed among mRNA expression endpoints for different DLC treatments in a given species (Table 3.2) was TCDD ≥ PCB 126 > PCB 77 ≥ PCB 118-RG ≈ PCB 118-P ≥ PCB 105-P ≈ PCB 105-RG in CEH and TCDD > PCB 126 ≥ PCB 105-P ≈ PCB 105-RG ≥ PCB 118-P ≈ PCB 118-RG > PCB 77 in JEH. ReP_{avg} values were calculated from $EC_{threshold}$-, $TCDD_{20}$-, $TCDD_{50}$-, $TCDD_{80}$- and $TCDD_{max}$-based ReP values. EC_{50}-based ReP values were excluded from the ReP_{avg} calculation since these values can often overestimate the potency of PCBs with lower maximal responses than TCDD (Kennedy *et al.*, 1996).

The rank order of DLC potency and the ReP values obtained from EROD and QPCR assays in chicken hepatocytes were generally similar to those suggested by the WHO-TEFs for birds (van den Berg *et al.*, 1998). However, the PCB 118 ReP was 1 to 2 orders of magnitude greater than its WHO-TEF of 0.00001, and therefore had approximately the same potency as PCB 105. This finding is consistent with the results of previous EROD studies where PCB 118 and PCB 105 were observed to be equally potent in avian hepatocyte

cultures from several species (Kennedy *et al.*, 1996). QPCR-based RePs for PCB 77 in CEH were 50 to 100 times lower than the WHO-TEF value of 0.05, however, this difference was not observed between the EROD-based ReP and TEF. QPCR-based RePs for PCB 77 were also 3 orders of magnitude lower than the corresponding EROD-based ReP in JEH. This could be due to higher degradation rates for CYP1A mRNA relative to CYP1A protein levels and CYP1A activity, which were previously observed in Sprague-Dawley rats, mummichog (*Fundulus heteroclitus*) and striped bass (*Morone saxatilis*) exposed to β-naphthoflavone (BNF) (Chen *et al.*, 2010; Durieux *et al.*, 2012; Kloepper-Sams and Stegeman, 1989). Treatment-related differences in CYP1A1 mRNA and protein level profiles were also observed in rainbow trout hepatocytes exposed to BNF and TCDD and were attributed to increased metabolism and inactivation of BNF, relative to TCDD (Pesonen *et al.*, 1992). Since PCB 77 is more easily metabolized than other DLCs, such as TCDD and PCB 126 (Bastien *et al.*, 1997), mRNA levels likely decline at a faster rate, which could explain why differences between EROD and QPCR-based ReP values are not observed to the same extent with the other PCBs tested.

EROD and QPCR-based RePs for PCB 126 in PEH and JEH were between 1 and 2 orders of magnitude lower than the ReP in CEH (Table 3.3) and WHO-TEF value of 0.1. LD_{50}-based ReP values for PCB 126 were also lower in the double-crested cormorant (*Phalacrocorax auritus*; type 3) than in the chicken; however the ReP values for each of these species were within one order of magnitude of each other (Farmahin *et al.*, in press; Powell *et al.*, 1996; Powell *et al.*, 1998). EROD-based RePs for PCB 77 in PEH and JEH were 1 to 2 orders of magnitude lower than the ReP in CEH (Table 3.3) and the WHO-TEF value of 0.05. The lower PCB 126 and 77 RePs observed in the ring-necked pheasant and Japanese quail indicate that the WHO-TEFs may overestimate the potency of PCB 126 and

77 in these species and other type 2 and type 3 species of birds. Although the TEQ concentrations observed in tree swallow (*Tachycineta bicolor*; type 2) eggs along the Hudson River of New York, USA (1.73-12.7 ng/g TEQ) were up to 10 times greater than the TCDD LD_{50} of 1.2 ng/g in ring-necked pheasant embryos (Cohen-Barnhouse *et al.*, 2011; Secord *et al.*, 1999), only slight effects on reproductive success and parental behaviour were observed (McCarty and Secord, 1999b). Since PCB 77 contributed 82 to 87% of the TEQs measured in tree swallow eggs and nestlings (Secord *et al.*, 1999), overestimation of the PCB 77 TEF by 1 to 2 orders of magnitude in type 2 species could explain the lack of significant embryolethal effects in tree swallows along the Hudson River.

While PCB 126 and 77 RePs in PEH and JEH were below the WHO-TEFs, EROD- and QPCR-based RePs for PCB 105 in JEH were 1 to 2 orders of magnitude greater than their corresponding TEF value of 0.0001 and the CEH RePs for PCB 105 (Table 3.3). PCB 118 RePs in CEH, PEH and JEH were also 1 to 2 orders of magnitude greater than the PCB 118 TEF. These *in vitro* results indicate that the current TEFs for birds may underestimate the relative potency of PCB 105 in Japanese quail and other type 3 species and the relative potency of PCB 118 in most avian species. Additional egg injection studies with PCB 105 and 118 could be performed to test whether this is also the case *in ovo*. The potency of PCBs relative to TCDD varies significantly between the chicken, ring-necked pheasant and Japanese quail, and is likely a result of differences in relative species sensitivity to different PCBs. Therefore, the application of one set of TEF values for calculating TEQs in all species of birds may not be appropriate.

3.4.4 Effects of contamination on RePs for mono-ortho PCBs

A previous study that investigated the effects of several purified mono-*ortho* PCBs on AHR-dependent gene expression in mouse and rat hepatoma cell lines found that PCB 105 and 118 RePs were 50 to 100 times lower than WHO mammalian TEFs (Peters *et al.*, 2006). The authors suggested that the differences between the ReP values obtained from their study and corresponding TEF values may have been due to contamination of mono-*ortho* PCB solutions with more potent AHR agonists in previous studies that were used to derive the WHO-TEFs (Peters *et al.*, 2006). We further tested this hypothesis by comparing induction of EROD activity and CYP1A4/5 mRNA expression in avian hepatocyte cultures exposed to either reagent-grade or purified PCB 105 and 118. PCB 77 was the major contaminant identified in PCB 105-P, 105-RG, 118-P and 118-RG. PCB 118-RG had the highest levels of PCB 77 (2470 ppm), whereas the PCB 77 concentrations found in PCB 105-P, 105-RG and 118-P were approximately 20 ppm (Chapter 2).

The maximal EROD response induced by PCB 118-RG (183 pmol/min/mg protein) in chicken hepatocytes was greater than that induced by PCB 118-P (94.6 pmol/min/mg protein) (Table 3.1, Figure 3.2) but no further differences in ReP_{avg}, EC_{50}, $EC_{threshold}$ or $TCDD_x$ values were observed between purified and reagent-grade PCBs (Tables 3.1 and 3.2, Figures 3.2 and 3.4). Furthermore, ReP_{avg} values between reagent-grade and purified PCBs differed by a maximum of 3-fold (Table 3.3), indicating that contamination of mono-*ortho* PCB solutions did not lead to significant overestimation of their ReP values for CYP1A induction in avian hepatocyte cultures.

3.4.5 Comparisons between CYP1A induction in avian hepatocyte cultures, luciferase reporter gene (LRG) activity and in ovo toxicity

EROD- and CYP1A4/5 mRNA expression-based ReS and ReP values for TCDD and PCB 126, 77, 105-P, 105-RG, 118-P and 118-RG in the chicken, ring-necked pheasant and Japanese quail were compared to values previously obtained from an LRG assay measuring induction of a CYP1A5 reporter gene (Table 3.3) (Chapter 2). ReS_{thr} values for a given species and DLC were generally within one order of magnitude between the EROD, QPCR and LRG assays. A greater range in Japanese quail ReS_{thr} values for TCDD was observed where EROD and LRG assay ReS_{thr} values were similar but were 30 to 100 times lower than the QPCR-based ReS_{thr} values. The rank order of species sensitivity obtained from EROD, QPCR and LRG assays was generally similar for TCDD, PCB 126 and PCB 77. However, ring-necked pheasant and Japanese quail AHR1 constructs exposed to PCB 105 and 118 were less sensitive than the chicken constructs in the LRG assay (Chapter 2), whereas PEH and JEH exposed to these PCBs were equally sensitive or more sensitive than CEH. Since little or no induction of luciferase activity was observed in cells transfected with type 2 and type 3 AHR1 constructs and exposed to PCB 105 and 118, the limited sensitivity of the LRG assay likely affected our ability to compare the effects of PCB 105 and 118 among AHR1 constructs.

The rank order of DLC potency observed from EROD and CYP1A4/5 mRNA expression in chicken and ring-necked pheasant hepatocyte cultures was similar to that observed for chicken and ring-necked pheasant, as well as other type 1 and type 2 AHR1 constructs in the LRG assay (Chapter 2). Although LRG assay results indicated that PCB 126 was more potent than PCB 77, 105 and 118 in the Japanese quail and other type 3 AHR1 constructs, the results of EROD and QPCR assays revealed that PCB 126 was approximately

as potent as these congeners in JEH. Limited sensitivity of the LRG assay could have also affected ReP estimates for PCB 77, 105 and 118 since little or no luciferase activity was induced by these PCBs in type 3 constructs (Chapter 2).

Linear regression analyses between reporter gene activity data and EROD activity or CYP1A4/5 mRNA expression data were conducted. The results of the linear regressions comparing $EC_{threshold}$ values from the LRG, EROD and QPCR assays are presented in Figure 3.5. LRG and EROD assay EC_{50}, $EC_{threshold}$ and $TCDD_{20}$ values were significantly correlated ($0.81 \leq R^2 \leq 0.92$, $p \leq 0.0061$), whereas weaker relationships were observed between LRG activity and CYP1A4 ($0.74 \leq R^2 \leq 0.82$, $p \leq 0.0622$) or CYP1A5 ($0.62 \leq R^2 \leq 0.84$, $p \leq 0.0285$) mRNA expression (Table A.3.3). This is consistent with the observation that ReS and ReP values obtained from the EROD and LRG assays were generally similar, whereas notable differences between EROD/LRG activity- and CYP1A4/5 mRNA expression-based ReS and ReP values were identified (Table 3.3).

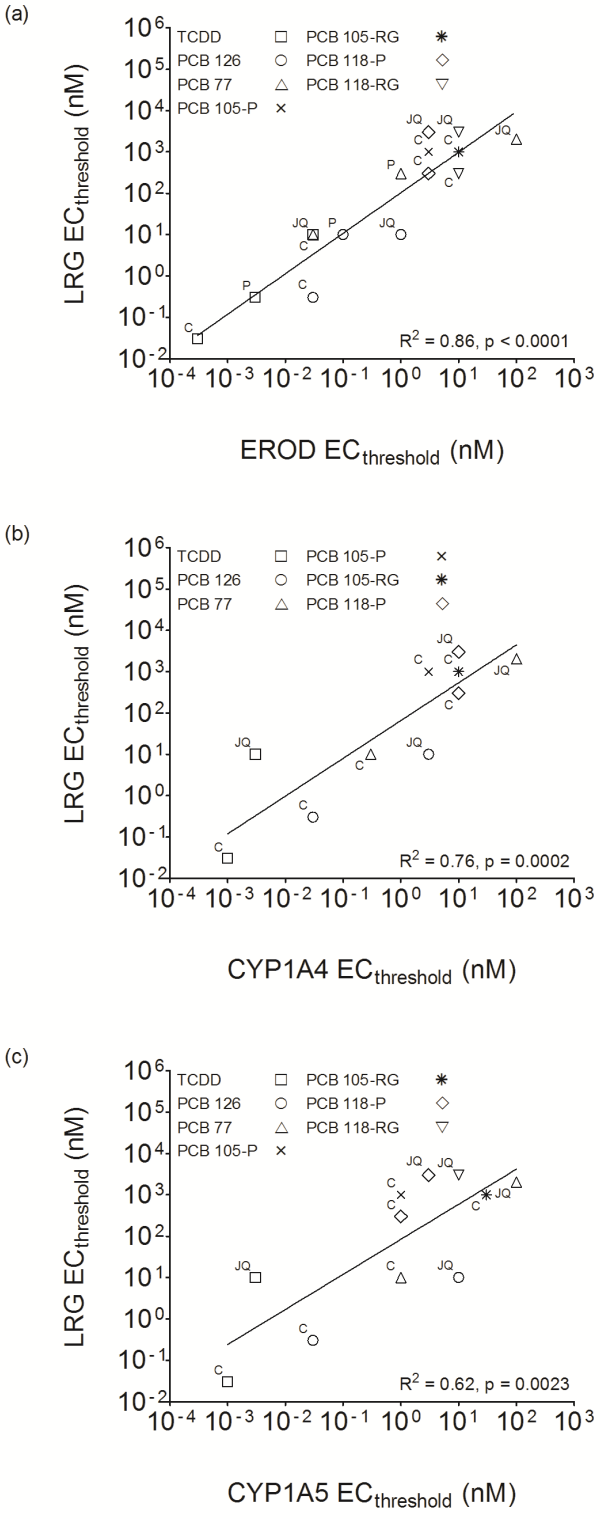

Figure 3.5. Correlation between $EC_{threshold}$ values from a luciferase reporter gene (LRG) assay (Chapter 2) and $EC_{threshold}$ values for (a) EROD activity, (b) CYP1A4 mRNA expression and (c) CYP1A5 mRNA expression in the chicken (C), ring-necked pheasant (P) and Japanese quail (JQ).

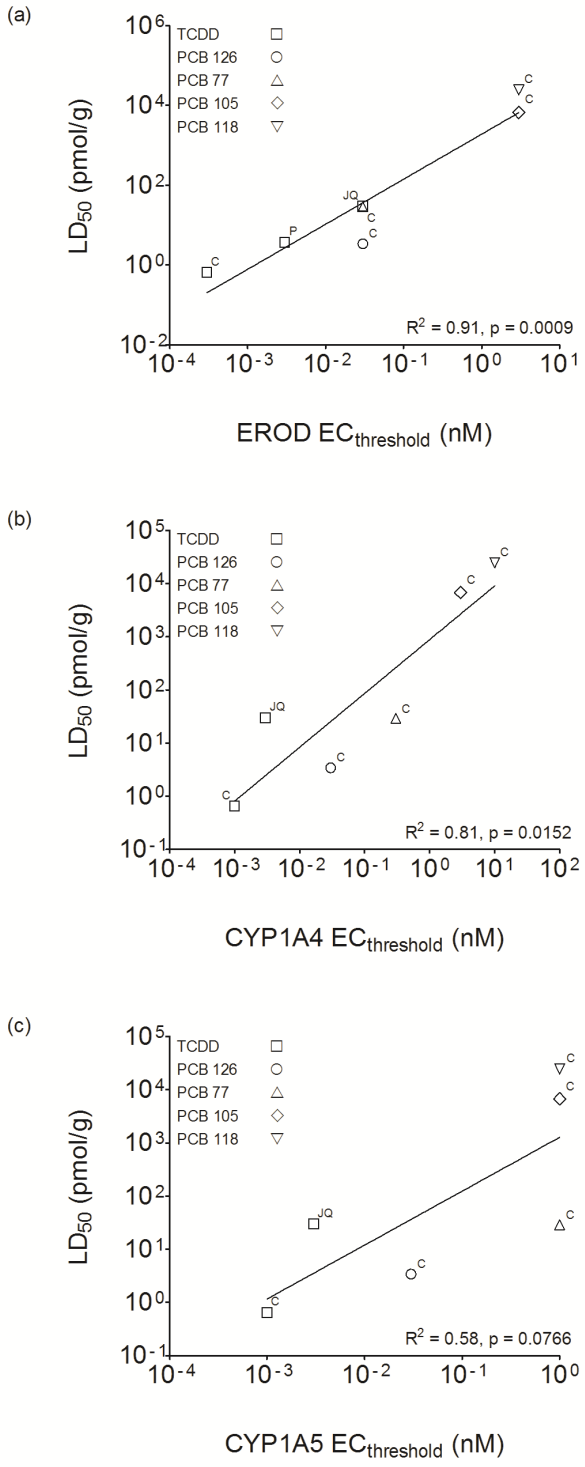

Figure 3.6. Correlation between LD_{50} values obtained from the literature and $EC_{threshold}$ values for (a) EROD activity (b) CYP1A4 mRNA expression and (c) CYP1A5 mRNA expression in the chicken (C), ring-necked pheasant (P) and Japanese quail (JQ).

Significant or near-significant relationships were also observed between *in ovo* LD_{50} data from the literature (Table A.3.2) and EC_{50}, $EC_{threshold}$ and $TCDD_{20}$ values measured for EROD activity ($0.91 \leq R^2 \leq 0.98$, $p \leq 0.0011$), CYP1A4 mRNA expression ($0.76 \leq R^2 \leq 0.81$, $p \leq 0.0229$) and CYP1A5 mRNA expression ($0.58 \leq R^2 \leq 0.81$, $p \leq 0.0766$) (Figure 3.6, Table A.3.4). Stronger relationships were observed between LD_{50} data and EROD activity than between LD_{50}s and CYP1A4/5 mRNA expression. LRG assay endpoints were also significantly associated with *in ovo* LD_{50} values (Chapter 2), indicating that AHR1-induced enzyme activity better predicts DLC-induced embryolethality than CYP1A4/5 mRNA expression. Thus, ReS and ReP values determined from EROD and LRG activity are likely to be more representative of *in ovo* toxicity-based ReS and ReP values than those based on CYP1A4/5 mRNA expression. The EROD assay is considerably quicker and cheaper to use than QPCR and therefore, represents a more economical and useful screening tool for predicting overt toxicity of DLCs compared to QPCR.

3.4.6 General conclusions

The results obtained for EROD activity and/or CYP1A4/5 mRNA expression in CEH, PEH and JEH support and add to the findings previously obtained from the LRG assay. As predicted by the LRG assay, the chicken was the most sensitive species to induction of CYP1A by TCDD, PCB 126 and PCB 77. However, the ring-necked pheasant and Japanese quail were either equally sensitive or more sensitive than the chicken to CYP1A induction by PCB 105 and 118.

Significant differences in PCB ReP values were observed among species. While the chicken RePs generally resembled the WHO-TEF values, ReP values for PCB 126 and 77 in ring-necked pheasant and Japanese quail hepatocytes were lower than those measured in

chicken hepatocytes. In contrast, RePs for PCB 105 and 118 in the Japanese quail were higher than those in the chicken. These findings can be extrapolated to other avian species based on their AHR1 genotype (Farmahin *et al.*, in press) and could be used, along with the results of additional *in vitro* and *in vivo* studies, to develop ReP values based on a species' AHR1 genotype.

Finally, EROD activity in avian hepatocyte cultures was more strongly associated with both luciferase activity in cells transfected with avian AHR1 constructs and *in ovo* PCB toxicity than CYP1A4/5 mRNA expression. This supports the selection of the EROD assay over the QPCR assay for relating changes in CYP1A gene expression to overt toxicity of PCBs. The results presented herein support those obtained from the LRG assay and indicate that AHR1-mediated induction of enzyme activity is strongly associated with PCB-induced embryolethality in several species of birds. To overcome problems associated with the limited sensitivity of the LRG assay that arise when testing the effects of weak AHR agonists, the EROD and LRG assays can be used in a complementary fashion to reduce the number of animals required for predicting DLC toxicity.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

The goals of this thesis were to measure *in vitro* aryl hydrocarbon receptor 1 (AHR1)-mediated gene expression using a luciferase reporter gene (LRG) assay in order to predict avian species sensitivity to polychlorinated biphenyls (PCBs) and the potency of PCB congeners relative to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in different species of birds. The results of the LRG assay were then compared with both *in ovo* toxicity and cytochrome P450 1A (CYP1A) induction measured in hepatocyte cultures from the chicken (*Gallus gallus domesticus*), ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Coturnix japonica*).

The major findings of this thesis were:

1) The amino acids at positions 324 and 380 in the AHR1 ligand binding domain (LBD) are the major determinants of avian species sensitivity to PCB toxicity.
2) The relative potencies of PCB 77, 105 and 118 differ between species, which could have significant implications in the application of the toxic equivalent (TEQ) approach to birds.
3) LRG and ethoxyresorufin *O*-deethylase (EROD) activity are more highly correlated with LD_{50} values ($0.87 \leq R^2 \leq 0.98$) than CYP1A4/5 mRNA expression ($0.58 \leq R^2 \leq 0.81$), and are therefore better predictors of PCB-induced embryolethality in birds.
4) The relative potency (ReP) values measured in the LRG, EROD or real-time quantitative polymerase chain reaction (QPCR) assays did not differ between reagent-grade and purified PCB 105 and 118. This result indicates that any impurities found in the reagent-grade PCB solutions did not affect the relative potency calculation for PCB 105 and 118.

Hypothesis 1: Amino acid residues at positions 324 and 380 in the avian aryl hydrocarbon receptor 1 ligand binding domain (AHR1 LBD) are predictive of avian species sensitivity to PCBs.

As hypothesized, amino acid residues at positions 324 and 380 in the avian AHR1 LBD were associated with differences in avian species sensitivity to PCBs, whereas amino acids at positions 256, 257, 297 and 337 did not appear to play any role in determining species sensitivity. However, the relative sensitivity (ReS) of type 1 (Ile324_Ser380), type 2 (Ile324_Ala380) and type 3 (Val324_Ala380) species varied among PCB congeners. For example, the chicken was the most sensitive species to induction of EROD activity by PCB 126 and PCB 77, whereas the ring-necked pheasant and Japanese quail were either equally sensitive or more sensitive than the chicken to PCB 105 and 118.

Hypothesis 2: The rank order of PCB potency and RePs for PCBs do not differ between species of birds.

The ReP values of PCBs did not differ significantly among AHR1 constructs in the LRG assay. However, ReP values for less potent PCBs were sometimes estimated based on the highest dose tested because of low assay sensitivity. Conversely, differences in EROD- and QPCR-based ReP values were observed between the chicken, ring-necked pheasant and Japanese quail. ReP values for PCB 126 and 77 in ring-necked pheasant and Japanese quail hepatocytes were lower than those measured in chicken hepatocytes, while RePs for PCB 105 and 118 in the Japanese quail were higher than those in the chicken. Therefore, the EROD and QPCR results reject the hypothesis that the rank order of PCB potency does not differ between species.

In contrast to the rank order of PCB potency predicted by the World Health Organization Toxic Equivalency Factors (WHO-TEFs) for birds (van den Berg *et al.*, 1998), results from the LRG, EROD and QPCR assays indicated that PCB 118 is a more potent inducer of *in vitro* AHR1-regulated gene expression than PCB 105. However, PCB 105 was more potent than PCB 118 based on *in ovo* toxicity, which could have been due to increased absorption or retention of PCB 105 (Brunström, 1990; Brunström *et al.*, 1990a; Drouillard *et al.*, 2007).

Hypothesis 3: The LRG assay along with the knowledge of a species' AHR1 genotype can be used to predict the embryotoxic effects of PCBs in that species.

LRG activity was significantly correlated with *in ovo* toxicity, supporting the hypothesis that the LRG assay along with the knowledge of a species' AHR1 genotype can be used to predict species sensitivity to the embryotoxic effects of PCBs. LRG activity and *in ovo* toxicity were also significantly correlated with EROD activity, whereas weaker relationships were observed with CYP1A4/5 mRNA expression. These results indicate that PCB-induced enzyme activity is a better predictor of embryolethality than mRNA expression.

Hypothesis 4: The LRG assay could be used instead of other in vitro techniques using primary hepatocyte cultures to predict species sensitivity to DLCs and the relative potency of DLCs in avian species.

The rank order of species sensitivity observed from the EROD, QPCR and LRG assays was generally similar for TCDD, PCB 126 and PCB 77, but differences in ReS values for type 2 and type 3 species exposed to PCB 105 and 118 were observed between the LRG assays and hepatocyte culture methods. The rank order of DLC potency in type 1 and type 2 species was

also similar between the EROD, QPCR and LRG assays, but inter-assay differences were observed for type 3 species. ReS and ReP values often differed between the LRG assay and EROD/QPCR assays in cases where little or no induction of luciferase activity was detected in the LRG assay. Thus, the LRG assay could be used instead of hepatocyte culture methods under certain circumstances but the reduced sensitivity of the LRG assay limits its ability to predict relative species sensitivity and relative potency for less sensitive avian species and less potent AHR agonists.

Hypothesis 5: The presence of AHR ligands found as impurities in reagent grade PCB 105 and PCB 118 preparations cause the potency of these congeners to be overestimated.

The maximal EROD response in chicken hepatocytes exposed to PCB 118-RG was significantly higher than the response for PCB 118-P. However, no differences in ReP values were observed between reagent-grade and purified solutions of PCB 105 and 118 in the LRG, EROD or QPCR assays. These findings reject the hypothesis that AHR ligands found as impurities in reagent grade solutions of mono-*ortho* PCBs lead to an overestimation of their potency.

4.2 Suggestions for future research

4.2.1 Improving luciferase reporter gene (LRG) assay sensitivity

The sensitivity of the LRG assay should be increased in order to improve its use with less potent AHR agonists and less sensitive AHR1 constructs. The selection of reporter constructs is one aspect of the LRG assay that could be investigated to improve assay sensitivity. Several chicken and Great cormorant (*Phalacrocorax carbo*) CYP1A5 reporter constructs were prepared

by (Lee *et al.*, 2009), one of which (pGL4-ccCYP1A5-7XREs) was employed in our assay. While the EC_{50} values for avian AHR1 constructs exposed to TCDD were not dependent on the CYP1A5 reporter construct used, luciferase activities were approximately 2 to 3 times higher when a chicken CYP1A5 reporter construct (pGL4-ckCYP1A5-6XREs) was used instead of the cormorant construct (pGL4-ccCYP1A5-7XREs) employed in our assay. These results indicate that use of the chicken CYP1A5 construct over the cormorant construct could allow for induction of luciferase activity by less potent PCBs (i.e. PCB 77, 105 and 118) to be observed for less sensitive AHR1 constructs in the LRG assay.

In a chemically activated luciferase expression (CALUX) system, the magnitude of induction and sensitivity of luciferase activity responses increased with the number of xenobiotic response elements (XREs) in reporter constructs (He *et al.*, 2011). The highest luciferase induction and sensitivity were observed in a cell line stably transfected with a reporter construct containing 20 XREs and the overall magnitude of induction was not changed by increasing the number of XREs to 40 (He *et al.*, 2011). Thus, increasing the number of XREs in CYP1A5 reporter constructs could also potentially increase LRG assay sensitivity.

Miniaturization for high-throughput screening requires high assay sensitivity in order to measure small quantities of analytes (Fan and Wood, 2007). Zhou *et al.*, (2003) reported that the use of microcarrier beads increased the sensitivity and reproducibility of an LRG assay by increasing surface area for cell adhesion, thus allowing introduction of more cells/well in a 96-well plate format. The applicability of this method could also be tested in our assay.

4.2.2 Other applications of the luciferase reporter gene (LRG) assay and follow-up studies

In addition to investigating the effects of single DLC congeners, the LRG assay could be used to determine the effects of DLC mixtures on induction of luciferase activity in different avian AHR1 constructs. This approach could be used to predict the embryotoxic effects of environmental mixtures in wild bird species. Furthermore, the validity of the WHO-TEFs and AHR1-specific ReP values obtained from this thesis could be tested by comparing observed induction of luciferase activity of a known DLC mixture with the response predicted by the toxic equivalents (TEQ) approach.

Aside from the chicken, 3 other wild avian species, namely the European starling (*Sturnus vulgaris*), ruby-throated hummingbird (*Archilochus colubris*) and the gray catbird (*Dumetella carolinensis*), were classified as type 1 species (Table A.1.1) (Farmahin *et al.*, in press). Furthermore, the results of the LRG assay indicated that these species are likely to be as sensitive to PCBs as the chicken. Fifty percent of European starlings fed a diet containing 1500 ppm Aroclor 1254 died after 4 days, whereas the 50% mortality points for red-winged blackbirds (*Agelaius phoeniceus*), brown-headed cowbirds (*Molothrus ater*) and common grackle (*Quiscalus quiscula*) were reached at 6, 7 and 8 days, respectively (Stickel *et al.*, 1984). PCB residues in the brains of European starlings that had died during the dosing study were also significantly lower than those found in red-winged blackbirds and grackles (Stickel *et al.*, 1984), supporting the hypothesis that starlings are more sensitive to PCB toxicity than other passerines. A recent study investigating the effects of dioxins and furans in European starling embryo hepatocyte cultures was conducted in our laboratory, and the results indicate that the starling is as sensitive to TCDD as the chicken (Farmahin *et al.*, unpublished data). Additional egg injection or hepatocyte culture studies could be performed to determine if the European starling,

ruby-throated hummingbird, or gray catbird are also as sensitive to the effects of PCBs as the chicken.

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APPENDIX A: SUPPLEMENTARY TABLES

A.1 Supplementary tables pertaining to chapter 1

Table A.1.1. AHR1 LBD subtypes determined in 86 avian species based on the identities of amino acids at sites 256, 257, 297, 324, 337 and 380. The two key amino acids (sites 324 and 380) used to classify species into 3 main AHR1 LBD types are indicated in bold. Avian AHR1 LBD sequences were obtained from GenBank or were determined from liver or blood samples obtained from the National Wildlife Research Centre, Ottawa, ON, commercial suppliers near Ottawa, ON, or the Tittabawassee River Basin, Michigan, USA. Modified from Farmahin *et al.* (in press).

	LBD Amino Acid Residues						
AHR Subtype	256	257	297	**324**[a]	337	**380**[a]	Species Identified
1A	A	A	T	**I**	V	**S**	domestic chicken (*Gallus gallus domesticus*)
1B	A	A	T	**I**	I	**S**	European starling (*Sturnus vulgaris*)
1B							ruby-throated hummingbird (*Archilochus colubris*)
1C	A	T	T	**I**	I	**S**	gray catbird (*Dumetella carolinensis*)
2A	A	A	I	**I**	V	**A**	ruffed grouse (*Bonasa umbellus*)
2A							wild turkey (*Meleagris gallopavo*)
2A							willow ptarmigan (*Lagopus lagopus*)
2B	A	A	T	**I**	I	**A**	American redstart (*Setophaga ruticilla*)
2B							Baltimore oriole (*Icterus galbula*)
2B							black-and-white warbler (*Mniotilta varia*)
2B							black-capped chickadee (*Poecile atricapilla*)
2B							brown-headed cowbird (*Molothrus ater*)
2B							cedar waxwing (*Bombycilla cedrorum*)
2B							chipping sparrow (*Spizella passerina*)
2B							common grackle (*Quiscalus quiscula*)
2B							common yellowthroat (*Geothlypis trichas*)
2B							indigo bunting (*Passerina cyanea*)
2B							Northern cardinal (*Cardinalis cardinalis*)
2B							ovenbird (*Seiurus aurocapilla*)
2B							red-winged blackbird (*Agelaius phoeniceus*)
2B							rose-breasted grosbeak (*Pheucticus ludovicianus*)
2B							song sparrow (*Melospiza melodia*)
2B							swamp sparrow (*Melospiza georgiana*)
2B							tufted titmouse (*Baeolophus bicolor*)
2B							white-throated sparrow (*Zonotrichia albicollis*)
2C	A	A	V	**I**	V	**A**	rock ptarmigan (*Lagopus muta*)

	LBD Amino Acid Residues						
AHR Subtype	256	257	297	**324**[a]	337	**380**[a]	Species Identified
2D	A	P	T	**I**	V	**A**	American woodcock (*Scolopax minor*)
2D							spotted sandpiper (*Actitis macularius*)
2E	A	T	T	**I**	I	**A**	American crow (*Corvus brachyrhynchos*)
2E							American goldfinch (*Carduelis tristis*)
2E							American robin (*Turdus migratorius*)
2E							bank swallow (*Riparia riparia*)
2E							barn swallow (*Hirundo rustica*)
2E							blue jay (*Cyanocitta cristata*)
2E							cliff swallow (*Petrochelidon pyrrhonota*)
2E							Eastern bluebird (*Sialia sialis*)
2E							hermit thrush (*Catharus guttatus*)
2E							house finch (*Carpodacus mexicanus*)
2E							house sparrow (*Passer domesticus*)
2E							house wren (*Troglodytes aedon*)
2E							Northern raven (*corvus corax*)
2E							red-eyed vireo (*Vireo olivaceus*)
2E							tree swallow (*Tachycineta bicolor*)
2E							veery (*Catharus fuscescens*)
2E							white-breasted nuthatch (*Sitta carolinensis*)
2F	A	T	T	**I**	V	**A**	black-footed albatross (*Phoebastria nigripes*)
2F							brown thrasher (*Toxostoma rufum*)
2F							Emu (*Dromaius novaehollandiae*)
2F							mourning dove (*Zenaida macroura*)
2G	T	A	I	**I**	V	**A**	bobwhite quail (*Colinus virginianus*)
2G							ring-necked pheasant (*Phasianus colchicus*)
3A	A	A	T	**V**	V	**A**	great blue heron (*Ardea herodias*)
3A							Japanese quail (*Coturnix japonica*)
3B	A	T	T	**V**	V	**A**	American kestrel (*Falco sparverius*)
3B							Arctic tern (*Sterna paradisaea*)
3B							bald eagle (*Haliaeetus leucocephalus*)
3B							barred owl (*Strix varia*)
3B							belted kingfisher (*Megaceryle alcyon*)
3B							common flicker (*Colaptes auratus*)
3B							common loon (*Gavia immer*)

AHR Subtype	LBD Amino Acid Residues 256	257	297	**324**[a]	337	**380**[a]	Species Identified
3B	A	T	T	**V**	V	**A**	common tern (*Sterna hirundo*)
3B							Cooper's hawk (*Accipiter cooperii*)
3B							double-crested cormorant (*Phalacrocorax auritus*)
3B							downy woodpecker (*Picoides pubescens*)
3B							Eastern kingbird (*Tyrannus tyrannus*)
3B							great cormorant (*Phalacrocorax carbo*)
3B							great horned owl (*Bubo virginianus*)
3B							herring gull (*Larus argentatus*)
3B							ivory gull (*Pagophila eburnea*)
3B							killdeer (*Charadrius vociferus*)
3B							osprey (*Pandion haliaetus*)
3B							red-tailed hawk (*Buteo jamaicensis*)
3B							ring-billed gull (*Larus delawarensis*)
3B							sandhill crane (*Grus canadensis*)
3B							saw whet owl (*Aegolius acadicus*)
3B							screech owl (*Megascops asio*)
3B							sharp shinned hawk (*Accipiter striatus*)
3B							thick-billed murre (*Uria lomvia*)
3B							turkey vulture (*Cathartes aura*)
3C	T	T	T	**V**	V	**A**	brant goose (*Branta bernicla*)
3C							Canada goose (*Branta canadensis*)
3C							common eider (*Somateria mollissima)*
3C							greater scaup (*Aythya marila*)
3C							mallard (*Anas platyrhynchos*)
3C							wood duck (*Aix sponsa*)
3C							wood thrush (*Hylocichla mustelina*)

[a] Amino acid residues at positions 324 and 380 were used to classify species into three major AHR1 types: type 1 (Ile324_Ser380), type 2 (Ile324_Ala380) and type 3 (Val 324_Ala380).

A.2 Supplementary tables pertaining to chapter 2

Table A.2.1. Endpoints determined for AHR1-mediated luciferase reporter gene activity in COS-7 cells transfected with (a) type 1, (b) type 2 and (c) type 3 AHR1 constructs exposed to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P or PCB 118-RG for 20 h. EC_{50}, PC_{20}, PC_{50}, PC_{80}, PC_{max} and maximal response values represent the average of three replicates ± standard error (SE). PC_{20}, PC_{50}, PC_{80} and PC_{max} values were not calculated if the highest observed response was below 20% of the positive control. $EC_{threshold}$ (EC_{thr}) values were determined from concentration-response curves averaged from 3 replicate curves.

(a)

AHR1	Compound	EC_{50}±SE (nM)	EC_{thr} (nM)	PC_{20}±SE (nM)	PC_{50}±SE (nM)	PC_{80}±SE (nM)	PC_{max}±SE (nM)	Max. response±SE (% PC)
chicken (1A)*	TCDD	0.40±0.08[a]	0.03	0.09±0.02[a]	0.33±0.04[a]	1.0±0.2[a]	N/A	105±7[a]
	PCB 126	4.7±0.9[b]	0.3	1.0±0.3[b]	4.8±0.8[b]	25±6[b]	N/A	99±7[a]
	PCB 77	74±21[c]	10	20±5[c]	438±190[c]		128300±128000	72±10[a,b]
	PCB 105-P	NC	1000				N/A	18±3†[c]
	PCB 105-RG	NC	1000				N/A	13±2†[c]
	PCB 118-P	NC	300	1773±714[d]			3349±1090	25±3†[c]
	PCB 118-RG	NC	300	1083±512[d]			6888±5563	38±13†[b,c]
1B	TCDD	0.34±0.08[a]	0.03	0.07±0.02[a]	0.30±0.07[a]	1.2±0.3[a]	N/A	105±5[a]
	PCB 126	4.2±1.6[b]	0.3	0.96±0.43[b]	4.9±2.8[b]	46±38[b]	N/A	104±9[a]
	PCB 77	51±13[c]	10	21±4[c]	458±203[c]		975±804	64±6[b]
	PCB 105-P	NC	1000				N/A	14±2†[c]
	PCB 105-RG	970±310[d]	1000				N/A	10±1[c]
	PCB 118-P	329±46[d]	300	584±49[d]			789±56	24±4[c]
	PCB 118-RG	NC	1000	1792±995[d]			2433±974	26±5†[c]
1C	TCDD	0.43±0.10[a]	0.03	0.08±0.03[a]	0.35±0.10[a]	1.3±0.4[a]	N/A	109±11[a]
	PCB 126	5.7±1.3[b]	0.3	1.0±0.3[b]	6.0±2.3[b]	47±33[b]	N/A	105±11[a]
	PCB 77	100±46[c]	3	32±9[c]	270±155[c]		12070±11900	76±12[a]
	PCB 105-P	788±246[d]	1000				N/A	11±1[b]
	PCB 105-RG	2258±936[d]	1000				N/A	18±5[b]
	PCB 118-P	771±392[d]	300	3562±2432[d]			4941±3456	25±1[b]
	PCB 118-RG	NC	1000	3346±419[d]			N/A‡	20±1†[b]

(b)

AHR1	Compound	EC_{50}±SE (nM)	EC_{thr} (nM)	PC_{20}±SE (nM)	PC_{50}±SE (nM)	PC_{80}±SE (nM)	PC_{max}±SE (nM)	Max. response±SE (% PC)
ring-necked pheasant	TCDD	4.6±0.5[a]	0.3	1.3±0.3[a]	3.7±0.3[a]	9.6±2.4[a]	N/A	114±8[a]
(2G)*	PCB 126	99±16[b]	10	21±9[b]	83±17[b]	669±401[b]	N/A	105±13[a]
	PCB 77	NC	300	1292±496[c]			2720±1031	36±10†[b]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	>3000	>3000				N/A	no induction
2A	TCDD	5.9±1.8[a]	0.3	1.7±0.3[a]	4.5±1.1[a]	9.4±2.6	N/A	117±5[a]
	PCB 126	63±7[b]	10	21±2[b]	146±45[b]		820±84	75±2[b]
	PCB 77	NC	100	731±170[c]			1407±579	31±5†[c]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	>3000	>3000				N/A	no induction
2B	TCDD	8.2±2.3[a]	0.3	1.8±0.2[a]	6.8±1.7[a]	23±9	N/A	108±3[a]
	PCB 126	164±76[b]	10	31±11[b]	290±119[b]	459§	N/A	91±25[a]
	PCB 77	NC	100	1876±479[c]			2697±756	24±4†[b]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	>3000	>3000				N/A	no induction
2C	TCDD	7.8±2.1[a]	0.3	1.2±0.1[a]	6.7±2.2[a]	36±22	N/A	107±4[a]
	PCB 126	63±9[b]	3	23±5[b]	341±205[b]		2361±2115	72±8[b]
	PCB 77	NC	100	1958±552[c]			4224±1447	25±6†[c]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	NC	3000				N/A	4.8±1.9†[c]

(b) (continued).

AHR1	Compound	EC_{50}±SE (nM)	EC_{thr} (nM)	PC_{20}±SE (nM)	PC_{50}±SE (nM)	PC_{80}±SE (nM)	PC_{max}±SE (nM)	Max. response±SE (% PC)
2D	TCDD	4.8±0.7[a]	0.3	0.86±0.24[a]	3.7±0.6[a]	13±1	N/A	109±5[a]
	PCB 126	84±20[b]	10	24±8[b]	2239±1965[b]		20140±19570	69±10[b]
	PCB 77	NC	100	1965±998[c]			3224±1847	24±4†[c]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	NC	3000				N/A	3.6±0.9†[c]
2E	TCDD	5.7±1.4[a]	0.3	1.5±0.7[a]	4.5±1.5[a]	12±4	N/A	123±13[a]
	PCB 126	86±38[b]	3	31±4[b]	636±361[b]		3743±3426	62±11[b]
	PCB 77	NC	100	1730±543[c]			N/A‡	20±2†[c]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	NC	3000				N/A	2.7±0.5†[c]
	PCB 118-RG	NC	3000				N/A	3.3±1.0†[c]
2F	TCDD	16±9[a]	0.3	1.4±0.3[a]	7.2±1.8[a]	33±17	N/A	123±9[a]
	PCB 126	75±30[a]	3	16±3[b]	129±26[b]		8685±7391	74±4[b]
	PCB 77	NC	100	1743±1050[c]			1917±1119	21±4†[c]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	NC	3000				N/A	1.6±0.5†[c]
	PCB 118-RG	>3000	>3000				N/A	no induction
2G	TCDD	4.9±2.3[a]	0.3	1.0±0.2[a]	3.8±1.6[a]	16±12	N/A	107±5[a]
	PCB 126	190±95[b]	3	26±9[b]	318±123[b]		4753±3286	70±6[b]
	PCB 77	NC	300	1677±351[c]			2315±589	25±4†[c]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	NC	3000				N/A	4.8±0.5†[d]

(c)

AHR1	Compound	EC_{50}±SE (nM)	EC_{thr} (nM)	PC_{20}±SE (nM)	PC_{50}±SE (nM)	PC_{80}±SE (nM)	PC_{max}±SE (nM)	Max. response±SE (% PC)
Japanese quail (3A)*	TCDD	86±35[a]	10	11±5[a]	37±12[a]	99±31[a]	N/A	135±20[a]
	PCB 126	445±36[b]	10	72±12[b]	450±91[b]	3409±1650[b]	N/A	100±5[a]
	PCB 77	NC	2065				N/A	13±4†[b]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	NC	3000				N/A	3.0±1.0†[b]
	PCB 118-RG	NC	3000				N/A	4.1±0.3†[b]
3A	TCDD	83±22[a]	1	16±3[a]	50±4[a]	126±24[a]	N/A	127±10[a]
	PCB 126	1487±1101[b]	30	153±60[b]	504±73[b]	2488±1256[b]	N/A	123±33[a]
	PCB 77	>2065	>2065				N/A	no induction
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	>3000	>3000				N/A	no induction
3B	TCDD	87±23[a]	10	19±3[a]	55±4[a]	130±22[a]	N/A	124±12[a]
	PCB 126	755±497[a]	30	118±45[b]	444±119[b]	1533±1000[b]	N/A	111±20[a]
	PCB 77	>2065	>2065				N/A	no induction
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	>3000	>3000				N/A	no induction
3C	TCDD	79±43[a]	10	18±7[a]	49±20[a]	107±41[a]	N/A	125±12[a,b]
	PCB 126	2537±2085[a]	30	113±35[b]	491±147[b]	1942±915[b]	N/A	155±54[a]
	PCB 77	NC	2065				N/A	13±6†[b]
	PCB 105-P	>3000	>3000				N/A	no induction
	PCB 105-RG	>3000	>3000				N/A	no induction
	PCB 118-P	>3000	>3000				N/A	no induction
	PCB 118-RG	>3000	>3000				N/A	no induction

Luciferase activity was normalized to response of 300 nM TCDD positive control (PC). Maximal response values were obtained from the curve fit, unless otherwise indicated.

Superscript letters indicate significant differences between DLC treatments ($p < 0.05$) within each AHR1 construct.

NC: EC_{50} was not calculated since maximal response was not reached, but significant induction of luciferase activity was observed.

N/A: not applicable since response either above 80% or less than 20% of positive control response.

*Wild-type AHR1 constructs containing the full length sequence for the chicken, ring-necked pheasant or Japanese quail AHR1.

§PC_{80} value obtained from one of three replicates. Other replicates had a maximal response below 80% TCDD.

†No maximal response was reached. Values represent highest observed response.
‡$PC_{max} = PC_{20}$

Table A.2.2. Sensitivity of wild-type (chicken, ring-necked pheasant and Japanese quail) and mutant (1B-C, 2A-G and 3A-C) AHR1 constructs to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG relative to chicken AHR1. Relative sensitivity values based on $EC_{threshold}$ (ReS_{thr}) and EC_{50} values (ReS_{EC50}) obtained from the luciferase reporter gene (LRG) assay are presented. If no induction of luciferase activity was observed, ReS values were estimated by dividing the chicken value by the highest concentration tested (2065 nM for PCB 77 and 3000 nM for all other compounds). When EC_{50} values for the chicken could not be calculated, ReS values were calculated relative to AHR1 constructs 1B or 1C.

AHR1 construct	TCDD		PCB 126		PCB 77		PCB 105-P		PCB 105-RG		PCB 118-P		PCB 118-RG	
	ReS_{thr}	ReS_{EC50}	ReS_{thr}	ReS_{EC50}	ReS_{thr}	ReS_{EC50}	ReS_{thr}	ReS_{EC50}	ReS_{thr}	ReS_{EC50}	ReS_{thr}	ReS_{EC50}	ReS_{thr}	ReS_{EC50}
chicken (1A)*	1.0	1.0[a]	1.0	1.0[a]	1.0	1.0[a]	1.0	NA	1.0	NA	1.0	NA	1.0	NA
1B	1.0	1.2[a]	1.0	1.1[a]	1.0	1.4[a]	1.0	NA	1.0	1.0[a]	1.0	1.0[a]	0.30	NA
1C	1.0	0.94[a]	1.0	0.82[a,b]	3.3	0.73[a]	1.0	1.0	1.0	0.43[a]	1.0	0.43[a]	0.30	NA
ring-necked pheasant (2G)*	0.10	0.086[b]	0.030	0.048[c,e]	0.033	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	<0.10	NA
2A	0.10	0.067[b]	0.030	0.076[b,c,d]	0.10	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	<0.10	NA
2B	0.10	0.049[b]	0.030	0.029[c,e]	0.10	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	<0.10	NA
2C	0.10	0.051[b]	0.10	0.075[b,c,d]	0.10	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	0.10	NA
2D	0.10	0.084[b]	0.030	0.057[c,f]	0.10	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	0.10	NA
2E	0.10	0.070[b]	0.10	0.055[c,f]	0.10	NA	<0.33	<0.26	<0.33	<0.32	0.10	NA	0.10	NA
2F	0.10	0.024[b,c]	0.10	0.063[b,c,f]	0.10	NA	<0.33	<0.26	<0.33	<0.32	0.10	NA	<0.10	NA
2G	0.10	0.08[b]	0.10	0.025[c,e]	0.033	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	0.10	NA
Japanese quail (3A)*	0.0030	0.0046[c,d]	0.030	0.011[d,e,f]	0.0048	NA	<0.33	<0.26	<0.33	<0.32	0.10	NA	0.10	NA
3A	0.030	0.0048[d]	0.010	0.0032[e,f]	<0.0048	<0.037	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	<0.10	NA
3B	0.0030	0.0046[d]	0.010	0.0063[d,e,f]	<0.0048	<0.037	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	<0.10	NA
3C	0.0030	0.0051[c,d]	0.010	0.0019[e]	0.0048	NA	<0.33	<0.26	<0.33	<0.32	<0.10	<0.11	<0.10	NA

Superscript letters indicate significant differences in EC_{50} values between AHR1 constructs ($p < 0.05$) for a given treatment.
NA: EC_{50} values not available to calculate ReS value.
*Wild-type AHR1 constructs containing the full length sequence for the chicken, ring-necked pheasant or Japanese quail AHR1.

Table A.2.3. Potency of PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG relative to TCDD in (a) type 1, (b) type 2 and (c) type 3 AHR1 constructs. Relative potency values were calculated based on EC_{50}, $EC_{threshold}$, PC_{20}, PC_{50}, PC_{80} and PC_{max} values (ReP_{EC50}, ReP_{thr}, ReP_{PC20}, ReP_{PC50}, ReP_{PC80} and ReP_{PCmax}) obtained from the luciferase reporter gene (LRG) assay. The average and range of ReP values are presented. If no induction of luciferase activity was observed, ReP values were estimated by dividing the TCDD value by the highest concentration tested (2065 nM for PCB 77 and 3000 nM for all other compounds).

(a)

AHR1	Chemical	ReP_{EC50}	ReP_{thr}	ReP_{PC20}	ReP_{PC50}	ReP_{PC80}	ReP_{PCmax}	ReP_{avg}	ReP range
chicken (1A)*	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.085	0.10	0.088	0.068	0.041	N/A	0.074	0.041-0.10
	PCB 77	0.0054	0.0030	0.0045	0.00074		0.0000045	0.0021	0.0000045-0.0045
	PCB 105-P	NC	0.000030				N/A	0.000030	0.000030-0.000030
	PCB 105-RG	NC	0.000030				N/A	0.000030	0.000030-0.000030
	PCB 118-P	NC	0.00010	0.000052			0.000036	0.000062	0.000036-0.00010
	PCB 118-RG	NC	0.00010	0.000084			0.000022	0.000069	0.000022-0.00010
1B	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.081	0.10	0.075	0.061	0.026	N/A	0.066	0.026-0.10
	PCB 77	0.0067	0.0030	0.0034	0.00066		0.00041	0.0019	0.00041-0.0034
	PCB 105-P	NC	0.000030				N/A	0.000030	0.000030-0.000030
	PCB 105-RG	0.00035	0.000030				N/A	0.000030	0.000030-0.000030
	PCB 118-P	0.0010	0.00010	0.00012			0.00010	0.00011	0.00010-0.00012
	PCB 118-RG	NC	0.000030	0.000040			0.000043	0.000038	0.000030-0.000043
1C	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.074	0.10	0.080	0.059	0.028	N/A	0.067	0.028-0.10
	PCB 77	0.0042	0.010	0.0026	0.0013		0.000069	0.0035	0.000069-0.010
	PCB 105-P	0.00054	0.000030				N/A	0.000030	0.000030-0.000030
	PCB 105-RG	0.00019	0.000030				N/A	0.000030	0.000030-0.000030
	PCB 118-P	0.00055	0.00010	0.000023			0.000018	0.000050	0.000018-0.00010
	PCB 118-RG	NC	0.000030	0.000025			N/A*	0.000027	0.000025-0.000030

(b)

AHR1	Chemical	ReP_{EC50}	ReP_{thr}	ReP_{PC20}	ReP_{PC50}	ReP_{PC80}	ReP_{PCmax}	ReP_{avg}	ReP range
ring-necked pheasant (2G)*	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.047	0.030	0.059	0.044	0.014	N/A	0.037	0.014-0.059
	PCB 77	NC	0.0010	0.00097	0.00089		0.00088	0.0010	0.00088-0.0010
	PCB 105-P	<0.0015	<0.00010				N/A		
	PCB 105-RG	<0.0015	<0.00010				N/A		
	PCB 118-P	<0.0015	<0.00010				N/A		
	PCB 118-RG	<0.0015	<0.00010				N/A		
2A	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.095	0.030	0.080	0.031		0.0093	0.038	0.0093-0.080
	PCB 77	NC	0.0030	0.0023			0.0019	0.0024	0.0019-0.0030
	PCB 105-P	<0.0020	<0.00010				N/A		
	PCB 105-RG	<0.0020	<0.00010				N/A		
	PCB 118-P	<0.0020	<0.00010				N/A		
	PCB 118-RG	<0.0020	<0.00010				N/A		
2B	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.050	0.030	0.059	0.023	0.051	N/A	0.041	0.023-0.059
	PCB 77	NC	0.0030	0.00098			0.00086	0.0016	0.00086-0.0030
	PCB 105-P	<0.0027	<0.00010				N/A		
	PCB 105-RG	<0.0027	<0.00010				N/A		
	PCB 118-P	<0.0027	<0.00010				N/A		
	PCB 118-RG	<0.0027	<0.00010				N/A		
2C	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.12	0.10	0.054	0.020		0.0080	0.045	0.0080-0.10
	PCB 77	NC	0.0030	0.00063			0.00042	0.0013	0.00042-0.0030
	PCB 105-P	<0.0026	<0.00010				N/A		
	PCB 105-RG	<0.0026	<0.00010				N/A		
	PCB 118-P	<0.0026	<0.00010				N/A		
	PCB 118-RG	NC	0.00010				N/A	0.00010	0.00010-0.00010

(b) (continued).

AHR1	Chemical	ReP_{EC50}	ReP_{thr}	ReP_{PC20}	ReP_{PC50}	ReP_{PC80}	ReP_{PCmax}	ReP_{avg}	ReP range
2D	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.057	0.030	0.036	0.0017		0.00034	0.017	0.00034-0.036
	PCB 77	NC	0.0030	0.00044			0.00035	0.0013	0.00035-0.0030
	PCB 105-P	<0.0016	<0.00010				N/A		
	PCB 105-RG	<0.0016	<0.00010				N/A		
	PCB 118-P	<0.0016	<0.00010				N/A		
	PCB 118-RG	NC	0.00010				N/A	0.00010	0.00010-0.00010
2E	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.067	0.10	0.048	0.0070		0.0013	0.039	0.0013-0.10
	PCB 77	NC	0.0030	0.00086			N/A†	0.0019	0.00086-0.0030
	PCB 105-P	<0.0019	<0.00010				N/A		
	PCB 105-RG	<0.0019	<0.00010				N/A		
	PCB 118-P	NC	0.00010				N/A	0.00010	0.00010-0.00010
	PCB 118-RG	NC	0.00010				N/A	0.00010	0.00010-0.00010
2F	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.22	0.10	0.088	0.056		0.0025	0.062	0.0025-0.10
	PCB 77	NC	0.0030	0.00079			0.00077	0.0015	0.00077-0.0030
	PCB 105-P	<0.0055	<0.00010				N/A		
	PCB 105-RG	<0.0055	<0.00010				N/A		
	PCB 118-P	NC	0.00010				N/A	0.00010	0.00010-0.00010
	PCB 118-RG	<0.0055	<0.00010				N/A		
2G	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.026	0.10	0.039	0.012		0.0019	0.038	0.0019-0.10
	PCB 77	NC	0.0010	0.00061			0.00057	0.00073	0.00057-0.0010
	PCB 105-P	<0.0016	<0.00010				N/A		
	PCB 105-RG	<0.0016	<0.00010				N/A		
	PCB 118-P	<0.0016	<0.00010				N/A		
	PCB 118-RG	NC	0.00010				N/A	0.00010	0.00010-0.00010

(c)

AHR1	Chemical	ReP_{EC50}	ReP_{thr}	ReP_{PC20}	ReP_{PC50}	ReP_{PC80}	ReP_{PCmax}	ReP_{avg}	ReP range
Japanese quail (3A)*	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.19	1.0	0.15	0.082	0.029	N/A	0.32	0.029-1.0
	PCB 77	NC	0.0048				N/A	0.0048	0.0048-0.0048
	PCB 105-P	<0.029	<0.0033				N/A		
	PCB 105-RG	<0.029	<0.0033				N/A		
	PCB 118-P	NC	0.0033				N/A	0.0033	0.0033-0.0033
	PCB 118-RG	NC	0.0033				N/A	0.0033	0.0033-0.0033
3A	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.056	0.033	0.11	0.099	0.051	N/A	0.072	0.033-0.11
	PCB 77	<0.040	<0.00048				N/A		
	PCB 105-P	<0.028	<0.00033				N/A		
	PCB 105-RG	<0.028	<0.00033				N/A		
	PCB 118-P	<0.028	<0.00033				N/A		
	PCB 118-RG	<0.028	<0.00033				N/A		
3B	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.11	0.33	0.16	0.12	0.085	N/A	0.18	0.085-0.33
	PCB 77	<0.042	<0.0048				N/A		
	PCB 105-P	<0.029	<0.0033				N/A		
	PCB 105-RG	<0.029	<0.0033				N/A		
	PCB 118-P	<0.029	<0.0033				N/A		
	PCB 118-RG	<0.029	<0.0033				N/A		
3C	TCDD	1.0	1.0	1.0	1.0	1.0	N/A	1.0	1.0-1.0
	PCB 126	0.031	0.33	0.16	0.10	0.055	N/A	0.16	0.055-0.33
	PCB 77	NC	0.0048				N/A	0.0048	0.0048-0.0048
	PCB 105-P	<0.026	<0.0033				N/A		
	PCB 105-RG	<0.026	<0.0033				N/A		
	PCB 118-P	<0.026	<0.0033				N/A		
	PCB 118-RG	<0.026	<0.0033				N/A		

NC: EC_{50} was not calculated since maximal response was not reached, but significant induction of luciferase activity was observed.
N/A: not applicable since response either above 80% or less than 20%.
*Wild-type AHR1 constructs containing the full length sequence for the chicken, ring-necked pheasant or Japanese quail AHR1.
†$ReP_{max} = ReP_{20}$

A.3 Supplementary tables pertaining to chapter 3

Table A.3.1. Sensitivity of ring-necked pheasant and Japanese quail to TCDD, PCB 126, PCB 77, PCB 105-P, PCB 105-RG, PCB 118-P and PCB 118-RG relative to the chicken. EC_{50}-based relative sensitivity values (ReS) obtained from EROD activity, CYP1A4/5 mRNA expression and luciferase reporter gene (LRG) activity are presented.

Compound	Species	ReS_{EC50}			
		EROD	CYP1A4	CYP1A5	LRG*
TCDD	Chicken	1.00[a]	1.00[a]	1.00[a]	1.00[a]
TCDD	Ring-necked pheasant	0.59[a]			0.086[b]
TCDD	Japanese quail	0.21[b]	0.11[b]	0.026[b]	0.0046[c]
PCB 126	Chicken	1.00[a]	1.00[a]	1.00[a]	1.00[a]
PCB 126	Ring-necked pheasant	0.10[b]			0.048[b]
PCB 126	Japanese quail	0.011[c]	0.0022[b]	0.0024[b]	0.011[b]
PCB 77	Chicken	1.00[a]	1.00	1.00	1.00
PCB 77	Ring-necked pheasant	0.10[b]			NA
PCB 77	Japanese quail	0.034[c]	NA	NA	NA
PCB 105-P	Chicken	1.00[a]	1.00[a]	1.00[a]	NA
PCB 105-P	Japanese quail	2.60[a]	143[a]	118[a]	<0.26
PCB 105-RG	Chicken	1.00[a]	1.00[a]	1.00[a]	NA
PCB 105-RG	Ring-necked pheasant	0.87[a]			<0.32
PCB 105-RG	Japanese quail	9.12[b]	21.8[b]	42.2[b]	<0.32
PCB 118-P	Chicken	1.00[a]	1.00[a]	1.00[a]	NA
PCB 118-P	Japanese quail	2.52[b]	2.07[a]	2.43[b]	NA
PCB 118-RG	Chicken	1.00[a]	1.00[a]	1.00[a]	NA
PCB 118-RG	Ring-necked pheasant	0.81[a]			NA
PCB 118-RG	Japanese quail	2.60[b]	1.61[a]	3.93[a]	NA

Superscript letters indicate significant differences between EC_{50} values among species ($p < 0.05$).
* LRG assay ReS values from Chapter 2.

Table A.3.2. *In ovo* LD_{50} values for TCDD, PCB 126, PCB 77, PCB 105 and PCB 118 in the chicken, ring-necked pheasant and Japanese quail.

Species	Compound	LD_{50} (ng/g egg)	LD_{50} (pmol/g egg)	Reference
chicken	TCDD	0.21	0.65	(Cohen-Barnhouse *et al.*, 2011)
	PCB 126	1.1[a]	3.4	(Head *et al.*, 2008)
	PCB 77	8.6	29	(Brunström and Andersson, 1988)
	PCB 105	2200	6700	(Brunström, 1990)
	PCB 118	8000[b]	25000	(Brunström, 1990)
ring-necked pheasant	TCDD	1.2	3.5	(Cohen-Barnhouse *et al.*, 2011)
Japanese quail	TCDD	9.7	30	(Cohen-Barnhouse *et al.*, 2011)

[a] LD_{50} calculated based on a review of several egg injection studies (Head *et al.*, 2008).
[b] LD_{50} taken from Giesy *et al.* (2006).

Table A.3.3. Equations, R^2 and *p* values associated with linear regression analyses between luciferase reporter gene activity and CYP1A induction measured as EROD activity or CYP1A4/5 mRNA expression in avian hepatocyte cultures.

Assay	Endpoint	Regression equation	R^2	*p* value
EROD	EC_{50}	$logEC_{50} = 1.257logEC_{50} + 2.303$	0.81	0.0061
	$EC_{threshold}$	$logEC_{threshold} = 0.980logEC_{threshold} + 2.014$	0.86	<0.0001
	$TCDD_{20}$	$logTCDD_{20} = 0.993logTCDD_{20} + 1.984$	0.92	<0.0001
CYP1A4	EC_{50}	$logEC_{50} = 0.775logEC_{50} + 1.344$	0.74	0.0622
	$EC_{threshold}$	$logEC_{threshold} = 0.914logEC_{threshold} + 1.819$	0.76	0.0002
	$TCDD_{20}$	$logTCDD_{20} = 0.928logTCDD_{20} -1.253$	0.82	0.0050
CYP1A5	EC_{50}	$logEC_{50} = 0.749logEC_{50} + 1.586$	0.84	0.0285
	$EC_{threshold}$	$logEC_{threshold} = 0.848logEC_{threshold} + 1.925$	0.62	0.0023
	$TCDD_{20}$	$logTCDD_{20} = 0.858logTCDD_{20} +1.712$	0.80	0.0068

Table A.3.4. Equations, R^2 and *p* values associated with linear regression analyses between *in ovo* LD_{50} values and CYP1A induction measured as EROD activity or CYP1A4/5 mRNA expression in avian hepatocyte cultures.

Assay	Endpoint	Regression equation	R^2	*p* value
EROD	EC_{50}	$logLD_{50} = 1.483logEC_{50} + 2.429$	0.98	<0.0001
	$EC_{threshold}$	$logLD_{50} = 1.129logEC_{threshold} + 3.283$	0.91	0.0009
	$TCDD_{20}$	$logLD_{50} = 1.368logTCDD_{20} + 3.228$	0.95	0.0011
CYP1A4	EC_{50}	$logLD_{50} = 0.894logEC_{50} + 1.426$	0.76	0.0229
	$EC_{threshold}$	$logLD_{50} = 1.012logEC_{threshold} + 2.944$	0.81	0.0152
	$TCDD_{20}$	$logLD_{50} = 0.851logTCDD_{20} + 1.921$	0.79	0.0186
CYP1A5	EC_{50}	$logLD_{50} = 0.861logEC_{50} + 1.700$	0.81	0.0151
	$EC_{threshold}$	$logLD_{50} = 1.013logEC_{threshold} + 3.106$	0.58	0.0766
	$TCDD_{20}$	$logLD_{50} = 0.927logTCDD_{20} + 2.427$	0.79	0.0171

APPENDIX B: INTRA-ASSAY VARIABILITY OF LUCIFERASE REPORTER GENE (LRG) AND ETHOXYRESORUFIN *O*-DEETHYLASE (EROD) ASSAYS

Table B.1. Intra-assay coefficients of variation (%) in the luciferase reporter gene (LRG) assay.

AHR1 construct	TCDD	PCB 126	PCB 77	PCB 105-P	PCB 105-RG	PCB 118-P	PCB 118-RG	Average
chicken (1A)	16.0%	14.4%	15.7%	18.5%	16.2%	17.9%	18.2%	**16.7%**
1B	19.1%	19.8%	16.9%	19.5%	19.2%	17.1%	21.0%	**18.9%**
1C	21.4%	19.0%	16.9%	18.9%	17.4%	20.0%	22.3%	**19.4%**
ring-necked pheasant (2G)	16.1%	14.7%	14.9%	24.4%	15.0%	13.7%	15.8%	**16.4%**
2A	16.8%	14.5%	19.2%	17.8%	20.3%	19.8%	18.8%	**18.2%**
2B	15.7%	14.2%	13.8%	20.6%	14.4%	16.6%	18.1%	**16.2%**
2C	17.9%	16.3%	20.0%	18.7%	20.8%	19.4%	17.2%	**18.6%**
2D	14.1%	13.6%	13.9%	18.8%	18.1%	19.3%	16.6%	**16.4%**
2E	17.1%	14.5%	17.0%	18.9%	17.8%	18.5%	20.1%	**17.7%**
2F	17.6%	14.5%	21.8%	18.1%	17.9%	15.4%	18.9%	**17.8%**
2G	20.4%	15.3%	21.8%	23.6%	20.3%	16.1%	20.4%	**19.7%**
Japanese quail (3A)	25.2%	18.9%	20.7%	17.4%	17.6%	14.8%	21.4%	**19.4%**
3A	18.4%	13.4%	21.5%	18.1%	13.7%	16.1%	18.1%	**17.0%**
3B	25.7%	16.7%	24.5%	19.6%	19.2%	15.5%	22.5%	**20.5%**
3C	20.1%	18.0%	15.6%	21.5%	15.3%	19.5%	19.8%	**18.5%**
Average	**18.8%**	**15.9%**	**18.3%**	**19.6%**	**17.6%**	**17.3%**	**19.3%**	

Table B.2. Intra-assay coefficients of variation (%) in the ethoxyresorufin *O*-deethylase (EROD) assay.

Species	TCDD	PCB 126	PCB 77	PCB 105-P	PCB 105-RG	PCB 118-P	PCB 118-RG	Average
chicken	16.9%	10.2%	9.5%	27.6%	32.6%	26.0%	27.2%	**21.4%**
ring-necked pheasant	14.1%	15.5%	25.0%	N/A	29.1%	N/A	21.7%	**21.1%**
Japanese quail	14.7%	19.2%	19.5%	21.7%	25.1%	18.5%	28.4%	**21.0%**
Average	**15.2%**	**14.9%**	**18.0%**	**24.6%**	**28.9%**	**22.2%**	**25.8%**	