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CYTOCHROME P4501A INDUCTION BY DIOXIN-LIKE COMPOUNDS IN AVIAN HEPATOCYTE CULTURES

Jessica Hervé

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Abstract

In this study, the potencies of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) to induce cytochrome P4501A (CYP1A) were determined. The concentration-dependent effects of TCDD, PeCDF and TCDF on ethoxyresorufin *O*-deethylase (EROD) activity, CYP1A4 mRNA and CYP1A5 mRNA expression were measured in primary hepatocyte cultures of chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*), Japanese quail (*Corturnix japonica*), and herring gull (*Larus argentatus*) embryos exposed for 24h. The results demonstrated that PeCDF was as potent as TCDD to induce CYP1A in chicken hepatocytes, but that PeCDF was more potent than TCDD in ring-necked pheasant, Japanese quail and herring gull hepatocytes. Moreover, the chicken was the most sensitive species to CYP1A induction by TCDD or TCDF, but all species were approximately equisensitive to CYP1A induction by PeCDF. Together these results showed that all species do not respond the same way to different DLCs, suggesting that interactions between a DLC and a species are structure- and cell type-dependent. These results raise interesting questions concerning the molecular mechanism of action of DLCs and the toxic equivalency factors that are broadly assigned to all avian species.

Résumé

Dans cette recherche, des cultures primaires d'hépatocytes de poulet (*Gallus gallus*), de faisan à collier (*Phasianus colchicus*), de caille japonaise (*Corturnix japonica*), et de goéland argenté (*Larus argentatus*) ont été exposées à la 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), au 2,3,4,7,8-pentachlorodibenzofurane (PeCDF) et au 2,3,7,8-tétrachlorodibenzofurane (TCDF) durant 24 h. La puissance relative d'induction du cytochrome P4501A (CYP1A) par la TCDD, le PeCDF et le TCDF a été mesurée par l'augmentation de l'activité de l'éthoxyresorufine *O*-deethylase, de l'expression de l'ARN messager (ARNm) du CYP1A4 et CYP1A5. Les résultats ont démontré que, chez le faisan à collier, la caille japonaise et le goéland argenté, le PeCDF était plus puissant que la TCDD pour induire le CYP1A, par contre, chez le poulet, la puissance des deux composés était équivalente. De plus, le poulet était l'espèce la plus sensible à l'induction du CYP1A par la TCDD ou le TCDF. Toutefois, toutes les espèces avaient une sensibilité similaire à l'induction du CYP1A par le PeCDF. Ces résultats démontrent que toutes les espèces ne répondent pas de la même manière à l'induction du CYP1A par les DLC et semblent indiquer que les interactions entre un DLC et une espèce sont dépendante de la structure du DLC et du contexte cellulaire. Ces résultats soulèvent des questions concernant le fonctionnement des mécanismes d'action moléculaires des DLC et concernant l'application des mêmes facteurs d'équivalence toxique à toutes les espèces aviaires.

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List of Abbreviations

AHR	aryl hydrocarbon receptor
Arnt	aryl hydrocarbon receptor nuclear translocator
bHLH-PAS	basic helix-loop-helix Per Arnt Sim
cDNA	complementary DNA
CFIA	Canadian Food Inspection Agency
Ct	threshold cycle
CYP1A	cytochrome P4501A
CYP1A4	cytochrome P4501A4
CYP1A5	cytochrome P4501A5
d	days
DLC	dioxin-like compound
DMSO	dimethyl sulfoxide
EC50	median effective concentration
ECthr	ECthreshold: threshold concentration for effect
ED50	median effective dose
EROD	ethoxyresorufin *O*-deethylase
HCP	highly carboxylated porphyrin
h	hours
LBD	ligand binding domain
LD50	lethal dose for 50% of the tested population
Log K_{ow}	log octanol-water partitioning coefficient
LOEL	lowest observable effect level
NWRC	National Wildlife Research Centre
PCB	polychlorinated biphenyl
PCB 77	3,3',4,4'-tetrachlorobiphenyl
PCB105	2,3,3',4,4'-pentachlorobiphenyl
PCB118	2,3',4,4',5-pentachlorobiphenyl
PCB126	3,3',4,4',5-pentachlorobiphenyl
PCB169	3,3',4,4',5,5'-hexachlorobiphenyl
PeCDF	2,3,4,7,8-pentachlorodibenzofuran
Q-PCR	quantitative RT-PCR
ReP	relative potency
ReS	relative sensitivity
RT-PCR	reverse transcriptase-polymerase chain reaction
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzofuran
TEF	toxic equivalency factor
TEQ	toxic equivalency
UROX	uroporphyrinogen oxidation
WHO	World Health Organization
XRE	xenobiotic response element

Statement of collaboration

The stock solutions of the chemicals used in this thesis were prepared, identified and quantified by Dr. Paul Jones of the University of Saskatchewan Toxicology Center (Section 2.3.3.).

Publication plan

Paper 1: Cytochrome P4501A induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin and two chlorinated dibenzofurans in primary hepatocyte cultures of three avian species

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Paper 2: Relative potencies of dioxin-like chemicals in primary herring gull hepatocytes: 2,3,4,7,8-PeCDF is a more potent CYP1A inducer than 2,3,7,8,-TCDD (In preparation)

Authors: Jessica C. Hervé *et al.*

Paper 3: Ethoxyresorufin O-deethylase induction by TCDD, PeCDF and TCDF: time-dependent effect on the concentration-dependant curve (In preparation

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Chapter 1.

Introduction

1.1. General introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is a highly toxic chemical that causes numerous adverse effects in vertebrates including edema, wasting, liver alterations, deformities in newborns and death (Ahlborg *et al.*, 1992; Boening, 1998; Poland and Knutson, 1982; Van den Berg *et al.*, 1998; Whitlock, Jr., 1990). Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and some polychlorinated biphenyls (PCBs) are referred to as 'dioxin-like chemicals' (DLCs), because they cause the same pattern of effects as TCDD. DLCs also alter biochemical responses; one of the best characterized biochemical response is the induction of cytochrome P450 1A (CYP1A). CYP1A induction is not a toxic endpoint *per se*, but it has been shown to be an indicator of toxic responses and is therefore used as a biomarker of susceptibility to the effects of DLCs (Brunström, 1991; Kennedy *et al.*, 1996; Poland and Glover, 1973; Poland and Knutson, 1982; Safe, 1987).

DLC emissions have decreased in the past 30 years, however, because of their environmental persistence, DLCs are still detected in human blood, animal tissues, soil and sediments worldwide (Fang *et al.*, 2007; Fernandes *et al.*, 2007; Hebert *et al.*, 1999a; Kannan *et al.*, 2007; Turrio-Baldassarri *et al.*, 2009). The assessment of the hazard of DLCs is arduous for at least two reasons: first, the **potency** with which DLCs can induce toxic effects varies greatly. This means that if the same species is exposed to different DLCs, one DLC can be highly potent (toxic effects will appear at low doses) while another DLC can be less

potent (toxic effects will occur at higher doses). Second, the **sensitivity** among species varies to a great extent. This means that when different species are exposed to the same chemical, one species can be highly sensitive (toxic effects will appear at low doses) and the other species can be less sensitive (toxic effects will appear at high doses).

Numerous studies assessed the potency of PCDDs and PCBs in birds (Blankenship *et al.*, 2003; Boening, 1998; Brunström and Halldin, 1998; Elliott *et al.*, 1990; Kennedy *et al.*, 1996; Nosek *et al.*, 1992b; Nosek *et al.*, 1992a; Nosek *et al.*, 1993; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). However, few assessed the potency of PCDFs (Bosveld *et al.*, 1992; Heid *et al.*, 2001; Kennedy *et al.*, 1996; Poland and Glover, 1977; Safe, 1993; Sanderson *et al.*, 1998).

The goal of my research was to measure CYP1A induction by TCDD and two PCDFs in domestic and wild avian species. There were two main objectives:

1) To compare the potency of TCDD and two PCDFs as CYP1A inducers in three domestic avian species (Chapter 2) and one free-living avian species (Chapter 3)
2) To compare the sensitivity of three domestic avian species (Chapter 2) and one free-living avian species (Chapter 3) to CYP1A induction by TCDD and two PCDFs

1.2. DLCs: A General Overview

Natural processes, such as forest fires, produce DLCs, but these contaminants come mainly from anthropogenic sources. In general, DLCs are lipophilic, semi-volatile, and chemically stable compounds (Ahlborg *et al.*, 1994; Lohmann and Jones, 1998), but chemical characteristics vary among the 419 existing PCDDs, PCDF and PCBs; thus they behave differently in the environment and in biota.

1.2.1. Sources of DLCs

Environmental contamination by DLCs comes mainly from anthropogenic sources. PCBs are man-made products that were widely commercialized for industrial purposes between 1929 and the late 1970s. Because of their low flammability, low solubility in water, resistance to acids and oxidation, thermal stability, high electrical resistance, and good insulating properties, PCBs were used as coolants and lubricants in capacitors and transformers (Ahlborg *et al.*, 1992; Poland and Knutson, 1982). They were also added to paints, oils, adhesives and pesticides (Ahlborg *et al.*, 1992; Poland and Knutson, 1982). Due to their toxicity and their classification as persistent organic pollutants, the production of PCBs was discontinued in the late 1970s, and levels in the environment declined substantially since then. For example, between 1974 and 2001, the concentration of PCBs in herring gull eggs from islands in the Great Lakes, decreased from about 80 μg/g wet weight (ww) to about 10 μg/g ww (Hebert *et al.*, 1999a; Jeremyn-Gee *et al.*, 2005).

PCDDs and PCDFs (PCDD/Fs) are by-products of different thermal activities involving chlorinated compounds - the incineration of domestic and chemical wastes, and the production of pesticides (e.g., chlorophenoxy herbicides) and PCBs (Everaert and Baeyens, 2002; Hutzinger *et al.*, 1985; Rappe *et al.*, 1991; Rappe, 1992). In the 1960s and 1970s, the contamination of technical products (herbicides and PCBs) was the main source of PCDD/Fs. These products are now regulated or banned, and PCDD/F emissions and levels in the environment are declining. In Sweden, for example, between 1985 and 1991, the total PCDD/F emissions decreased between 2- and 6-fold (Rappe, 1992), and between 1981 and 1991, the level of TCDD in herring gull eggs from the Great Lakes decreased 2- to 10-fold (Bishop *et al.*, 1992; Hebert *et al.*, 1999a; Jeremyn-Gee *et al.*, 2005; Pettit *et al.*, 1996).

Despite the regulation of PCBs and the decrease in levels of PCBs and PCDD/Fs in the environment, these chemicals are still detected in high concentrations in certain areas due to their persistence. For example, in the early 1900s, PCDD/Fs were released from industrial activities into Saginaw Bay (Michigan State, US) and its tributaries (U.S.EPA., 1995). A century later, levels of between 1,260 and 14,800 pg /g dry weight PCDD/Fs are still detected in sediments and flood plain soils of the Tittabawassee River, the main tributary of Saginaw Bay (Hilscherova *et al.*, 2003). Relatively high concentrations were also measured in bird eggs from the same area (Zwiernik *et al.*, manuscripts in preparation).

1.2.2. The role of chemical structure in the characteristics of DLCs

The structure of DLCs is highly variable. PCDDs have two benzene rings linked by two oxygen atoms (Figure 1.1). PCDFs are also composed of two benzene rings, but they are connected by one oxygen atom and a single bond (Figure 1.1). PCBs have two biphenyl rings linked by a single bond (Figure 1.1). Each of these three structures can be substituted with variable numbers of chlorine atoms that can be positioned at various locations. There are more than 75 possible PCDDs, 135 possible PCDFs, and 209 possible PCBs, and the variable structures modulate the physical properties of DLCs and influence the response of biological systems to them.

The low water solubility of DLCs causes them to bind to soil and sediments instead of entering the aqueous phase (Lohmann and Jones, 1998). In the air, depending on the degree of chlorination, they adsorb to particles of different sizes. For example, hexa- to octa-CDD/Fs are distributed on small particles, while the lighter congeners (substituted with less chlorines) are found on larger particles (Lohmann and Jones, 1998).

In living systems, the steps of DLC disposition are influenced by the ring structure and the number and position of the chlorines on the ring structure. Universal rules are rare,

but some trends can be observed (Birnbaum, 1985). As the chlorination increases, so does the lipid solubility, which influences the absorption in the blood. Indeed, the higher the solubility in lipids, the easier it is for the compound to get through the phospholipid cell membrane. Once in the blood, DLCs are distributed mainly in liver, adipose tissue and skin, and the ring structure affects where DLCs are preferentially stored. There are high levels of PCBs in fat compared to levels measured in liver. On the other hand, PCDD/Fs are preferentially stored in liver. In order to be excreted, DLCs have to be metabolised. In a general manner, metabolism will decrease with increasing substitution, and two adjacent unsubstitued carbons will increase metabolism. Finally, the major determinant of excretion seems to be the size of the molecule; the bigger the molecule (higher chlorination), the greater the excretion via the biliary-fecal route compared to the urinal route.

Structure also impacts the downstream effects of DLCs, such as thymic atrophy, the inhibition of body weight gain, and enzyme induction (Poland and Knutson, 1982; Safe, 1987). For example, in immature male Wistar rats, the dose of 2,3,7,8-TCDD that caused thymic atrophy in half of the tested population (ED50) was 0.09 μmol/kg, while the ED50 of 1,3,7,8-TCDD was 100 μmol/kg.

1.2.3. Toxic equivalency factor (TEF) and toxic equivalency (TEQ)

The TEF and TEQ concepts were developed to "facilitate risk assessment and regulatory control of exposure to DLC mixtures" (Van den Berg *et al.*, 1998). Indeed, there are at least two limitations to the risk assessment of DLCs: 1) the potency of DLCs varies greatly, and 2) there are many different DLCs present simultaneously in the environment.

The TEF is the toxicity of a given compound relative to that of TCDD, which has a TEF of 1. To be assigned a TEF, a compound should meet four criteria: 1) show a structural relationship to PCDDs and PCDFs, 2) bind to the aryl hydrocarbon receptor (AHR), 3) induce

dioxin-like biochemical and toxic responses, and 4) be persistent and accumulate in the food chain (Van den Berg *et al.*, 1998; Van den Berg *et al.*, 2006). The TEF value is determined based on the approximated ratio of a given endpoint, such as the dose that kills 50% of the tested population (Lethal dose 50 [LD50]). The value of the endpoint for TCDD is divided by the value of the compound of interest (e.g. $LD50_{TCDD}$ / $LD50_{compound\ X}$ = TEF). For example, in chicken embryos, the LD50 of TCDD is 0.15 μg/kg and of 3,3',4,4',5-pentachlorinated biphenyl (PCB 126) is 2.3 μg/kg. Thus, the value obtained for PCB 126 was 0.07 (0.15/2.3 = 0.07), therefore the TEF was rounded to 0.1, indicating that PCB 126 is 10-times less potent than TCDD in chicken (Powell *et al.*, 1996; Van den Berg *et al.*, 1998). Based on the assumption that the effects of individual congeners are additive, the TEQ of a mixture is the sum of the amount of each chemical in the mixture, multiplied by its respective TEF. Thus the TEQ of a mixture containing 100 μg/kg of TCDD and 100 μg/kg of PCB 126 is 110 μg/kg. It should be noted that, if the potency of a compound relative to TCDD is derived from a single study, the term "relative potency" (ReP) should be used instead of TEF.

The TEF and TEQ concepts were criticized because: 1) additive effects are not always observed, 2) the TEFs assigned by the World Health Organization (WHO) are not always consistent with the RePs and TEFs derived from experimental studies, and 3) dose-response curve shapes are different among compounds (Harper *et al.*, 1995; Harris *et al.*, 1993; Toyoshiba *et al.*, 2004). Moreover, TEFs are assigned for broad groups of organisms (e.g. mammals, birds, fishes), but are based on experiments carried upon a limited number of species (e.g. laboratory rodents, chicken, and rainbow trout) (Van den Berg *et al.*, 1998). Sensitivities to DLCs vary greatly among species of the same group and the relevance of TEFs for specific species can be contested. TEFs are used worldwide to assess the effects of

dioxin-like mixtures to human and wildlife health, but they should be used with caution when extrapolating the results from one species to another.

1.2.4. Toxicity of DLCs in birds

Effects of DLCs from environmental exposure on wild birds were investigated in the 1940s in the Great Lake area, but most studies were carried out in the laboratory with chicken. Some studies have been carried out with pheasant, turkey, and certain wildlife species.

Environmental exposure

Starting in the late 1940s, birds in the Great Lakes began to show signs of reproductive impairment and the avian population began to decline. Researchers observed numerous signs of toxicity including embryonic and chick mortality, growth retardation, developmental abnormalities such as bill, eye and foot deformities, as well as liver enlargement and necrosis, edema, and porphyria (Gilbertson *et al.*, 1991; Gilbertson and Fox, 1997). These signs of toxicity, designated as the Great Lakes Embryo Mortality, Edema and Deformities Syndrome (GLEMEDS), were linked to the presence of organochlorine contaminants, such as TCDD and PCBs, in that area (Gilbertson, 1983; Gilbertson *et al.*, 1991).

Laboratory exposure

Eye and beak deformities, edema, liver lesions, thymus atrophy, and death were observed in chicken, ring-necked pheasant, and turkey embryos exposed to TCDD or 3,3',4,4'-tetrachlorobiphenyl (PCB 77) (Blankenship *et al.*, 2003; Brunström and Darnerud, 1983; Brunström and Lund, 1988; Nosek *et al.*, 1993; Poland and Knutson, 1982). TCDD also reduced egg production and hatchability in female ring-necked pheasants (Nosek *et al.*, 1992a). Sanderson and Bellward (1995) also observed edema and death in pigeon, great blue heron, and double-crested cormorant embryos exposed to TCDD.

1.3. A short history of DLCs

1.3.1. Events that raised concerns about DLCs

DLCs have been contaminating the environment for the last century, but they were brought to the attention of the public and scientific community around the 1960s. Three events stimulated research on TCDD and other DLCs. First, in 1957, there was an outbreak of chicken death caused by the "chick edema factor", later found to be 1,2, 3, 7, 8, 9-hexachlorodibenzo-*p*-dioxin (HxCDD) (Cantrell *et al.*, 1969; Firestone, 1973). Second, between 1961 and 1971, the US army sprayed millions of litres of the herbicide Agent Orange, which was contaminated with TCDD, on Vietnam forests and crops, and many American soldiers as well as Vietnamese soldiers and civilians were exposed to it (Stellman *et al.*, 2003). Third, in 1976, the workers and residential population of the town of Seveso (Italy) were exposed to TCDD after a spill from a chemical manufacturing plant (Bertazzi *et al.*, 1998). This incident is commonly referred to as the "Seveso disaster". Following these events, numerous epidemiological and laboratory studies were conducted on DLCs and most researchers agreed that TCDD was highly toxic and should be considered the most potent DLC.

1.3.2. TCDD considered as the most potent DLC

Chicken edema disease and the first comparative laboratory studies

In the late 1950s, a great number of chickens were poisoned through feed fats that were contaminated with HxCDD, the "chick edema factor" (Cantrell *et al.*, 1969; Firestone, 1973). Death was accompanied with the presence of fluid in the heart sac and in the abdominal cavity, subcutaneous edema, and liver necrosis (Firestone, 1973). The first comparative study on DLCs, which assessed chicken embryo mortality, demonstrated that TCDD was the most toxic among the tri-, tetra-, hexa-, hepta- and octa-chlorodibenzo-*p*-

dioxin congeners (Higginbotham *et al.*, 1968). Subsequent comparative studies and reviews on DLCs also came to the conclusion that TCDD was the most potent DLC to induce biochemical and toxic responses in mammals, fishes and birds (Brunström *et al.*, 1991; Kennedy *et al.*, 1996; McConnell *et al.*, 1978; Poland and Glover, 1973; Safe, 1987; Schwetz *et al.*, 1973; Van den Berg *et al.*, 1998).

Toxicity in humans

Researchers investigated several potential effects of TCDD and other DLCs in humans including dermatologic, cardiac, pulmonary and digestive troubles, endocrine and reproductive impairment, and carcinogenicity. It is difficult to draw strong conclusions from human exposure due to the limitations of epidemiological studies including variable exposure among individuals, unknown exposure levels, small sample sizes, inconsistent follow-up, early death in highly exposed populations, and simultaneous exposure with other chemicals. Reviews and meta-analyses demonstrated that exposure to TCDD and other DLCs was linked with toxic effects in humans, but chloracne was the only symptom found to be clearly correlated with TCDD exposure (Ahlborg *et al.*, 1992; Bertazzi *et al.*, 1998; Crump *et al.*, 2003; Guo *et al.*, 2004; Ngo *et al.*, 2006; Pesatori *et al.*, 2003; Steenland *et al.*, 2004; Sweeney and Mocarelli, 2000). No study compared the potency of DLCs to induce toxic effects in humans, but based on findings in human cell lines and in other mammals, TCDD has generally been considered the most potent DLC in humans (Carlson *et al.*, 2009; Poland and Knutson, 1982; Schwetz *et al.*, 1973; Silkworth *et al.*, 2005; Van den Berg *et al.*, 1998).

1.4. The mechanism of action of DLCs

DLCs elicit biological responses through a common mechanism of action (Figure 1.2). The first step consists of the binding of a DLC to the AHR[†], a receptor that is a member of the bHLH-PAS[‡] family of transcription factors (Bock, 1994; Bohonowych and Denison, 2007; Mimura and Fujii-Kuriyama, 2003; Okey *et al.*, 2005; Poland *et al.*, 1976). Upon binding with a DLC, the AHR translocates into the nucleus. Subsequently, the DLC-AHR complex dimerizes with the aryl hydrocarbon receptor nuclear translocator and binds to the xenobiotic responsive element (XRE) on DNA (also called dioxin response enhancer [DRE]). The heterodimer, attached to DNA, interacts with coactivators and alter the transcription of genes of phase I and phase II metabolism as well as genes involved in cell proliferation and cell cycle regulation (Landers and Bunce, 1991; Mimura and Fujii-Kuriyama, 2003; Okey *et al.*, 2005).

Although the mechanism of AHR binding and activation is well described, the mechanism by which these compounds induce harmful effects has not yet been elucidated. However, the involvement of the AHR in DLC toxicity has been shown. Indeed, the foetuses of AHR-null mice were insensitive to the teratogenic effects of TCDD, such as cleft palate and hydronephrosis (Mimura *et al.*, 1997). Moreover, AHR activation by DLCs has been shown to correlate with toxic endpoints such as cardiotoxicity (Heid *et al.*, 2001), embryotoxicity (Head and Kennedy, 2009; Kennedy *et al.*, 1996), body weight loss, and thymic atrophy (Safe, 1987).

[†] Mammalian species possess a single AHR, but there are at least two forms of the AHR in birds and fishes: AHR1 and AHR2 (Hahn *et al.*, 2006). In birds, AHR1 is the most transcriptionally active form, but in fishes AHR2 is the dominant form (Hahn *et al.*, 2006; Yasui *et al.*, 2007). Therefore, AHR1 will be used for details about birds, but the term 'AHR' will be used to refer to other taxa and for general comments

[‡] Basic helix-loop-helix Per-Arnt-Sim homology

1.5. Biomarkers of DLCs

Biomarkers are defined as "xenobiotically induced alterations in cellular or biochemical components or processes, structures or functions that are measurable in a biological system or sample," by the National Academy of Sciences (Klassen, 2001). They can be used to assess the exposure to a chemical (biomarker of exposure), the effect of the chemical (biomarker of effect), or the susceptibility of the biological system or sample to the adverse effects caused by this chemical (biomarker of susceptibility). In the area of DLCs, cytochrome P450 1A induction and porphyrin accumulation are used for this purpose.

1.5.1. CYP1A: a predictor of toxic effects in birds

CYPs are membrane-bound hemoproteins mostly found in the liver, but they are also present in the heart and kidneys and small intestine (Gilday *et al.*, 1996; Nebert and Gonzalez, 1987). The CYP1A isoforms are phase I metabolizing enzymes involved in the oxidation of various endogenous substrates such as steroids, fatty acids and prostaglandins (Denison and Nagy, 2003; Nakai *et al.*, 1992; Nebert and Gonzalez, 1987). Numerous xenobiotics induce these enzymes, including drugs, environmental pollutants, and plant metabolites (Conney, 1967; Denison and Nagy, 2003; Guengerich, 2008; Waterman and Estabrook, 1983).

TCDD induces two isoforms of CYP1A in mammals (CYP1A1 and CYP1A2) (Silkworth *et al.*, 2005; Whitlock, Jr., 1990) and in birds (CYP1A4 and CYP1A5) (Gilday *et al.*, 1996; Rifkind *et al.*, 1994). The avian CYPs are not directly orthologuous to the mammalian CYPs, because neither CYP1A4 nor CYP1A5 is more similar to CYP1A1 or CYP1A2 with respect to: 1) amino acid sequence, 2) enzymatic and immunological characteristics, 3) constitutive and TCDD-induced expression, and 4) half-life (Sinclair *et al.*, 1997). In birds, CYP1A4 is responsible for TCDD-induced 7-ethoxyresorufin *O*-deethylase

(EROD) (Figure 1.3) and arylhydrocarbon hydroxylase (AHH) activities, while CYP1A5 is involved in the TCDD-induced arachinodate metabolism as well as in uroporphyrinogen oxidase activity (Rifkind *et al.*, 1994; Sinclair *et al.*, 1997).

CYP1A induction can be measured through enzymatic assays (Bosveld *et al.*, 1992; Hahn *et al.*, 1996; Kennedy *et al.*, 1996; Poland and Knutson, 1982), through CYP1A protein induction (Hahn *et al.*, 1996; Kennedy *et al.*, 1995), and through the expression of CYP1A messenger RNA (mRNA) (Head and Kennedy, 2007b; Hestermann *et al.*, 2000; Jones and Kennedy, 2009; Watanabe *et al.*, 2005). The direct involvement of CYP1A in toxicity is not yet clear; CYP1A was shown to contribute to TCDD-induced lethality and wasting syndrome (Uno *et al.*, 2004), but not in hepatotoxicity (Nukaya *et al.*, 2009). However, it was shown that the potencies of compounds to induce CYP1A were predictive of their potencies to elicit toxic effects in birds (Brunström, 1991; Head and Kennedy, 2009; Kennedy *et al.*, 1996). CYP1A induction is therefore considered a biomarker of susceptibility to the toxic effects DLCs in avian species.

Studies in wild birds

CYP1A induction has been shown to correlate with DLC levels in tree swallows (Papp *et al.*, 2005) and double-crested cormorants (Custer *et al.*, 2001). However, the effectiveness of EROD as a biomarker can be affected by the length and route of exposure, diet, age and sex (Ahlborg *et al.*, 1992; Augspurger *et al.*, 2008; Custer *et al.*, 2001; Head, 2006; Poland and Knutson, 1982; Toyoshiba *et al.*, 2004). For example, no correlation between levels of PCBs and EROD activity was seen in 10-day-old double-crested cormorant nestlings (Custer *et al.*, 2001) or in adult herring gulls (Kennedy *et al.*, 2003). However, EROD induction was shown to correlate with DLC exposure in double-crested cormorant embryos (Custer *et al.*, 2001) and in herring gull embryonic cell cultures

(Kennedy *et al.*, 1996). Thus, EROD induction in embryos and embryonic cell cultures has a good potential as a biomarker of exposure to DLCs.

Studies in laboratory

In ovo and *in vitro* studies demonstrated that the compounds that were the most potent at inducing EROD activities were also the most toxic in chicken embryos (Brunström, 1992; Kennedy *et al.*, 1996). Kennedy *et al.* (1996) compared the EROD-inducing potencies of PCBs in avian embryonic cell cultures to their potency to cause lethal effects in embryos. They found that the rank order of potency was the same for EROD induction and embryo mortality: PCB 126 > PCB 77 > PCB 169 > PCB 105 > PCB 118. A recent review showed the correlation between the *in vitro* and *in ovo* potency of TCDD, PCB126, PCB77, PCB169, PCB 105, PCB 157 and PCB 156 in chicken was significant ($r^2 = 0.93$, $p < 0.005$) (Head and Kennedy, 2009). These results demonstrate that the EROD-inducing potencies of DLCs in cell cultures are predictive of their potency to cause death *in ovo.*

The same study by Kennedy *et al.* (1996) also showed that there was a correlation between sensitivity to EROD induction in hepatocyte cultures of different bird species and their sensitivity to lethal effects *in ovo.* Indeed, the study found that ring-necked pheasants were 5- to 10-times less sensitive than chickens to EROD induction and to lethal effects induced by TCDD. Moreover, the rank order of sensitivity of four species of birds exposed to PCB 77 was the same for embryonic mortality and for EROD induction: chicken > turkey > duck ≥ herring gulls. The review mentioned above also found that the correlation between EROD EC50 and the embryonic LD50 of TCDD and PCD126 in chicken, ring-necked pheasant, double-crested cormorant, turkey and common tern was significant ($r^2 = 0.92$, $p < 0.005$). Therefore, EROD induction in embryonic hepatocyte cultures can predict the sensitivity of avian species to embryo-lethality induced by DLCs (Head and Kennedy, 2009).

CYP1A induction in avian cell cultures can also be assessed by measuring the mRNA expression of the CYP1A4/5 transcripts. Investigators exposed embryonic chicken hepatocyte cultures to TCDD and measured EROD induction and mRNA expression of CYP1A4 and CYP1A5 (Head and Kennedy, 2007b). They showed that the dose-response curves for EROD and mRNA induction were superimposable. However, while CYP1A mRNA induction reached a plateau, EROD activity showed a decrease at higher concentrations. This decrease is caused by the competitive inhibition of the enzyme by the inducer (TCDD in this case) (Gooch *et al.*, 1989; Petrulis and Bunce, 1999). In some cases, this could lead to an overestimation of the potency of a compound (see Appendix 1). Because mRNA induction is not affected by the competitive inhibition, in certain circumstance, it could be a more accurate method than the EROD assay to measure the potency of DLCs (Head and Kennedy, 2007b).

1.5.2. Porphyrins: Biomarkers of exposure

Porphyrins are tetrapyrrolic pigments found in eggshells and feathers. They are also involved in the heme biosynthesis pathway (De Matteis and Lim, 1994). Porphyrins are either intermediates in the heme pathway (protoporphyrins) or oxidized by-products (uroporphyrins and coproporphyrins) of other intermediates (uroporphyrinogen and coproporphyrinogen) and are naturally present in blood, liver, kidney, and excreta (Casini *et al.*, 2003; De Matteis and Lim, 1994). Low concentrations of porphyrins can be detected in tissues and exposure to chemicals can alter their profile (Ahlborg *et al.*, 1992; Lorenzen and Kennedy, 1995; Sano *et al.*, 1985; Whitlock, Jr., 1993). Therefore, they are considered as biomarkers of exposure (De Matteis and Lim, 1994).

The abnormal accumulation and excretion of porphyrins due to the alteration of heme biosynthesis results in a disorder called porphyria (Kennedy and Fox, 1990). It can be caused

by an inborn defect or chemically induced by numerous compounds including unsaturated chemicals (e.g. ethylene), drugs (e.g. griseofluvin), metals (e.g. lead and mercury) and halogenated aromatic hydrocarbons (e.g., hexachlorobenzene, TCDD, PCBs) (De Matteis and Lim, 1994; Kennedy and Fox, 1990). In order to identify DLC-induced porphyria in birds, elevated levels of highly carboxylated porphyrins (HCPs), such as uroporphyrins, hepta- and hexa-carboxylic acid porphyrins, need to be measured (Kennedy and Fox, 1990). Elevated levels of HCPs were measured in wild birds from contaminated areas (Kennedy and Fox, 1990), but also in embryonic cell cultures exposed to DLCs (Lorenzen *et al.*, 1997). Using AHR knock-out mice, it was recently shown that the AHR pathway was involved in TCDD-induced uroporphyria in mice (Davies *et al.*, 2008). Indeed, when exposed to TCDD, the $AHR^{+/+}$ and the $AHR^{+/-}$ mice showed an increase in hepatic uroporphyrin of 964- and 60-fold, respectively, while no increase was measured in the $AHR^{-/-}$ mice.

1.6. Differential potencies among DLCs and differential sensitivity among species

As mentioned earlier, different DLCs demonstrate considerable variability with respect to potency (e.g., some compounds are more potent than others when tested in a particular species) and sensitivity (e.g., some species are more sensitive than others to the effects of a given compound).

1.6.1. Differential potency: which compound is the most potent?

Studies looking at embryo mortality or EROD-inducing potencies highlighted that, when tested in a given species, different DLCs display different potencies. In general, TCDD, 2,3,7,8-tetrachlorodibenzofuran (TCDF) and non-ortho substituted PCBs (all of which exhibit a co-planar conformation), are more potent CYP1A inducers than the mono- or di-

ortho substituted PCBs, a condition that induces non-planarity (Hestermann *et al.*, 2000; Kennedy *et al.*, 1996; Poland and Knutson, 1982).

In chicken, TCDF was shown to be equipotent to TCDD, and PCB 126 and PCB 77, two non-ortho substituted PCBs, were shown to be 3 to 6- and 28 to 33-times less potent than TCDD at inducing toxic or biochemical effects, respectively (Head *et al.*, 2008; Kennedy *et al.*, 1996). On the other hand 2,3',4,4',5-pentachlorobiphenyl (PCB 118), a mono-ortho substituted PCB, was shown to be at least 1,250-times less potent than TCDD with respect to EROD induction (Kennedy *et al.*, 1996).

This variation in potency can be explained, at least in part, by the binding affinity of the compound to a cytosolic receptor; that is, the strength by which the ligand (the DLC in this case) binds to its receptor (the AHR in this case) (Hestermann *et al.*, 2000; Poland *et al.*, 1976; Poland and Knutson, 1982; Safe, 1986; Whitlock, Jr., 1990). Hestermann *et al.* (2000) tested ten DLCs in a fish cell line (HLPC-1) and compared their CYP1A-inducing potencies with their binding affinities. They demonstrated that the compounds that exhibited a greater CYP1A-inducing potency were also those that had a greater AHR binding affinity. Other factors, such as intrinsic efficacy and events downstream of AHR-binding (e.g., transactivation), can also influence the potency of DLCs (Carlson *et al.*, 2009; Hestermann *et al.*, 2000).

As mentioned earlier, TCDD is generally recognized as the most potent DLC. However, certain studies found that 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 1,2,3,7,8,-pentachlorodibenzo-*p*-dioxin (PeCDD) were more potent EROD inducers than TCDD (Rankouhi *et al.*, 2005; Sanderson *et al.*, 1998). Sanderson *et al.* (1998) found that in double-crested cormorant and Forster's tern hepatocyte cultures, PeCDD and PeCDF were 10- to 20-times more potent than TCDD at inducing EROD activity. However, the maximal

EROD activity of PeCDF was lower than TCDD in double-crested cormorants, and the maximal EROD activity of PeCDF was not provided for the Forster's tern. Because maximal activity can influences EC50 and relative potency values (see Appendix 1), another method, such as the Lowest Observed Effect Concentration (LOEC), EC10 or the ECthreshold (ECthr; see Appendix 1) could have been used to compare the potency among compounds. In green frog hepatocytes, PeCDF was also found to be about 10-times more potent than TCDD in terms of EROD induction (Rankouhi *et al.*, 2005). In this case, the comparison between EC50 values was more accurate as PeCDF reached the same maximal activity as TCDD.

1.6.2. Differential sensitivity: which species is the most sensitive?

Sensitivities to the toxic effects of DLCs range over three orders of magnitude among tested vertebrate species. One of the most cited examples is the case of the guinea pig and the hamster; the guinea pig is 580- to 2000-times more sensitive than the hamster. Indeed, the acute oral LD50 for the guinea pig is between 0.6 and 2 μg/kg compared to 1157 μg/kg for the hamster (Olson *et al.*, 1980; Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982).

Among birds, the chicken is the most sensitive species to DLCs tested to date (Brunström and Lund, 1988; Head *et al.*, 2008; Hoffman *et al.*, 1998; Kennedy *et al.*, 1996), Differential sensitivity among bird species can partially be explained by the differential AHR binding affinity and transactivation among species (Brunström and Lund, 1988; Karchner *et al.*, 2006; Sanderson and Bellward, 1995). A molecular study identified two sites in the AHR1 ligand binding domain (LBD), corresponding to Ile324 and Ser380 in the chicken, that were responsible for the variable binding affinity of TCDD to the AHR1 and for the variable AHR1 transactivation by TCDD (Karchner *et al.*, 2006). According to a review of the literature on egg injection studies, the chicken was the most sensitive species and was the only species that had the Ile/Ser genotype (Head *et al.*, 2008). Moderately sensitive species

(turkey, ring-necked pheasant and eastern bluebird) had the Ile/Ala genotype and the least sensitive species had the Val/Ala genotype (American kestrel, common tern, double-crested cormorant, Japanese quail, herring gull and duck). The authors suggested that the AHR1 LBD sequence could be predictive of three broad groups of DLC sensitivity: 1) high sensitivity (Ile/Ser), 2) moderate sensitivity (Ile/Ala) and 3) low sensitivity (Val/Ala) (Head *et al.*, 2008). It was also suggested that other non-molecular species-specific characteristics could be involved in differential sensitivity, such as uptake, metabolism and excretion rates (Head *et al.*, 2008). It is also worth noting that wild species are pre-exposed to DLCs, which could affect their response to DLCs when exposed in the laboratory. An interesting point that this review highlighted is that chicken, ring-necked pheasant and Japanese quail, three species of the Galliform order, show differential sensitivity. Indeed, ring-necked pheasant and Japanese quail are respectively 12- and more than 218-times less sensitive than chicken to the embryotoxic effects of the DLCs studied to date, and their AHR1 LBD genotypes correspond to the three groups of sensitivity listed above (Head *et al.*, 2008).

1.7. The disposition of DLCs

The disposition of DLCs has been shown to vary among compounds. For example, in orally administered rats, TCDD had a whole body half-life of about 31 days (Rose *et al.*, 1976), while TCDF half-life was shorter than two days (Birnbaum *et al.*, 1980). Moreover, when injected intravenously in rats, the half-life of 2,3,4,7,8-PeCDF was found to be 64 days (Brewster and Birnbaum, 1987), compared to less than two days for 1,2,3,7,8-PeCDF (Brewster and Birnbaum, 1988). Because metabolism is a prerequisite for the excretion of these compounds, differences in half-lives were attributed to differences in metabolism.

Difference in metabolism among compounds was also seen in embryonic cell cultures. A decrease of 90-fold in the apparent potency of PCB 77 as an EROD inducer was observed between 24 and 72h, compared to a decrease of less than 10-fold for TCDD, TCDF and PCB 126 (Bastien *et al.*, 2007). After 48h, only 7% of PCB 77 was recovered, along with metabolites, while most of the PCB 126 was still present in the cell culture well. The authors of the study concluded that the decrease in the potency of PCB 77 as an EROD inducer, compared to TCDD, TCDF and PCB 126, was due to a higher metabolism of this compound.

1.8. Thesis overview

1.8.1. Rationale

Due to the high concentrations of DLCs monitored in the Tittabawassee River area, Michigan (USA), a large risk assessment was undertaken by a multi-disciplinary team from different universities. PCDFs are the chemicals contributing most to the TEQ concentrations in this area, yet little is know about their potency as CYP1A inducers in birds.

My research assessed the downstream effects of PeCDF and TCDF on AHR-mediated responses (i.e. CYP1A induction) in avian embryonic hepatocyte cultures of three laboratory species and one wild species: the chicken (*Gallus gallus*), the ring-necked pheasant (*Phasianus colchicus*), the Japanese quail (*Coturnix japonica*), and the herring gull (*Larus argentatus*).

1.8.2. Objectives and hypotheses

The two main objectives of my research were as follows:

1) To determine the potencies of TCDD, PeCDF, and TCDF as CYP1A inducers in chicken, ring-necked pheasant, Japanese quail and herring gull hepatocytes.

2) To determine the sensitivity of chicken, ring-necked pheasant, Japanese quail and herring gull hepatocytes to CYP1A induction when exposed to TCDD, TCDF or PeCDF.

The hypotheses pertaining to these objectives are as follows:

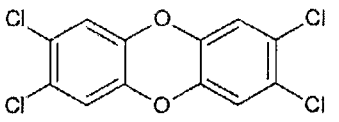
1) TCDD, PeCDF and TCDF will be approximately equipotent in each species.
2) The rank order of sensitivity will be as follows for each compound: chicken > ring-necked pheasant > Japanese quail = herring gull

1.8.3. Species selected

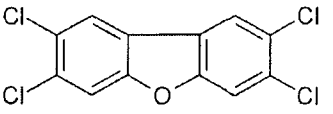
The species were chosen based on work from Head *et al.* (2008) and Karchner *et al.* (2006). They showed that, based on their *in ovo* sensitivity to DLCs and their AHR1 LBD genotype, chickens are highly sensitive to DLCs (type 1), ring-necked pheasants are moderately sensitive to DLCs (type 2) and Japanese quails have low sensitivity to DLCs (type 3). Thus, they are representative of the range of sensitivities found in birds. The herring gull, a type 3 species, is a good bio-indicator species due to its high trophic level and history of exposure to chemicals (Hebert *et al.*, 1994; Hebert *et al.*, 1999a; Hebert *et al.*, 1999b).

1.8.4. Cell culture and CYP1A induction

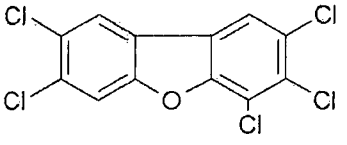
CYP1A induction in embryonic hepatocyte cultures is a convenient and pertinent endpoint to evaluate the potencies of DLCs in birds. First, the use of cells reduces the number of individuals needed to carry out the experiment. Second, it is time- and cost-effective compared to egg injection studies. Third, factors that could affect CYP1A induction, such as age, diet and stress, can be controlled. Finally, CYP1A induction in embryonic cell cultures has been shown to correlate with embryo-lethality, which confirms its relevance as a biomarker of susceptibility to the effects of DLCs.



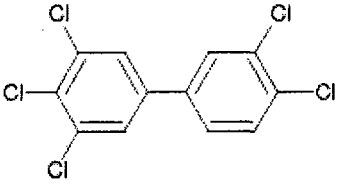
TCDD



TCDF



PeCDF



PCB 126

Figure 1.1. Chemical structures of 2,3,7,8-terachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), 2,3,7,8-tetrachlorodibenzofuran (TCDF) and 3,3',4,4',5-pentachlorinated biphenyl (PCB 126)

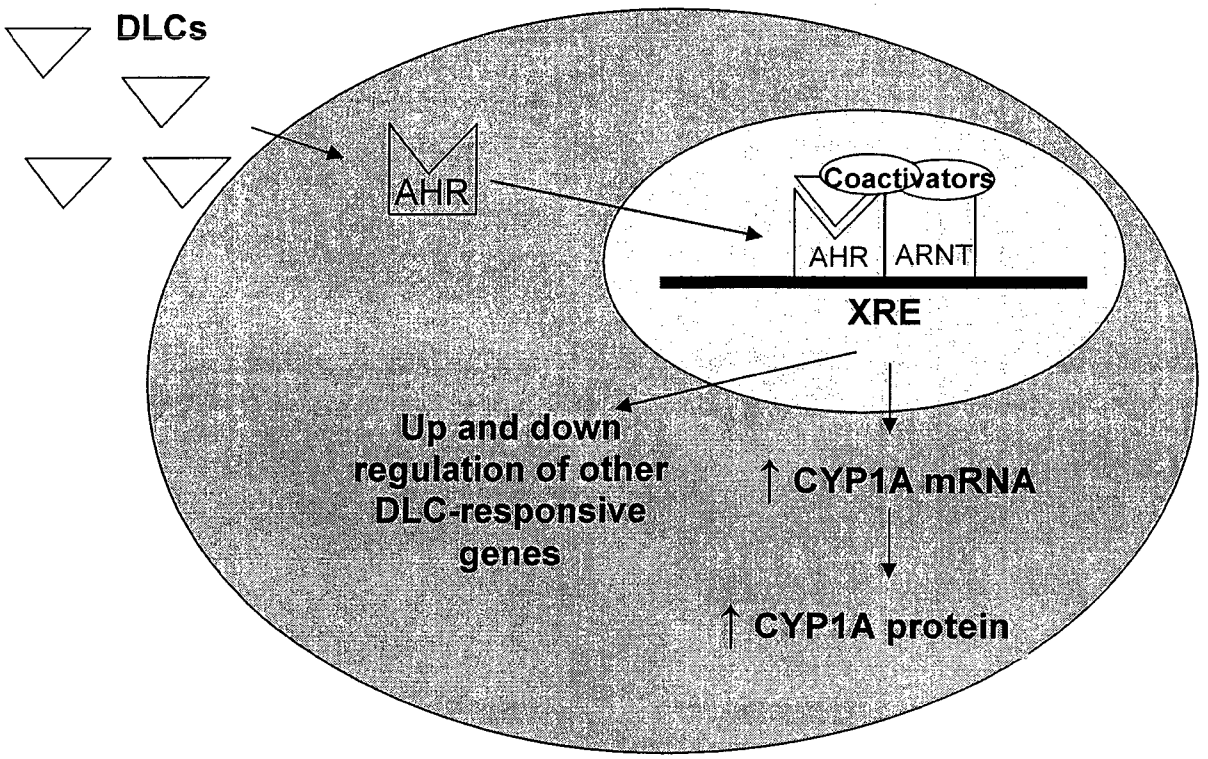


Figure 1.2. Simplified diagram of the aryl hydrocarbon receptor (AHR) pathway. When bound to a dioxin-like compound (DLC), the AHR translocates into the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). Subsequently, this heterodimer binds to the xenobiotic responsive element (XRE) on DNA. The complex, attached to DNA, interacts with coactivators and induces the transcription of various genes of phase I and phase II metabolism, such as cytochrome P4501A (CYP1A), as well as genes involved in cell proliferation and cell cycle regulation. This diagram is adapted from a figure in Okey *et al.* (2005).

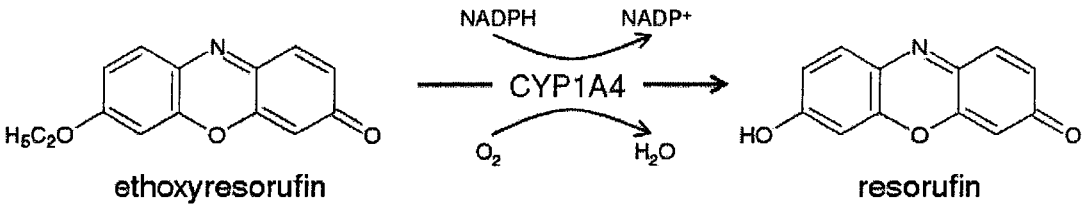


Figure 1.3. Conversion of ethoxyresorufin by cytochrome P450 1A4 (CYP1A4) in the ethoxyresorufin *O*-deethylase (EROD) assay

Chapter 2

Cytochrome P4501A induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and two chlorinated dibenzofurans in cultured hepatocytes of three avian species[†]

2.1 Abstract

Potencies of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were determined in primary hepatocyte cultures of chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Corturnix japonica*) embryos. Concentration-dependent effects on ethoxyresorufin *O*-deethylase (EROD) activity, and expression of cytochrome P4501A4 and 1A5 (CYP1A4 and CYP1A5) mRNA were determined in hepatocytes exposed to serial dilutions of TCDD, PeCDF or TCDF for 24 h. In chicken hepatocytes, the three compounds were equipotent inducers of EROD activity and CYP1A4/5 mRNA expression. However, in ring-necked pheasant and Japanese quail hepatocytes, PeCDF was more potent than TCDD (3- to 5-fold in ring-necked pheasant; 13- to 30-fold in Japanese quail). Among species, the rank order of sensitivity (most to least) to EROD and CYP1A4/5 mRNA expression for TCDD and TCDF was: chicken > ring-necked pheasant > Japanese quail. In contrast, the three species were approximately equisensitive to EROD and CYP1A4/5 mRNA induction by PeCDF. It has generally been assumed that TCDD is the most potent 'dioxin-like compound' (DLC) and that the chicken is the most sensitive avian species to CYP1A induction by all DLCs. This study indicates that PeCDF is

[†] Adapted from Hervé, J.C., Crump, D.E., Mundy, L.J., Jones, S.P., Zwiernik, M.J., Bursian, S.J. Giesy, J.P., Jones, P.D., Wiseman, S.B, Wan,Y., Kennedy, S.W. [submitted for publication to Toxicological Sciences]

more potent than TCDD in ring-necked pheasant and Japanese quail hepatocytes, and that ring-necked pheasant, Japanese quail and chicken hepatocytes are equally sensitive to CYP1A induction by PeCDF. These results raise questions regarding (a) the molecular mechanisms of DLC toxicity in birds, and (b) the currently accepted avian-specific toxic equivalency factors assigned by the World Health Organization.

2.2. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are toxic and persistent contaminants generated unintentionally during the incineration of wastes and during the production of chlorine, polychlorinated biphenyls (PCBs) and some pesticides (Hutzinger *et al.*, 1985; Rappe, 1992). PCDD/Fs and some PCB congeners are referred to as 'dioxin-like chemicals' (DLCs) because they induce similar toxic and biochemical effects to those caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). It is generally accepted that TCDD is the most biologically potent DLC. In avian species, effects of DLC exposure include: reduced egg production and hatchability, teratogenicity, edema, liver toxicity, porphyria and enzyme induction (Casini *et al.*, 2003; Kennedy *et al.*, 1996; Poland and Knutson, 1982).

The molecular mechanisms of DLC toxicity are not fully understood, but the involvement of the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, has been demonstrated in various studies (Bohonowych and Denison, 2007; Mimura and Fujii-Kuriyama, 2003; Poland and Knutson, 1982). When DLCs bind to the AHR, the DLC-AHR complex enters the nucleus, binds to the xenobiotic response element on DNA, and interacts with transcriptional cofactors to alter the transcription of numerous genes, including increased transcription of cytochrome P4501A (CYP1A) genes (Mimura and Fujii-Kuriyama,

2003). CYP1A induction is not a toxic response *per se*, because the relationship between CYP1A gene expression and the occurrence of toxic effects is not clear (Nukaya *et al.*, 2009; Okey *et al.*, 2005; Uno *et al.*, 2008). Nonetheless, CYP1A induction has been shown to be predictive of the relative toxicity of DLCs in avian embryos (Brunström *et al.*, 1991; Kennedy *et al.*, 1996) and other species (Safe, 1987).

Two major CYP1A isoforms induced by TCDD in chicken embryo liver were characterized several years ago by Rifkind and colleagues (Gilday *et al.*, 1996; Rifkind *et al.*, 1994; Sinclair *et al.*, 1997). CYP1A4 exhibits catalytic specificity for aryl hydrocarbon hydroxylase and ethoxyresorufin *O*-deethylase (EROD). CYP1A5 is principally responsible for the epoxygenation of the endogenous fatty acid arachidonic acid and also exhibits catalytic specificity for uroporphyrinogen oxidase. Measurement of EROD activity (Kennedy *et al.*, 1993; Kennedy *et al.*, 1996; Sanderson *et al.*, 1998) and the characterization of CYP1A4 and CYP1A5 messenger RNA (mRNA) expression (Head and Kennedy, 2007a; Head and Kennedy, 2007b; Jones and Kennedy, 2009; Mahajan and Rifkind, 1999) are practical methods to quantify CYP1A induction in birds.

Despite the fact that all DLCs cause a similar pattern of effects and that they act through the same mechanism of action, there is a wide range of potency among compounds. For example, in chicken, the potency of compounds to induce embryotoxic or biochemical responses ranges over three orders of magnitude (Head *et al.*, 2008; Kennedy *et al.*, 1996; Van den Berg *et al.*, 1998); TCDD is generally considered to have the greatest potency to elicit these responses (Brunström, 1991; Kennedy *et al.*, 1996; Poland and Knutson, 1982; Van den Berg *et al.*, 1998). Furthermore, there is a wide range of sensitivities to DLCs among species. For example, the chicken (*Gallus gallus*), which is considered the most sensitive avian species, is over 1000-times more sensitive than the mallard duck (*Anas*

platyrhynchos) to the embryo-lethal effects of DLCs (Head *et al.*, 2008). As mentioned previously, a range of sensitivities is also observed among species within the order of Galliformes; for the DLCs selected for review by Head *et al.* (2008), the rank order of sensitivity was chicken > ring-necked pheasant (*Phasianus colchicus*) > Japanese quail (*Coturnix japonica*).

In this study CYP1A induction by TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) was measured in chicken, ring-necked pheasant and Japanese quail embryonic hepatocytes. These species were chosen because they are representative of the broad range of sensitivities to DLCs among birds. PeCDF and TCDF were the DLCs selected for this study because little is known regarding their potency to induce CYP1A in birds and because their possible effects on birds could be of concern in certain parts of North America (Hilscherova *et al.*, 2003) and elsewhere (Kiguchi *et al.*, 2007). The objectives of this study were to use embryonic primary hepatocyte cultures to: 1) compare the potencies of TCDD, PeCDF and TCDF as CYP1A inducers in each species; 2) compare the sensitivities of chicken, ring-necked pheasant and Japanese quail hepatocytes when exposed to TCDD, PeCDF or TCDF; and, 3) characterize the relative expression of CYP1A4 mRNA and CYP1A5 mRNA in the three species when exposed to TCDD, PeCDF or TCDF.

2.3. Materials and methods

2.3.1. Source of eggs and incubation conditions

Chicken eggs were obtained from the Canadian Food Inspection Agency (Ottawa, ON, Canada); ring-necked pheasant and Japanese quail eggs were obtained from Couvoir Simetin (Mirabel, QC, Canada). Eggs from each species were incubated at 37.5 °C and 60%

relative humidity until 1-3 d pre-hatch. Chicken eggs were incubated for 19 d, ring-necked pheasant eggs for 23 d and Japanese quail eggs for 16 d. Eggs were candled periodically and infertile eggs and eggs containing dead embryos were discarded.

2.3.2. Preparation and dosing of cultured hepatocytes

Primary cultures of hepatocytes were prepared from avian embryos using methods described elsewhere (Kennedy *et al.*, 1995) including subsequent modifications (Head and Kennedy, 2007b). All procedures were conducted according to protocols approved by the animal care committee at the National Wildlife Research Centre. Reagents were obtained from Sigma (St. Louis, MO, USA) unless another supplier is indicated. In brief, embryos were decapitated and livers were removed, pooled and digested with collagenase. Pools of chicken, ring-necked pheasant or Japanese quail livers were prepared from 50, 63 and 100 embryos, respectively. Percoll (Amersham Bioscience, Uppsala, Sweden) was used to separate erythrocytes from hepatocytes and DNAse (Roche, Laval, QC, Canada) treatment was carried out to prevent cell clumping. Cells were plated in 48-well culture plates containing 500 µl of cell culture medium 199, supplemented with insulin (1 µg/ml) and thyroxine (1 µg/ml), and incubated for 24 h at 37 °C in a humidified incubator with 5% CO_2, allowing them to form a monolayer attached to the bottom of the wells. Cells were treated in triplicate with in-well concentrations of TCDD, PeCDF or TCDF ranging from 0.0003 to 10 nM (2.5 µl of chemical/well in DMSO). Cells were incubated for 24 h, medium was removed and plates were flash frozen on dry ice and stored at -80° C until they were analysed. Plates used for the EROD assays were rinsed with phosphate buffered saline-ethylenediaminetetraacetic acid (PBS-EDTA; 200 µl/well) before they were flash frozen on dry ice.

2.3.3. Preparation of TCDD, PeCDF and TCDF solutions

TCDD, PeCDF and TCDF solutions were administered to hepatocyte cultures such that concentrations in cell culture medium ranged from 0.0003 nM to 10 nM. Serial dilutions of PCDD/Fs were prepared in dimethyl sulfoxide (DMSO) from stock solutions prepared in DMSO with concentrations that ranged between 40 and 100 μg/ml. Concentrations of PCDD/Fs in the DMSO stock solutions were confirmed by isotope dilution following EPA method 1613 (U.S.EPA, 1994a) with ^{13}C surrogate standards (DF-CS-C100, Wellington Laboratories, Guelph, ON, Canada). Identification and quantification of TCDD, PeCDF and TCDF was performed using a Hewlett-Packard 5890 series high-resolution gas chromatograph interfaced with a Micromass® Autospec® high-resolution mass spectrometer (HRGC-HRMS) (Micromass®, Beverly, MD, USA). The mass spectrometer was operated in a Selected Ion-Monitoring (SIM) mode, resolution for all reference gas peaks in all time windows was more than 10,000. Concentrations of TCDD, PeCDF and TCDF were quantified by the internal standard isotope-dilution method using mean relative response factors determined from standard calibration runs. Recoveries of ^{13}C-labeled PCDD/Fs internal standards and all other QA/QC criteria were within ranges specified by the EPA methods (U.S.EPA, 1994b).

2.3.4. EROD Assays

EROD assays were conducted as described previously (Head *et al.*, 2006; Kennedy *et al.*, 1995). Reagents were obtained from Sigma (St. Louis, MO, USA) unless another supplier is indicated. Briefly, hepatocytes were incubated at 37.5 °C in the presence of nicotinamide adenine dinucleotide phosphate (NADPH, reduced) and 7-ethyoxyresorufin for 7 min. The reaction was stopped by the addition of cold acetonitrile containing fluorescamine. Resorufin and protein standard curves were prepared for each run. Plates

were analyzed for both EROD activity (excitation wavelength: 530, emission wavelength: 590 nm) and total protein concentration (excitation wavelength: 400 nm, emission wavelength 460 nm) using a fluorescence plate reader (Cytofluor 2350, Millipore, Bedford, MA, USA).

2.3.5. RNA isolation and cDNA synthesis

Total RNA was extracted from 48-well plates using RNeasy 96 kits (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. An on-column DNAse treatment was performed; the only modification was the use of 50% ethanol solution for RNA isolation instead of the suggested 70% because previous tests in our laboratory showed that greater RNA yields were obtained with 50% ethanol. To ensure the maximal removal of genomic DNA, total RNA was treated a second time with DNAse from the Ambion DNA-free kit (Ambion, Austin, TX, USA) according to manufacturer's instructions. Total RNA (11.5 µl from each well) was reverse transcribed to complementary DNA (cDNA) with Superscript II and random hexamers (Invitrogen, Burlington, ON, 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.

2.3.6. Quantitative reverse transcription polymerase chain reaction (QPCR)

A multiplex QPCR assay, using dual-labelled fluorescent hydrolysis probes (Head and Kennedy, 2007b), was used to quantify chicken CYP1A4, CYP1A5 and beta-actin mRNA abundance. Assays similar to the chicken assay were optimized for ring-necked pheasant and Japanese quail gene targets. Primers and probes were designed with Primer3 (Rozen and Skaletsky, 2000) based on gene sequences from Genbank: ring-necked pheasant CYP1A4 (accession no. FJ872527) and CYP1A5 (accession no. FJ872528) and Japanese

quail CYP1A4 (accession no. GQ906939) and CYP1A5 (accession no. GQ906938). Beta-actin is highly conserved among chicken, ring-necked pheasant and Japanese quail and therefore primers and probes were the same for all three species. Primers were obtained from Invitrogen and probes were obtained from Biosearch Technologies (Novato, CA, USA). The amplicons of ring-necked pheasant and Japanese quail CYP1A4, CYP1A5 and beta-actin were cloned and sequenced to demonstrate the specificity of the primers. Brilliant QPCR Core Reagent kits (Stratagene, La Jolla, CA, USA) were used to carry out each assay. Each 25 µl reaction contained 1x PCR buffer, 5 mM $MgCl_2$, 0.8 mM dNTPs, 0.08 v/v glycerol, 0.05 U Surestart Taq polymerase and 25 nM reference dye (ROX). Validation studies for all QPCR assays were conducted as indicated elsewhere (Head and Kennedy, 2007b). In brief, each gene target was amplified alone and multiplexed with the other targets to determine the optimal primer concentrations (Table 2.1). The lowest concentration of each CYP1A4 and CYP1A5 primer that generated a relatively small and consistent cycle threshold (Ct) value with an elevated fluorescence value was selected for the assay. Based on previous assay optimization studies for chicken hepatocytes (Head and Kennedy, 2007b), the concentrations of beta-actin primers were limited to 50 nM in the master mix. Standard curves for ring-necked pheasant and Japanese quail target genes, alone and multiplexed, were produced from a 1:2 dilution series of concentrated cDNA, covering 2 orders of magnitude. Quantification of CYP1A4/5 mRNA fold induction by TCDD, PeCDF and TCDF was assessed using the 2^{-ddCt} method (Livak and Schmittgen, 2001).

2.3.7. Cell viability

Cell viability was determined by use of the Calcein-AM assay (Invitrogen - Molecular Probes, Eugene, Oregon, 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 PBS-EDTA. The culture medium was removed and 200 μL of the Calcein-AM solution was added to each well. The plates were incubated in the dark for 45 min and fluorescence was measured using a Cytofluor 2350 fluorescence plate-reader (Millipore, Billerica, MA, USA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.3.8. 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 were fit to a modified Gaussian curve as explained elsewhere (Kennedy *et al.*, 1993). For each treatment, three EROD curves were generated from data originating from separate cell culture plates. EC50, ECthreshold, and maximal values are presented as the mean value of replicates obtained from three 48-well plate replicates ± standard error. The ECthreshold represents the concentration of a compound producing a response equivalent to 10% of the maximal response produced by TCDD in the same species (see Discussion for more details). EROD activity data were also fitted to a four-parameter equation (see below) in order to compare hillslopes (describes the steepness of the curve). The hillslopes of EROD activity are presented as the mean value obtained from three plates ± standard error.

CYP1A mRNA expression data were fitted to a four parameter logistic model as described previously (Head and Kennedy, 2007b). The equation integrates the hillslope, EC50, baseline response and maximal response as parameters. A single curve fit was generated for mRNA induction, using data from the average of three wells from the same cell culture plate. EC50, maximal values and hillslopes are presented as the values calculated from the curve fit ± standard error.

Statistical differences among EC50, ECthreshold and hillslope values were tested using a one-way analysis of variance (ANOVA) with a Bonferroni correction. Significance was set at $p<0.05$ for all tests.

2.3.9. Calculation of relative potencies and relative sensitivities

The concept of using relative potency (ReP) values to compare the potencies of DLCs is well established (Van den Berg *et al.*, 1998). In this study, RePs are defined as: [EC50 (or ECthreshold) of TCDD in species A] ÷ [EC50 (or ECthreshold) of the compound of interest in species A]. There is also value in comparing the sensitivities of avian species to a particular compound (for example, the comparison of the EC50 of chicken, ring-necked pheasant and Japanese quail when exposed to TCDD). Because the chicken is the most sensitive species tested to date, comparisons are based on this species. For such comparison, the relative sensitivity (ReS) is defined as: [EC50 (or ECthreshold) of compound A in chicken] ÷ [EC50 (or ECthreshold) of compound A in the species of interest].

2.4. Results

2.4.1. Concentration-dependent effects of TCDD, PeCDF and TCDF on EROD activity

General observations

TCDD, PeCDF and TCDF induced EROD activity in a concentration-dependent manner in chicken, ring-necked pheasant and Japanese quail hepatocytes (Figure 2.1). In most cases, maximal EROD activity was followed by a decrease in activity at greater concentrations of the inducer. The decrease in EROD activity at high concentrations of TCDD, PeCDF or TCDF was not due to overt toxicity as measured by the Calcein-AM assay (results not shown). Maximal EROD activities for TCDD, PeCDF and TCDF in chicken and ring-necked pheasant hepatocytes were similar (approximately 440 – 550 pmol/min/mg

protein), but maximal EROD activity was lower in Japanese quail hepatocytes (approximately 250-285 pmol/min/mg protein). The steepness of the curves (hillslope values) was similar among compounds and species (Figure 2.1, Table 2.4; statistics not shown).

Intercompound comparisons: relative potencies of TCDD, PeCDF and TCDF

There was no statistical difference in EC50 or ECthreshold values among TCDD, TCDF and PeCDF (Table 2.2) in chicken hepatocytes and the EROD concentration-response curves for the three compounds were essentially identical (Figure 2.1). Therefore, RePs based on the EC50 for PeCDF and TCDF were 1.0 in chicken hepatocytes, and RePs based on the ECthreshold were 0.7 and 0.6 for PeCDF and TCDF, respectively (Table 2.2). The three compounds were essentially equipotent (PeCDF = TCDD = TCDF) in chicken hepatocytes.

In ring-necked pheasant hepatocytes, the EROD concentration-response curve for PeCDF was shifted slightly to the left of the TCDD and TCDF curves (Figure 2.1). The EC50 and ECthreshold values for PeCDF were statistically lower than the values for TCDD, and the EC50 and ECthreshold values for TCDF were similar to the values for TCDD (Table 2.2). Consequently, the RePs for PeCDF based on the EC50 and ECthreshold were 3.0 and 4.5, respectively, and the RePs for TCDF based on the EC50 and ECthreshold were 0.9 and 0.5, respectively. In contrast with the chicken, the rank order of potency (in the order of the most to the least potent) in ring-necked pheasant hepatocytes was: PeCDF > TCDD = TCDF.

In Japanese quail hepatocytes, there were clear differences in the EROD concentration-response curves. From left to right the order was: PeCDF, TCDD and TCDF (Figure 2.1). The EC50 and ECthreshold values for PeCDF were statistically lower than the values for TCDD. The EC50 and ECthreshold values for TCDF were statistically higher than

the values for TCDD (Table 2.2). Consequently, the RePs for PeCDF based on EC50 and ECthreshold were 13 and 30, respectively, and the RePs for TCDF based on the EC50 and ECthreshold were 0.1 and 0.3, respectively. In Japanese quail hepatocytes, the rank order of potency was: PeCDF > TCDD > TCDF.

Interspecies comparisons: relative sensitivities of chicken, ring-necked pheasant and Japanese quail

The EC50 values for chicken, ring-necked pheasant and Japanese quail hepatocytes exposed to TCDD were 0.018 nM, 0.085 nM and 0.19 nM, respectively; these were statistically different among the three species (Table 2.3). The ECthreshold values for TCDD-exposed hepatocytes were also different (0.00081 nM, 0.0051 nM and 0.020 nM, respectively). Consequently, the ReS values were 0.2 for ring-necked pheasant, and for Japanese quail the ReS values based on EC50 and ECthreshold were 0.1 and 0.04, respectively. The rank order of sensitivity to EROD induction by TCDD (in the order of most to least sensitive) was: chicken > ring-necked pheasant > Japanese quail.

The EC50 values for chicken, ring-necked pheasant and Japanese quail hepatocytes exposed to PeCDF were not statistically different from one another (Table 2.3), but there were small differences in ECthreshold values among the species. The EC50- and ECthreshold-based ReS values for ring-necked pheasant were 0.8 and 1.1, respectively. The ReS values based on the EC50 and ECthreshold for Japanese quail were 0.8 and 1.8, respectively. These results generally indicate that the rank order of sensitivity to EROD induction by PeCDF was: chicken = ring-necked pheasant ≥ Japanese quail.

The EC50 and ECthreshold values for ring-necked pheasant and Japanese quail hepatocytes exposed to TCDF were statistically greater than the values for chicken hepatocytes (Table 2.3). Consequently, the ReS values based on the EC50 and ECthreshold

for ring-necked pheasant were 0.11 and 0.009, respectively, and for Japanese quail they were 0.1 and 0.04, respectively. The rank order of sensitivity to EROD induction by TCDF was: chicken > ring-necked pheasant > Japanese quail.

2.4.2. Concentration-dependent effects of TCDD, PeCDF and TCDF on CYP1A4/5 mRNA expression

Assay optimization

Standard curves for CYP1A4, CYP1A5 and beta-actin mRNA expression in chicken, ring-necked pheasant and Japanese quail hepatocytes were obtained using triplex assays (i.e., measuring the mRNA expression of the three genes in the same tube). The Ct values were plotted against relative cDNA concentrations and data were fit to a linear regression model. The efficiencies of the reactions for CYP1A4, CYP1A5 and beta-actin mRNA expression were similar within each species. In addition, standard curves for the three transcripts were nearly parallel (slopes were within 0.1 for each gene for the three species), and r^2 values for all curves were between 0.983 and 0.999. Beta-actin expression was not affected by TCDD, PeCDF or TCDF in hepatocytes from any of the species. Therefore, changes in mRNA expression were a result of changes in CYP1A4 and CYP1A5 mRNA expression, and not in changes in beta-actin mRNA expression, which validates the use of beta-actin as a housekeeping gene.

General observations

The mRNAs of both CYP1A4 and CYP1A5 isoforms were induced in a concentration-dependent manner by TCDD, PeCDF and TCDF in chicken, ring-necked pheasant and Japanese quail hepatocytes (Figure 2.2). In the three species, the CYP1A4 isoform had a greater maximal induction than the CYP1A5 isoform (Figure 2.2, Table 2.4). The maximal induction of CYP1A4/5 mRNA differed among species: chicken hepatocytes

showed a higher CYP1A4/5 mRNA induction (47- to 502-fold change) than ring-necked pheasant (16- to 30-fold change) and Japanese quail hepatocytes (6- to 33-fold change; Figure 2.2 and Table 2.4).

Comparisons between EROD activity, CYP1A4/5 mRNA expression

Concentration-dependent responses of EROD activity and CYP1A4/5 mRNA expression to TCDD, PeCDF and TCDF were compared after normalizing the maximal responses to 100% maximal response for each compound and for each endpoint (Figure 2.3). The shapes of the concentration-response curves were similar and there was general concordance in EC50, ECthreshold and hillslope values for each compound (Table 2.4).

2.5. Discussion

The present experiments measured CYP1A induction by TCDD, PeCDF and TCDF exposure in primary hepatocyte cultures, prepared from chicken, ring-necked pheasant and Japanese quail embryos. These three species of Galliforms were chosen because they demonstrate differential overt and biochemical sensitivities to DLCs; the chicken is very sensitive, the ring-necked pheasant is moderately sensitive and the Japanese quail is the least sensitive (Head *et al.*, 2008). Comparison of the potencies of compounds within each species (relative potency; ReP) and comparison of sensitivities among species when exposed to each compound (relative sensitivity; ReS) were made. Concentration-dependent effects of TCDD, PeCDF and TCDF on EROD activity and CYP1A4/5 mRNA expression were compared in hepatocytes from the three species to (a) compare the concentration-response curves and (b) determine if there were differences in the ratios of CYP1A4 and CYP1A5 mRNA expression among the species.

TCDD and TCDF were approximately equipotent EROD inducers in both chicken and ring-necked pheasant hepatocytes, a finding that is consistent with an earlier study (Kennedy *et al.*, 1996). In contrast, TCDF was a less potent inducer of EROD activity (ReP = 0.1 to 0.3) than TCDD in Japanese quail hepatocytes. PeCDF was a more potent inducer of EROD activity and CYP1A4/5 mRNA than TCDD in ring-necked pheasant (ReP = 3.0 to 4.5) and Japanese quail (ReP = 13 to 30) hepatocytes. This is the first report that PeCDF is a more potent inducer of EROD activity and CYP1A4/5 mRNA in Japanese quail and ring-necked pheasant hepatocytes. However, approximately ten years ago, it was discovered that PeCDF was a more potent inducer of EROD activity than TCDD in double-crested cormorant (*Phalacrocorax auritus*) and Forster's tern (*Sterna forsteri*) hepatocytes (Sanderson *et al.*, 1998). A more recent study indicated that PeCDF was also a more potent EROD inducer than TCDD in green frog (*Rana esculenta*) hepatocytes (Rankouhi *et al.*, 2005).

Despite our previous knowledge that PeCDF was a more potent EROD inducer than TCDD in some species, our initial hypothesis was that the rank order of species sensitivity to EROD induction by all three compounds (TCDD, PeCDF and TCDF) would be: chicken (most sensitive), ring-necked pheasant (moderately sensitive) and Japanese quail (least sensitive). This hypothesis was based upon (a) earlier studies with TCDD- and TCDF-exposed chicken and ring-necked pheasant hepatocytes (Kennedy *et al.*, 1996), and (b) the identity of key amino acid residues within the ligand binding domains of AHR1 for the three species (Head *et al.*, 2008). As mentioned in Chapter 1, amino acid residues corresponding to Ile324 and Ser380 in chicken were compared to amino acid residues in ring-necked pheasant, Japanese quail and several other avian species, and it was found that the identity of the amino acid residues at sites 324 and 380 were predictive of broad categories of dioxin sensitivity

among species (Head *et al.*, 2008). The chicken was unique in having the Ile/Ser genotype, the ring-necked pheasant has the Ile/Ala genotype and was classified as moderately sensitive and the Japanese quail has the Val/Ala genotype, which places it in the least sensitive category. The results of the present study supported our hypothesis for TCDD and TCDF. Indeed, the EC50-based ReS values were 1.0, 0.2 and 0.1 for chicken, ring-necked pheasant and Japanese quail exposed to TCDD, respectively, and EC50-based ReS values were 1.0, 0.2 and 0.02 for chicken, ring-necked pheasant and Japanese quail exposed to TCDF, respectively (Table 2.3). In contrast, the ReS values for PeCDF were similar (within 2-fold) for all three species, regardless of whether EC50 or ECthreshold values were used to compare EROD curves.

The molecular mechanism(s) underlying the reason(s) why PeCDF is a more potent inducer of EROD activity and CYP1A4/5 mRNA expression than TCDD in some species, and why hepatocytes from the three species were approximately equisensitive to EROD and CYP1A4/5 mRNA induction when exposed to PeCDF is not yet known, but the data presented here allow for the testing of several hypotheses. For example, variation in potency among compounds might be partially explained by the binding affinity of the ligand to the AHR - the greater the binding affinity, the greater the potency (Hestermann *et al.*, 2000; Poland and Knutson, 1982; Safe, 1986). Therefore, in ring-necked pheasant and Japanese quail, PeCDF might have a greater binding affinity to AHR1 than TCDD. It would also be interesting to investigate the influence of four additional amino acids within the ligand binding domain that are of interest (Head *et al.*, 2008) on AHR1 binding affinity and *in vitro* expression of AHR1-dependent responses for PeCDF, TCDD and other DLCs in avian species. However, factors other than AHR1 binding should also be considered in future studies with avian species. For example, differences in the relative potency of PCB 126 to

induce or repress a number of genes in rat (ReP was 0.06) and human (ReP was 0.002) hepatocytes could not be explained by differences in their AHR binding affinity (Carlson *et al.*, 2009). The authors suggested that intrinsic efficacy and events downstream of AHR-binding (for example transactivation) could be involved in the differences in relative potency among rats and humans. Among other suggestions is the possibility of differences in ligand-specific AHR coactivator interactions among species (Zhang *et al.*, 2008). It is possible that in the ring-necked pheasant and Japanese quail, the interactions between the PeCDF-AHR1 complex and the coactivators could lead to greater CYP1A responses than the interactions with the TCDD-AHR1 complex. On the other hand, the interactions of PeCDF-AHR1 and TCDD-AHR1 with coactivators could lead to similar CYP1A responses in the chicken.

ReP and ReS values are often calculated by comparing the EC50 values from EROD concentration-response curves. As mentioned in Chapter 1, there is competition between the substrate (ethoxyresorufin) and the inducer (the DLC) for the CYP1A enzyme (Petrulis and Bunce, 1999), it is why a decrease in EROD activity is observed at greater concentrations of DLCs. The inhibition of EROD activity can, in certain situations (mainly with weak AHR ligands), decrease the maximal activity. As such, there is a leftward shift of the EC50, creating an overestimation of the potency of a compound (Hahn *et al.*, 1993; Head and Kennedy, 2007b; Hestermann *et al.*, 2000). A less biased estimate can be derived from the threshold concentration for effect (ECthreshold), which, as mentioned before, represents the concentration of a compound that produces a response equivalent to 10% of the maximal response produced by TCDD in the same species (Kennedy *et al.*, 1996) (Appendix 1). In this study, relative potencies and relative sensitivities calculated with EC50 and ECthreshold were similar, and the major conclusions are not changed by the measure of potency used. The results of both approaches were included to allow careful examination of the data by

others. In this study, maximal EROD activity for the three compounds was less in Japanese quail hepatocytes compared to chicken and ring-necked pheasant hepatocytes. It is possible that the binding affinity of DLCs to the Japanese quail AHR1 is lower than the binding affinity of DLCs to the AHR1 of the two other species due to the Val/Ala substitution in the Japanese quail AHR1 (Head *et al.*, 2008; Karchner *et al.*, 2006). As explained for the weak AHR ligands, this 'weak AHR1' in Japanese quail could lead to a decreased maximal activity in Japanese quail. There are also indications that maximal activities vary among cell culture preparations. Indeed, a 3-fold difference in maximal EROD activity was observed among different cell culture preparations of the same species that were conducted in the same laboratory, with the same procedures (Kennedy *et al.* unpublished results).

Both CYP1A4 and CYP1A5 mRNA were induced in a concentration-dependent manner by TCDD, PeCDF and TCDF in the three species, but differences in maximal induction were observed within and among species. CYP1A4 mRNA was up-regulated more than CYP1A5 mRNA in all species. It was previously shown that in chicken hepatocytes exposed to TCDD, CYP1A4 mRNA was preferentially up-regulated relative to CYP1A5 mRNA (Head and Kennedy, 2007a; Head and Kennedy, 2007b). The maximal fold induction of CYP1A4/5 mRNA was greater in chicken than in ring-necked pheasant and Japanese quail. Differences in the maximal responses of mRNA induction had been observed previously between chicken and herring gull (*Larus argentatus*) hepatocytes treated with TCDD (Head and Kennedy, 2007a). The investigators hypothesised that TCDD could regulate mRNA half-life differently among species. They tested this hypothesis by tracking the decay of relative transcript quantity at different time points 24h after the addition of actinomycin D, but did not find strong evidence for half-life regulation by TCDD in chicken or herring gull.

Finally, as shown in a previous study with chicken (Head and Kennedy, 2007b), EROD activity and CYP1A4/5 mRNA expression can be used interchangeably to predict *in ovo* toxicity of DLCs. Indeed, concentration-response curves of the three endpoints have similar curve slopes and EC50 values for each compound in each species. It is therefore unlikely that the use of any of the three endpoints would lead to large differences in estimates of relative potency or relative sensitivity among species. Both methods have their advantages and disadvantages. The EROD assay is cost- and time-effective and can be applied to virtually all species, unlike the CYP1A4/5 assay that is cost- and time-consuming and that must be optimized for each species. However, the CYP1A4/5 mRNA assay has a lower detection limit and provides useful information when a limited amount of tissue is available. As well, it can be important to know the relative expression levels of both CYP1A4 and CYP1A5 for developing hypotheses regarding the downstream effects of CYP1A induction.

In summary, this study demonstrated that TCDD, PeCDF and TCDF were equipotent EROD and CYP1A4/5 mRNA inducers in chicken embryo hepatocyte cultures. However, PeCDF was a more potent inducer than TCDD in ring-necked pheasant and Japanese quail hepatocytes. The rank order of sensitivity among the three avian species when exposed to TCDD and TCDF was: chicken > ring-necked pheasant > Japanese quail, but when exposed to PeCDF, the three species were essentially equisensitive to EROD and CYP1A4/5 mRNA induction. The results obtained in this study raise interesting questions about species-specific responses to DLCs. It is expected that on-going studies in our laboratory will contribute to new data with respect to the rank order of potency for other DLCs in the species studies here, and also in other species of interest (especially, wildlife species). Some of our research is being conducted with the goal of revealing important insights into the molecular mechanisms

that underlie species differences in sensitivity and response to DLCs, but there are also practical reasons for this research. For example, the current avian toxic equivalency factor (TEF) values established by the World Health Organization for PCDDs, PCDFs and PCBs are based upon a limited number of studies (*in vitro* and *in ovo*) that were largely conducted with chickens. The avian TEF values for TCDD, PeCDF and TCDF are currently 1.0 for all three compounds. The results of the present study and concurrent egg injection studies using Japanese quail (Cohen-Barnhouse, 2008) indicate that a re-evaluation of the TEFs for PeCDF and TCDF in birds should be considered.

2.6 Acknowledgements

This research was supported, in part, by an unrestricted grant from the Dow Chemical Company to the University of Ottawa, Michigan State University and the University of Saskatchewan and by Environment Canada's Strategic Applications of Genomic Technologies to the Environment (STAGE) program. Jessica Hervé was supported by a scholarship from le Fond Québécois de la Recherche sur la Nature et les Technologies. Prof. Giesy was supported by the Canada Research Chair program, an at large Chair Professorship at the Department of Biology and Chemistry and Research Centre for Coastal Pollution and Conservation, City University of Hong Kong.

Table 2.1. Nucleotide sequence of primers and probes, fluorescent dye/quencher and amplicon size in base pairs (bp) for ring-necked pheasant and Japanese quail cytochrome P450 1A4 (CYP1A4), CYP1A5 and beta-actin. Final primer and probe concentrations used for QPCR are shown in brackets.

Ring-necked pheasant	
CYP1A4	
Dye/Quencher	FAM/BHQ-1
Forward	5'-GAGCACATTCGGGATGTCA-3' (300 nM)
Reverse	5'-CAGAGAGTTGGACACGGACA-3' (300 nM)
Probe[a]	5'-CCTGGCAGTGCCCAATCAACG-3' (200 nM)
Amplicon size	85 bp
CYP1A5	
Dye/Quencher	Quasar 670/BHQ-3
Forward	CATCCGAGATGTCACCGACT (300 nM)
Reverse	TTGGGATCTGTGTGGCACTA (300 nM)
Probe[b]	CCCTCATTGAGCAGTGCATGGA (200 nM)
Amplicon size	80 bp
Japanese quail	
CYP1A4	
Dye/Quencher	FAM/BHQ-1
Forward	5'-GGATGTCAATACCCGTTTCG-3' (300 nM)
Reverse	5'-CTGCCCAATCAATGAGTCTG-3' (300 nM)
Probe[a]	5'-TGACGTCCCGAATGTGCTCCT-3' (200 nM)
Amplicon size	109 bp
CYP1A5	
Dye/Quencher	Quasar 670/BHQ-3
Forward	5'-TACAGGCAGCTGTGGATGAG-3' (300 nM)
Reverse	5'-ACTGCTCAATGAGGGAGTCG-3' (300 nM)
Probe[b]	5'-GACAAGAACAGCATCCGAGACGTCA-3' (200 nM)
Amplicon size	81 bp
Ring-necked pheasant and Japanese quail	
Beta-actin	
Dye/Quencher	HEX/BHQ-1
Forward primer	5'-AAATTGTGCGTGACATCAAGGA-3' (50 nM)
Reverse primer	5'-GAGGCAGCTGTGGCCATCT-3' (50 nM)
Probe[b]	5'-TGCTACGTCGCACTGGATTTCGAGC-3' (50 nM)
Amplicon size	76 bp

Note: primer and probes used for the chicken are presented elsewhere (Head and Kennedy, 2007b).

Beta-actin primer and probe sequences and concentrations are the same for both species.

[a] probe anneals to the same strand as the reverse primer

[b] probe anneals to the same strand as the forward primer

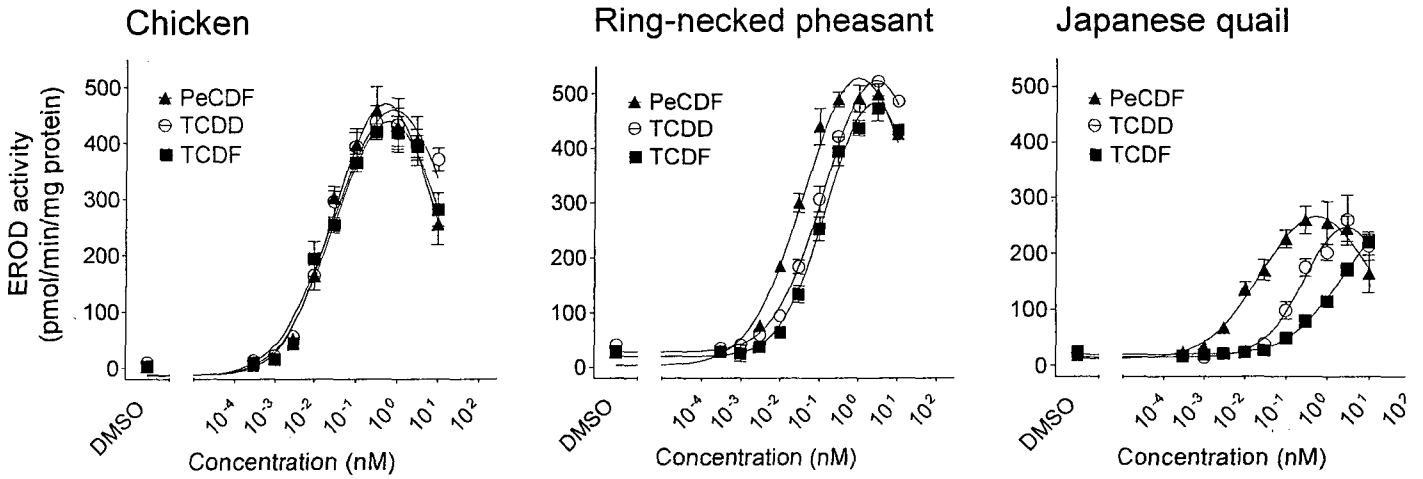


Figure 2.1. Concentration-dependent effects of PeCDF (▲), TCDD (○) or TCDF (■) on EROD activity in chicken, ring-necked pheasant and Japanese quail embryo hepatocyte cultures exposed for 24 h. Points represent mean EROD activity from three replicate cell culture plates; values before the axis break indicate EROD activity observed for control (DMSO-treated hepatocytes). Bars represent standard errors.

Table 2.2. Maximal EROD activity, EC50, ECthreshold (ECthr) and relative potency (ReP) values in chicken, ring-necked pheasant (R-n. pheasant) and Japanese quail (J. quail) hepatocyte cultures exposed to TCDD, PeCDF or TCDF for 24 h.

Species	Chemical	EC50 (nM)	EC50-based ReP	ECthr (nM)	ECthr-based ReP	Max EROD activity (pmol/min/mg protein)
Chicken	TCDD	0.018 ± 0.0007 [a]	1.0	0.00081 ± 0.00002 [a]	1.0	460 ± 36 [a]
Chicken	PeCDF	0.019 ± 0.0003 [a]	1.0	0.0013 ± 0.0002 [a]	0.7	471 ± 38 [a]
Chicken	TCDF	0.021 ± 0.001 [a]	1.0	0.0014 ± 0.0003 [a]	0.6	443 ± 17 [a]
R-n. pheasant	TCDD	0.085 ± 0.01 [a]	1.0	0.0051 ± 0.001 [a]	1.0	525 ± 6 [a]
R-n. pheasant	PeCDF	0.025 ± 0.002 [b]	3.0	0.0011 ± 0.0001 [b]	4.5	530 ± 15 [a]
R-n. pheasant	TCDF	0.11 ± 0.02 [a]	0.8	0.0090 ± 0.002 [a]	0.5	486 ± 12 [a]
J.quail	TCDD	0.19 ± 0.02 [a]	1.0	0.020 ± 0.001 [a]	1.0	248 ± 31 [a]
J.quail	PeCDF	0.015 ± 0.0009 [b]	13	0.00073 ± 0.0001 [b]	30	267 ± 27 [a]
J.quail	TCDF	1.57 ± 0.4 [c†]	0.1[†]	0.077 ± 0.01 [c]	0.3	285 ± 24 [a]*

Notes: Mean values were derived from three replicate cell culture plates ± standard error. Different superscript letters indicate significant differences among treatments ($p<0.05$) within each species. EROD data were fit to a modified Gaussian curve.

[†] Because no maximal value was reached when J. quail hepatocytes were exposed to TCDF (Figure 2.1), the EC50 for TCDF might be underestimated and EC50-based ReP might be overestimated

* No maximal response was reached

Table 2.3. Maximal EROD activity, EC50, ECthreshold (ECthr), and relative sensitivity (ReS) values in chicken, ring-necked pheasant (R-n. pheasant) and Japanese quail (J. quail) hepatocyte cultures exposed to TCDD, PeCDF or TCDF for 24 h.

Chemical	Species	EC50 (nM)	EC50-based ReS	ECthr (nM)	ECthr-based ReS
TCDD	Chicken	0.018 ± 0.0007^{a}	1.0	0.00081 ± 0.00002^{a}	1.0
TCDD	R-n. pheasant	0.085 ± 0.01^{b}	0.2	0.0051 ± 0.001^{b}	0.2
TCDD	J.quail	0.19 ± 0.02^{c}	0.1	0.020 ± 0.001^{c}	0.04
PeCDF	Chicken	0.019 ± 0.0003^{a}	1.0	0.0013 ± 0.0002^{a}	1.0
PeCDF	R-n. pheasant	0.025 ± 0.002^{a}	0.8	$0.0011 \pm 0.0001^{a,b}$	1.1
PeCDF	J.quail	0.015 ± 0.0009^{a}	1.3	0.00073 ± 0.0001^{b}	1.8
TCDF	Chicken	0.021 ± 0.001^{a}	1.0	0.0014 ± 0.0003^{a}	1.0
TCDF	R-n. pheasant	0.11 ± 0.02^{b}	0.2	0.0090 ± 0.002^{b}	0.1
TCDF	J.quail	$1.57 \pm 0.4^{c\dagger}$	$0.02^{\dagger}$	0.077 ± 0.01^{c}	0.02

Notes: Mean values were derived from three replicate cell culture plates ± standard error. Different superscript letters indicate significant differences among species ($p<0.05$) exposed to the same chemical. EROD data were fit to a modified Gaussian curve.

† Because no maximal value was reached when J. quail hepatocytes were exposed to TCDF (Figure 2.1), the EC50 might be underestimated and ReS based on EC50 might be overestimated.

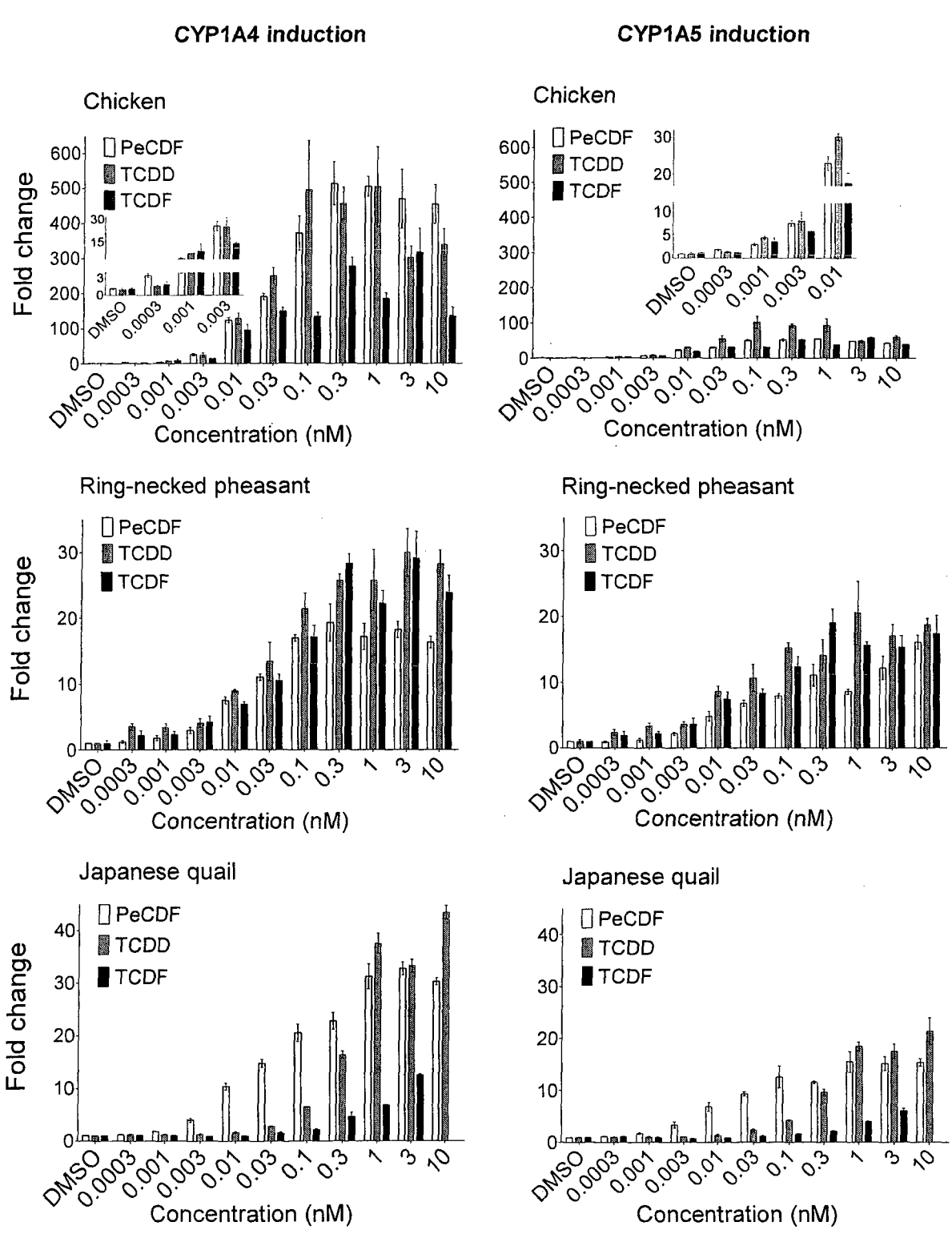


Figure 2.2.Concentration-dependent effects of PeCDF, TCDD and TCDF on CYP1A4 and CYP1A5 mRNA induction in chicken, ring-necked pheasant and Japanese quail hepatocyte cultures exposed for 24 h. Bars represent mean fold induction (± SEM) of three replicate cell culture wells from the same cell culture plate.

Table 2.4. Curve fitting parameters for concentration-dependent effects of TCDD, PeCDF, and TCDF on EROD activity and CYP1A4/5 mRNA induction in chicken, ring-necked pheasant (R-n. pheasant) and Japanese quail (J.quail) hepatocyte cultures exposed for 24 h.

Species	Compound	End point	EC50 (nM)	Hillslope	Maximal response (EROD: pmol/min/mg protein, CYP1A4/5: fold induction)
Chicken	TCDD	EROD	0.018 ± 0.0007 [a]	1.18 ± 0.2 [a]	460 ± 36
Chicken	TCDD	CYP1A4	0.026 ± 0.008 [a]	1.48 ± 0.7 [a]	502 ± 47
Chicken	TCDD	CYP1A5	0.021 ± 0.005 [a]	1.52 ± 0.5 [a]	78 ± 6
Chicken	PeCDF	EROD	0.019 ± 0.0003 [a]	1.09 ± 0.2 [a]	471 ± 38
Chicken	PeCDF	CYP1A4	0.037 ± 0.008 [a]	1.28 ± 0.3 [a]	490 ± 23
Chicken	PeCDF	CYP1A5	0.014 ± 0.003 [a]	1.22 ± 0.2 [a]	50 ± 2
Chicken	TCDF	EROD	0.021 ± 0.001 [a]	1.1 ± 0.1 [a]	443 ± 17
Chicken	TCDF	CYP1A4	0.017 ± 0.01 [a]	1.00 ± 0.5 [a]	222 ± 22
Chicken	TCDF	CYP1A5	0.020 ± 0.006 [a]	0.93 ± 0.2 [a]	47 ± 2
R-n. pheasant	TCDD	EROD	0.085 ± 0.01 [a]	1.00 ± 0.08 [a]	525 ± 6
R-n. pheasant	TCDD	CYP1A4	0.038 ± 0.01 [a]	0.87 ± 0.2 [a]	30 ± 6
R-n. pheasant	TCDD	CYP1A5	0.022 ± 0.01 [a]	0.69 ± 0.2 [a]	21 ± 8
R-n. pheasant	PeCDF	EROD	0.025 ± 0.002 [a]	1.32 ± 0.3 [a]	530 ± 15
R-n. pheasant	PeCDF	CYP1A4	0.017 ± 0.004 [a]	1.24 ± 0.2 [a]	18 ± 2
R-n. pheasant	PeCDF	CYP1A5	N/A[‡]	N/A[‡]	N/A[‡]
R-n. pheasant	TCDF	EROD	0.11 ± 0.02 [a]	1.10 ± 0.01 [a]	486 ± 12
R-n. pheasant	TCDF	CYP1A4	0.048 ± 0.01 [a]	1.16 ± 0.03 [a]	29 ± 7
R-n. pheasant	TCDF	CYP1A5	0.026 ± 0.01 [a]	0.89 ± 0.1 [a]	17 ± 5
J.quail	TCDD	EROD	0.19 ± 0.02 [a]	1.02 ± 0.3 [a]	248 ± 31
J.quail	TCDD	CYP1A4	0.36 ± 0.03 [a]	1.83 ± 0.3 [a]	40 ± 1.3
J.quail	TCDD	CYP1A5	0.32 ± 0.04 [a]	1.44 ± 0.3 [a]	20 ± 0.9
J.quail	PeCDF	EROD	0.015 ± 0.0009 [a]	0.78 ± 0.2 [a]	267 ± 27
J.quail	PeCDF	CYP1A4	0.047 ± 0.01 [a]	0.65 ± 0.09 [a]	33 ± 1.4
J.quail	PeCDF	CYP1A5	0.019 ± 0.006 [a]	0.71 ± 0.2 [a]	16 ± 0.8
J.quail	TCDF	EROD	1.57 ± 0.4 [a†]	0.97 ± 0.2 [a]	285 ± 24 *
J.quail	TCDF	CYP1A4	0.69 ± 0.2 [a†]	1.30 ± 0.3 [a]	12 ± 0.3 *
J.quail	TCDF	CYP1A5	0.69 ± 0.1 [a†]	1.53 ± 0.3 [a]	6 ± 0.5 *

Notes (for table 2.4). Mean values were derived from three replicate cell culture plates ± standard error (EROD parameters) or from the average of three different wells of the same cell culture plate (mRNA expression). Different superscript letters indicate significant differences between endpoints ($p<0.05$). EROD data were fit to a modified Gaussian curve and CYP1A4/5 mRNA expression data were fit to a four-parameter logistic curve. EROD data were also fit to the four-parameter equation in order to compare the hillslope values of EROD activity and mRNA induction.

‡ Despite the concentration-dependent increase in mRNA, the points could not be fitted to the four-parameter equation (Figure 2.3)

† Because no maximal value was reached, EC50 might be underestimated

* No maximal response was reached

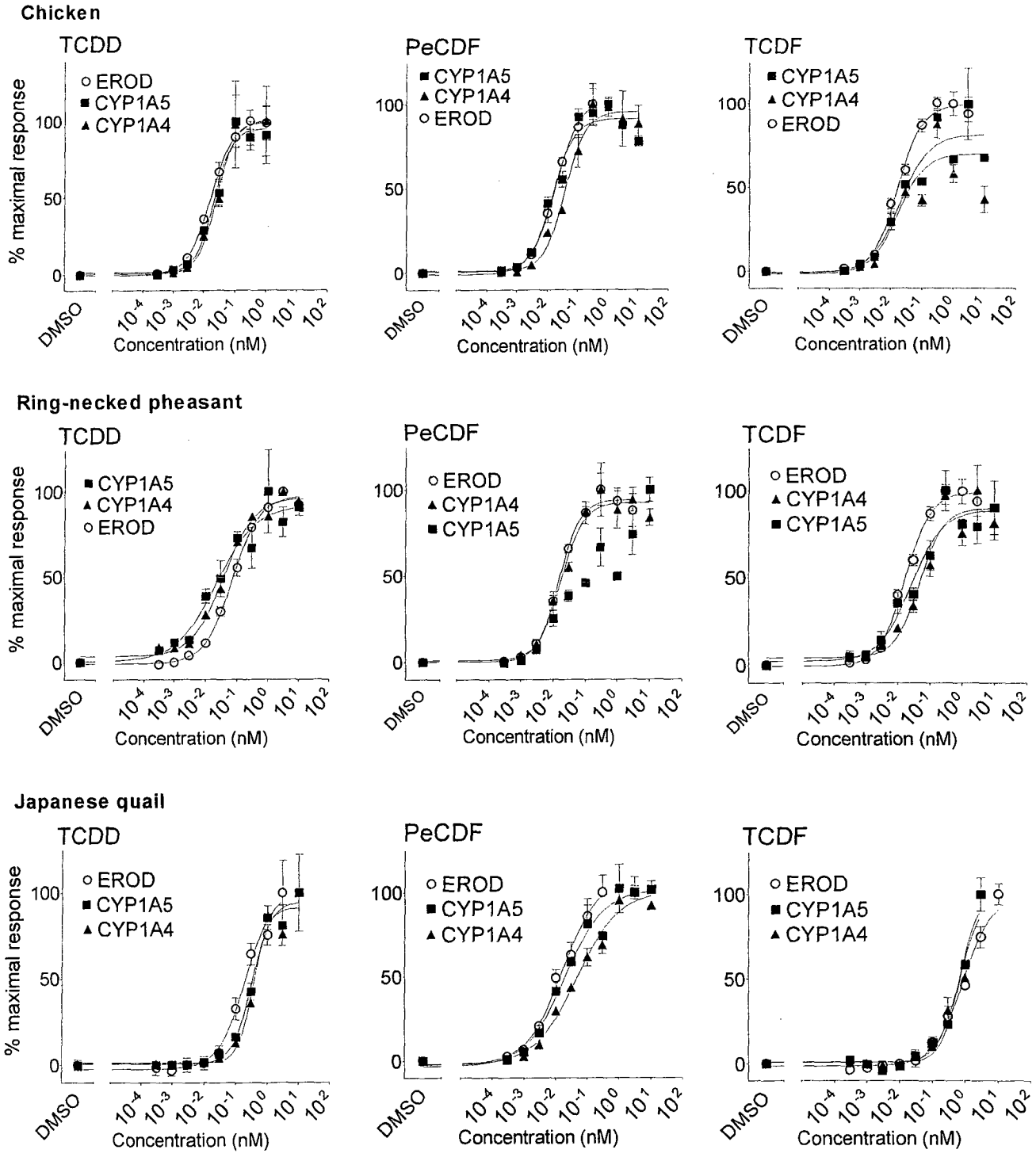


Figure 2.3. Concentration-dependent effects of TCDD, PeCDF and TCDF on EROD activity (○), CYP1A4 mRNA expression (▲) and CYP1A5 mRNA expression (■) in chicken, ring-necked pheasant, and Japanese quail hepatocyte cultures exposed for 24 h. For comparison purposes, data are expressed as percent maximal response. For EROD activity, each point represents the mean of three different cell culture plates. For mRNA expression each point represents the mean of three different wells from the same cell culture plate. Bars represent standard errors of the means.

Chapter 3

Relative potencies of dioxin-like chemicals in primary herring gull hepatocytes: 2,3,4,7,8-PeCDF is a more potent CYP1A inducer than 2,3,7,8,-TCDD[†]

3.1. Abstract

Cytochrome P4501A (CYP1A)-inducing potencies of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF) were determined in herring gull (*Larus argentatus*) embryonic hepatocytes after 24 h exposure. Concentration-dependent effects on ethoxyresorufin *O*-deethylase (EROD) activity and CYP1A4 and CYP1A5 messenger RNA (mRNA) expression were measured. TCDF and TCDD were equipotent inducers of EROD activity and CYP1A4/5 mRNA expression. In contrast, PeCDF was 10- to 21-times more potent than TCDD at inducing EROD activity and CYP1A4/5 mRNA expression. This finding, combined with data reporting levels of TCDD and PeCDF in herring gull eggs during the 1980s, suggests that the contribution of PeCDF to the toxic equivalency was greater than the contribution of TCDD. This study with cultured herring gull hepatocytes, along with previous *in vitro* research, indicates that PeCDF is more potent than TCDD in some avian species. This finding raises questions about the molecular mechanisms of the effects of dioxin-like compounds action and the accuracy of toxic equivalency factors (TEFs) that are assigned to avian species.

[†] Adapted from Hervé, J.C., *et al.* [manuscript in preparation]

3.2. Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and some polychlorinated biphenyls (PCBs) are referred to as 'dioxin-like chemicals' (DLCs) because they elicit similar toxic and biochemical effects to those caused by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Ahlborg *et al.*, 1992; Poland and Knutson, 1982; Van den Berg *et al.*, 1998). Adverse effects of DLCs in birds have been observed in both the laboratory (Boening, 1998; Brunström, 1991; Nosek *et al.*, 1992a; Poland and Knutson, 1982) and the environment. During the 1970s, signs of toxicity in colonial fish-eating birds of the Great Lakes were associated with high levels of TCDD and other organochlorine contaminants, such as PCBs (Gilbertson, 1983; Gilbertson *et al.*, 1991). Adverse effects included embryonic and chick mortality, developmental abnormalities, liver impairment, edema and porphyria; the effects were grouped under the name Great Lakes Embryo Mortality, Edema and Deformity Syndrome (GLEMEDS) (Gilbertson, 1983; Gilbertson *et al.*, 1991).

The molecular mechanisms of how DLCs induce toxic effects toxicity have not been discovered yet. However, various studies showed that toxic effects are linked to the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor (Bohonowych and Denison, 2007; Mimura *et al.*, 1997; Mimura and Fujii-Kuriyama, 2003; Poland and Knutson, 1982). Subsequent to binding the AHR, DLCs affect biochemical responses, including the induction of cytochrome P450 1A (CYP1A). The relationship between CYP1A induction and the occurrence of toxic effects is not constant for all toxic effects (Nukaya *et al.*, 2009; Okey *et al.*, 2005; Uno *et al.*, 2008). Nonetheless, as mentioned previously, CYP1A induction has been shown to be predictive of the relative toxicity of DLCs in avian embryos (Brunström, 1991; Kennedy *et al.*, 1996; Poland and Glover, 1973). Each DLC has a unique

chemical structure which influences how an organism will respond to exposure. Indeed, the potency to induce biochemical and toxic effects varies among compounds, and TCDD is generally considered to have the greatest ability to elicit these responses. Forty years ago, a study showed that TCDD was the most toxic among the tri-, tetra-, hexa-, hepta- and octa-chlorodibenzo-*p*-dioxin congeners, in respect to chicken embryo mortality (Higginbotham *et al.*, 1968). The same conclusion was reached by following studies and reviews on toxic and biochemical effects of PCDDs, PCDFs and PCBs (Brunström *et al.*, 1991; Kennedy *et al.*, 1996; McConnell *et al.*, 1978; Poland and Glover, 1973; Safe, 1987; Schwetz *et al.*, 1973; Van den Berg *et al.*, 1998). In addition to the wide range of potency observed among compounds, sensitivity of avian species is also very broad. For example, the herring gull (*Larus argentatus*) is over 200-times less sensitive than the chicken to the embryotoxic effects of DLCs (reviewed in Head *et al.*, 2008).

Risk assessments involving DLCs are difficult to conduct because the different compounds exhibit different potencies and are found as mixtures in the environment. To overcome these difficulties, the World Health Organization developed the following concepts for comparing toxicity: toxic equivalency factors (TEFs) and toxic equivalency (TEQ). The TEF represents the toxicity of a compound compared to TCDD. Because TCDD is the reference chemical, it has a TEF of 1.0. The TEQ of a mixture is the sum of the amount of each chemical in the mixture, multiplied by their respective TEFs. When the TEF is derived from a single study, the term relative potency (ReP) should be used.

In this study, primary cultures of herring gull embryonic hepatocyte were used to measure CYP1A induction by TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDF). High concentration of PeCDF were measured in certain parts of North America (Hilscherova *et al.*, 2003; Kannan *et al.*, 2007) and elsewhere

(Kiguchi *et al.*, 2007; Salo *et al.*, 2008), and their possible effects on birds could still be of concern. The herring gull is a good bio-indicator species because it feeds at a high trophic level and has a history of exposure to DLCs (Hebert *et al.*, 1999a; Hebert *et al.*, 1999b).

The objectives of this study were to use herring gull embryonic hepatocyte cultures to (a) compare the potencies of TCDD, PeCDF and TCDF as CYP1A inducers in a wild species, (b) compare the sensitivity of the herring gull to the sensitivity of species tested previously with respect to CYP1A induction, and (c) estimate the contribution of PeCDF to the TEQ in herring gull eggs of the Great Lakes.

3.3. Materials and methods

3.3.1. Source of eggs and incubation conditions

Fertile, unincubated herring gull eggs were collected from nests containing one egg on April 28, 2009 from Chantry Island, Lake Huron (44°29′22″N, 81°24′7″W). Chantry Island is considered to be a suitable reference colony within the Great Lakes due to the relatively low contaminant levels detected in herring gull eggs by the Canadian Wildlife Service in recent years (Jeremyn-Gee *et al.*, 2005). Eggs were transported to the laboratory in insulated coolers and then artificially incubated at 37.5 °C and 60% relative humidity, for 26 days (1-2 days pre-hatch). Eggs were candled periodically, and eggs that were infertile or contained dead embryos were discarded.

3.3.2. Preparation and dosing of cultured hepatocytes

Primary hepatocyte cultures were prepared from 60 herring gull embryos using the methods described in Chapter 2. Cells were dosed in triplicate with in-well concentrations ranging from 0.003 to 100 nM (TCDD and TCDF) or 0.0003 to 100 nM (PeCDF) (2.5 µl chemical/well in DMSO). Cells were incubated for 24 h in the presence of the chemical,

medium was removed and plates were flash frozen on dry ice and stored at -80 °C until they were analysed.

3.3.3. Preparation of TCDD, PeCDF and TCDF solutions

Serial dilutions of PCDD/Fs were prepared from stock dimethyl sulfoxide (DMSO) solutions, as described in Chapter 2.

3.3.4. EROD assays

EROD assays were conducted as described in Chapter 2.

3.3.5. RNA isolation and cDNA synthesis

Total RNA was extracted from 48-well plates using RNeasy 96 kits (Qiagen, Mississauga, ON) according to the manufacturer's instructions and total RNA was reverse transcribed to complementary DNA (cDNA) as described in Chapter 2.

3.3.6. Quantitative reverse transcription polymerase chain reaction (QPCR)

A multiplex QPCR assay, using dual-labelled fluorescent hydrolysis probes (Head and Kennedy, 2007a), was used to quantify herring gull CYP1A4, CYP1A5 and beta-actin mRNA abundance. Primers were obtained from Invitrogen and probes were obtained from Biosearch Technologies (Novato, CA, USA). Brilliant QPCR Core Reagent kits (Stratagene, La Jolla, CA, USA) were used to carry out each assay. Each 25 µl reaction contained 1x PCR buffer, 5 mM $MgCl_2$, 0.8 mM dNTPs, 0.08 v/v glycerol, 0.05 U Surestart Taq polymerase and 25 nM reference dye (ROX).

3.3.7. Cell viability

Cell viability was determined using the Calcein-AM assay (Invitrogen - Molecular Probes, Eugene, Oregon, USA), as described in Chapter 2.

3.3.8. EROD and CYP1A4/5 mRNA data analysis

Data were analysed as described in Chapter 2.

3.3.9. Calculation of relative potencies

The concept of using relative potency (ReP) values to compare the potencies of DLCs is well established (Van den Berg *et al.*, 1998). The relative potency is the comparison of CYP1A-inducing potency of a compound relative to the CYP1A-inducing potency of TCDD in the same species (herring gull in this case). In this study, RePs are defined as $[EC50_{TCDD}] \div [EC50_{PeCDF}]$ for PeCDF and, $[EC50_{TCDD}] \div [EC50_{TCDF}]$ for TCDF.

3.4. Results

3.4.1. Characteristics of the concentration-response curves

EROD activity. TCDD, PeCDF and TCDF induced EROD activity in a concentration-dependent manner. Maximal activity was followed by a decrease in EROD activity at greater concentrations of the inducer (Figure 3.1). The decrease in EROD activity at high concentrations of TCDD, PeCDF or TCDF was not due to overt toxicity (determined by the Calcein-AM assay; data not shown). There were clear differences between the EROD concentration-response curves of PeCDF and the two other compounds; the curve for PeCDF was shifted to the left compared to TCDD and TCDF (Figure 3.1). The concentration-response curve for TCDF was shifted slightly to the left compared to TCDD (Figure 3.1), which may simply reflect the fact that the maximal EROD responses for TCDD and TCDF were different. The maximal EROD induction of TCDD was significantly lower than the maximal EROD induction of PeCDF or TCDF (Figure 3.1, Table 3.1). It should be noted that EROD activity was not normalized against protein content, because protein content was highly variable among wells (up to 30%; data not shown).

mRNA induction. CYP1A4 and CYP1A5 mRNA expression was up-regulated in a concentration-dependent manner by TCDD, PeCDF and TCDF and mRNA expression reached a plateau at high concentrations of the inducers (Figure 3.1). For CYP1A4 mRNA expression, the concentration-response curve for PeCDF was shifted to the left compared to the TCDD and TCDF curves, which were superimposed (Figure 3.1). For CYP1A5, the concentration-response curve for PeCDF was also shifted to the left of the TCDD curve. However, the concentration-response curve for TCDF was shifted slightly to the right compared to the TCDD curve (Figure 3.1). CYP1A4 and CYP1A5 mRNA were induced to similar maximal levels by TCDD, PeCDF and TCDF (< 1.2-fold and 1.4-fold difference, respectively; Table 3.1, Figure 3.1). No amplification was observed in no-RT controls or no-template controls, confirming the absence of contamination. Changes in mRNA expression were a result of changes in CYP1A4 and CYP1A5 mRNA expression, not in beta-actin mRNA expression, thus validating its use as a housekeeping gene.

3.4.2. Relative potencies

For all three endpoints, the EC50 of PeCDF was lower than the EC50 of TCDD (up to 40-fold difference), while the EC50 of TCDD and TCDF were similar (less than 3.5-fold difference). As such, the ReP values for PeCDF were 40, 21 or 9.8, based on EROD activity, CYP1A4 or CYP1A5 mRNA expression, respectively (Table 3.1). The ReP values for TDCF were 2.0, 1.0 or 0.3, based on EROD activity, CYP1A4 or CYP1A5 mRNA expression, respectively (Table 3.1). The rank order of potency was: PeCDF > TCDD ≈ TCDF.

3.5. Discussion

The present experiments determined the CYP1A-inducing potencies of TCDD, PeCDF and TCDF in herring gull hepatocytes. There were three main objectives: (a) to

compare the potencies of TCDD, PeCDF and TCDF to induce EROD activity, CYP1A4 and CYP1A5 mRNA in herring gull, and (b) to compare the sensitivity of the herring gull to CYP1A4 induction to the sensitivity of species tested previously, and (c) to estimate the contribution of PeCDF to the TEQ in herring gulls eggs of the Great Lakes.

EROD activity is usually expressed relative to the protein content within cell culture wells (Head and Kennedy, 2007b; Kennedy *et al.*, 1996; Sanderson *et al.*, 1998). In the present experiments, the variability in protein content among wells of the same culture plate was over 30 %. This variability resulted in fluctuation of EROD activity among replicate wells of the same plate when activity was normalized against protein content. This high variation in protein content was due to residual medium in the wells. Herring gull hepatocytes did not adhere effectively to the bottom of the wells and therefore no rinse was carried out. As such, an uneven amount of medium was left in each well. Amino acids present in the residual medium could therefore alter total protein measurements. The protein content of hepatocytes is usually stable (variation of about 10 % among all the wells of a cell culture plate; results of multiple studies conducted in the Kennedy laboratory). While this chapter presents EROD activity in units of pmol/min/well rather than pmol/min/mg protein, general observations and conclusions are considered to be valid.

ReP values are often calculated by comparing the EC50 values from EROD concentration-response curves. As mentioned in Chapter 2, there is competition between the substrate (ethoxyresorufin) and the inducer (the DLC) for the CYP1A enzyme (Petrulis and Bunce, 1999), it is why a decrease in EROD activity is observed at high concentrations of DLCs. The inhibition of EROD activity can, in certain situations (usually with weak AHR ligands), decrease the maximal activity. As such, there is a leftward shift of the EC50, creating an overestimation of the potency of the compound (Hahn *et al.*, 1993; Hahn *et al.*,

1996; Head and Kennedy, 2007b; Hestermann *et al.*, 2000). When maximal responses are different among compounds, a less biased estimate can be derived from the threshold concentration for effect (ECthreshold), which represents the concentration of a compound that produces a response equivalent to 10% of the maximal response produced by TCDD in the same species (Kennedy *et al.*, 1996) (Appendix 1). The reason why TCDD, which is a strong AHR ligand, reached a lower maximal activity than PeCDF and TCDF is not known, but the fact that the activity was not normalized against protein content could be part of the explanation. Due to the low response of TCDD in this study, the ECthreshold calculation could not be achieved. Therefore, the major conclusions of this research are based mainly on the expression of CYP1A4 and CYP1A5 mRNA.

Similar maximal responses and parallel curves were observed for mRNA induction; therefore, the conclusions based on EC50 values derived from CYP1A4/5 mRNA expression allow for careful comparisons of relative potencies. Based on CYP1A4 mRNA expression, TCDF and TCDD were equipotent; based on CYP1A5 mRNA expression, TCDF was 3-times less potent than TCDD. The CYP1A4 data were consistent with previous findings of Kennedy *et al.* (1996) which showed that TCDD and TCDF were equipotent in terms of EROD induction in herring gulls. In contrast, PeCDF was 21- times more potent than TCDD at inducing CYP1A4 mRNA and 9.8-times more potent than TCDD at inducing CYP1A5 mRNA. This is the first study to show that PeCDF is more potent than TCDD in herring gull hepatocytes. Previous studies reported similar findings in double-crested cormorant (*Phalacrocorax auritus*) and Forster's tern (*Sterna forsteri*) hepatocytes (Sanderson and Bellward, 1995), ring-necked pheasant (*Phasianus colchicus*) and Japanese quail (*Corturnix japonica*) hepatocytes (Chapter 2), and green frog (*Rana esculenta*) hepatocytes (Rankouhi *et al.*, 2005) exposed to PeCDF and TCDD. Moreover, *in ovo* data suggest that PeCDF is

approximately 9-times more potent than TCDD at causing embryonic death in Japanese quail after injection at day 0 of incubation (Cohen-Barnhouse, 2008).

Variation of potency among compounds can be explained by different factors, mentioned in Chapter 2. It is known that the greater the binding affinity of the compound to the AHR, the greater the potency (Hestermann *et al.*, 2000; Poland and Glover, 1973; Poland and Knutson, 1982; Whitlock, Jr., 1990). The intrinsic efficacy of a DLC can also be a determinant for its potency, but that holds true only for weak AHR ligands, such as the mono- or di-ortho substituted PCBs (Hestermann *et al.*, 2000), and is an unlikely explanation for what was observed here. Ligand-specific interactions between coactivators and the AHR could also be involved in variable potency of DLCs. Indeed, when the AHR binds to DNA, it interacts with coactivators to increase gene transcription and recent findings indicate that interactions with these co-activators are structure-dependent and influence AHR transactivation (Zhang *et al.*, 2008).

It is well established that different species exhibit different sensitivities to DLCs (Head *et al.*, 2008), and it was of interest to compare our results with previous findings. As mentioned in the previous chapters, the difference in sensitivity among avian species has been attributed, in part, to substitutions in specific amino acids in the AHR1 ligand binding domain. The amino acid residues corresponding to Ile324/Ser380 in the chicken were compared among several avian species, and it was found that their identity was predictive of broad categories of dioxin sensitivity among species (Head *et al.*, 2008). The herring gull and Japanese quail have the Val/Ala genotype which corresponds to the lowest sensitivity to embryotoxic effects of DLCs. This study and the study with the Japanese quail in Chapter 2 also found a lower sensitivity of the species with the Val/Ala genotype for CYP1A induction by TCDD and TCDF, but not by PeCDF. The EC50 values for CYP1A4 induction of herring

gull and Japanese quail hepatocytes exposed to TCDD were 56- and 13-times higher than the EC50 of chicken hepatocytes exposed to TCDD (Table 3.2). Similar to the observations for TCDD, the EC50 values of herring gull and Japanese quail hepatocytes exposed to TCDF were 100- and 40-times higher than the EC50 of chicken hepatocytes exposed to TCDF (Table 3.2). On the other hand, when hepatocytes were exposed to PeCDF, the sensitivity of the herring gull and Japanese quail was similar to that of chicken. Indeed, EC50s of herring gull and Japanese quail hepatocytes exposed to PeCDF were 3.5- and 1.2-times lower than the EC50 of chicken hepatocytes exposed to PeCDF (Table 3.2).

There are possibly factors other than the 324/380 amino acid residues that contribute to the difference in CYP1A sensitivity. This is suggested by (a) the difference in rank order of sensitivity when the species are exposed to different DLCs, and (b) the variation observed between the herring gull and Japanese quail EC50 values when exposed to the same compound (up to 5-fold; Table 3.2). As mentioned in Chapter 2, differences in ligand-specific AHR-coactivator interactions among species (Zhang *et al.*, 2008), metabolism (Head *et al.*, 2008) or in four additional amino acids of interest in the AHR1 (Head *et al.*, 2008, Kennedy *et al.* manuscript in preparation), are possible explanations that are worth investigating.

It is interesting to note that, in addition to the double-crested cormorant (Sanderson *et al.*, 1998) and Japanese quail (Chapter 2), the herring gull is the third avian species with the Val/Ala substitution for which PeCDF was found to be a more potent CYP1A inducer than TCDD (Table 3.2). The substitution in amino acids in the AHR1 was shown to be responsible for differences in binding affinity of TCDD to the AHR (Karchner *et al.*, 2006). Thus, it would be interesting to see if the high binding affinity of TCDD to the Ile/Ser

substituted AHR1 and the low binding affinity of TCDD to the Val/Ala substituted AHR1 also holds true for other DLCs, such as PeCDF.

It should be noted that previous exposure of wild bird eggs to DLCs during their embryonic development could affect the CYP1A response measured in this and other studies. However, CYP1A measurements from 25 eggs collected from Chantry Island in 2003, were not affected by maternally deposited DLCs (Head, 2006), and exposure of chicken embryos to environmentally relevant DLC levels did not affect CYP1A induction in chicken (Head *et al.*, 2006).

The finding that PeCDF is a more potent CYP1A inducer than TCDD in herring gull hepatocytes could have consequences on how the history of the GLEMEDS is interpreted. The Canadian Wildlife Service's Great Lakes Herring Gull Monitoring Program has been measuring the levels of contaminants in herring gull eggs for the last 40 years and relatively high levels of PeCDF were measured between 1971 and 1991 (Bishop *et al.*, 1992; Hebert *et al.*, 1999a; Jeremyn-Gee *et al.*, 2005; Pettit *et al.*, 1996). For example, between 1984 and 1991, 5 – 12 ng PeCDF/g wet weight (ww) was reported in herring gull eggs from geographically distinct locations in the Great Lakes (Table 3.3) (Hebert *et al.*, 1994). The ratio of the TEQs ($PeCDF_{TEQ}/TCDD_{TEQ}$) suggests that PeCDF could have contributed 2- to 17-times more to the TEQ than TCDD in herring gull eggs analyzed during this period (Table 3.3). Repetition of these experiments, *in vivo* experiments and a meticulous revision of the literature are needed before any conclusion can be made, but our results suggest that PeCDF could have contributed to the GLEMEDS and the poor reproductive success of colonial fish-eating bird species of the Great Lakes to a greater extent than previously thought.

The present study established that (a) PeCDF was a more potent CYP1A inducer than TCDD in herring gull hepatocytes, (b) when exposed to TCDD or TCDF, the herring gull was less sensitive than the chicken to CYP1A induction, and (c) when exposed to PeCDF, the two species were approximately equisensitive. The results presented here raise questions about the molecular mechanisms of DLCs and on-going molecular studies in our laboratory will contribute to our understanding of why there are species-specific responses to DLCs. Based on a limited number of studies (*in ovo* and *in vitro*), conducted principally with chicken, PeCDF was assigned a TEF of 1.0 (Van den Berg *et al.*, 1998). The findings of the present study, Chapter 2 of this thesis, and *in ovo* studies (Cohen-Barnhouse, 2008), suggest that a TEF of 1.0 might underestimate the potency of PeCDF in certain species. A more comprehensive approach could include different avian species in the TEF concept.

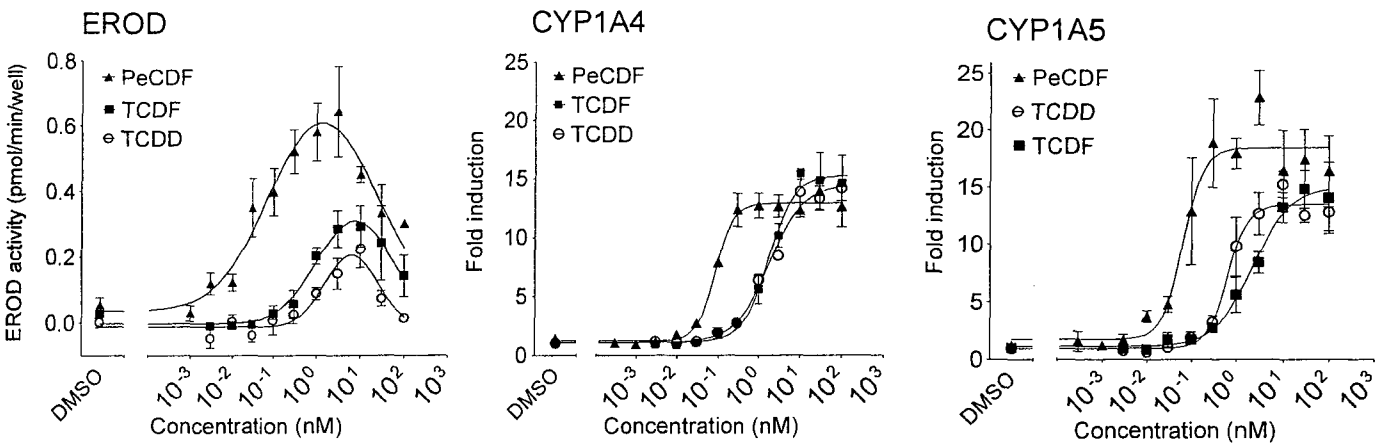


Figure 3.1. Concentration-dependent effects of PeCDF (▲), TCDD (○) or TCDF (■) on EROD activity, CYP1A4 mRNA expression and CYP1A5 mRNA expression in herring gull hepatocyte cultures exposed for 24h. For EROD activity, each point represents the mean of three different cell culture plates. For mRNA expression each point represents the mean of three different wells from the same cell culture plate. Values before the axis break indicate response observed for control (DMSO-treated hepatocytes). Bars represent standard errors.

Table 3.1. Maximal response, EC50 and relative potency values (ReP) for EROD activity, CYP1A4 mRNA expression and CYP1A5 mRNA expression measured in herring gull hepatocyte cultures exposed to TCDD, PeCDF or TCDF for 24 h.

Species	Compound	End point	EC50 (nM)	EC50-based ReP	Maximal response (EROD: pmol/min, CYP1A4/5: fold change)
HERG	TCDD	EROD *	1.61 ± 0.6 [a]	1.0	0.20 ± 0.03 [a]*
HERG	PeCDF	EROD *	0.04 ± 0.01 [b]	40	0.61 ± 0.04 [b]*
HERG	TCDF	EROD *	0.79 ± 0.3 [a]	2.0	0.31 ± 0.03 [b]*
HERG	TCDD	CYP1A4	1.80 ± 0.37	1.0	15 ± 0.7
HERG	PeCDF	CYP1A4	0.084 ± 0.012	21	13 ± 0.3
HERG	TCDF	CYP1A4	1.74 ± 0.37	1.0	15 ± 0.8
HERG	TCDD	CYP1A5	0.64 ± 0.1	1.0	13 ± 0.6
HERG	PeCDF	CYP1A5	0.065 ± 0.02	9.8	18 ± 0.8
HERG	TCDF	CYP1A5	2.23 ± 0.7	0.3	15 ± 1.1

* EROD EC50 values and maximal responses are derived from data not normalized against protein content

Note: Means are derived from the average of three replicate cell culture plates ± standard error (EROD parameters) or from data originating from the average of three different wells of the same cell culture plate (mRNA expression). Superscript letters indicate significant differences between endpoints ($p<0.05$). No statistical tests could be performed on the EC50 of mRNA expression because only one curve fit was generated and therefore only one value of EC50 was obtained. EROD data were fit to a modified Gaussian curve and CYP1A4/5 mRNA expression data were fit to a four-parameter logistic curve.

Table 3.2. EC50 and relative potency values (ReP) for CYP1A4 induction activity measured in herring gull, Japanese quail and chicken hepatocyte cultures exposed to TCDD, PeCDF or TCDF for 24 h.

		Herring gull		Japanese quail		Chicken	
Endpoint	Compound	EC50 (nM)	ReP	EC50 (nM)	ReP	EC50 (nM)	ReP
CYP1A4	TCDD	1.80 ± 0.37	1.0	0.36 ± 0.03	1.0	0.026 ± 0.008	1.0
CYP1A4	PeCDF	0.084 ± 0.012	21	0.047 ± 0.01	7.7	0.037 ± 0.008	0.7
CYP1A4	TCDF	1.74 ± 0.37	1.0	0.69 ± 0.2 [†]	0.5[†]	0.017 ± 0.01	1.5

Note: Means are derived from the average of three wells of the same cell culture plate. Superscript letters indicate significant differences between treatments ($p<0.05$). CYP1A4 mRNA expression data were fit to a four-parameter logistic curve. EC50s and RePs for Japanese quail and chicken come from Chapter 2.

[†] Because no maximal response was reached, EC50 might be underestimated and ReP might be overestimated

Table 3.3. Geometric mean for TCDD and PeCDF concentrations (conc.) in pg/g wet weight (ww) and TCDD toxic equivalent (TCDD-TEQ) in herring gull eggs collected from 13 Great Lakes colonies from 1984 to 1991.

	TCDD		PeCDF		
Colony	Conc. (pg/g ww)	TEQ (pg/g ww)	Conc. (pg/g ww)	TEQ (pg/g ww)	Ratio ($PeCDF_{TEQ}$ / $TCDD_{TEQ}$)
Granite	16.03	16.03	10.38	217.98	13.60
Agawa	20.09	20.09	11.43	240.03	11.95
Big Sister	14.16	14.16	10.50	220.50	15.57
Gull	14.09	14.09	11.80	247.80	17.59
Double	24.99	24.99	8.63	181.23	7.25
Chantry	18.32	18.32	9.08	190.68	10.41
Channel/Shelter	86.50	86.50	23.28	488.88	5.65
Fighting	16.75	16.75	6.38	133.98	8.00
Middle	15.81	15.81	8.11	170.31	10.77
Port Colborne	17.13	17.13	6.05	127.05	7.42
Niagara	23.77	23.77	7.10	149.10	6.27
Muggs	43.55	43.55	5.71	119.91	2.75
Snake	68.87	68.87	6.98	146.58	2.13

Concentrations measured in eggs are from Hebert *et al.* (1994)

Note: the TEQ was obtained by multiplying the concentration measured in eggs by the TEF or ReP value of the compounds. TCDD TEF = 1, PeCDF CYP1A4-based ReP = 21

Chapter 4

Conclusions, general discussion and future research

The experiments presented in this thesis investigated the cytochrome P4501A (CYP1A) response of avian hepatocytes upon exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and two polychlorinated dibenzofurans (PCDFs). The findings provide useful information regarding dioxin-like compounds (DLCs) that were not studied in detail previously, and suggest that there are species-specific compound interactions that influence CYP1A induction.

The first finding of these experiments demonstrated that the rank order of potency of DLCs varies among species, or in other words, that the relative potency of DLCs varies among species. Indeed, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is as potent as 2,3,7,8-tetrachlrodibenzo-*p*-dioxin (TCDD) to induce CYP1A in chicken, but PeCDF is a more potent CYP1A inducer than TCDD in ring-necked pheasant (Chapter 2) and Japanese quail (Chapter 2) herring gull hepatocytes (Chapter 3). To my knowledge, this is the first time that there are clear evidences that showed that some compounds can be more potent than TCDD.

The second finding demonstrated that the rank order of sensitivity of species changes according to the identity of the chemical that the species are exposed to, or in other words, that the relative sensitivity of species changes according to chemical they are exposed to (Chapter 2). Indeed, when exposed to TCDD or 2,3,7,8-tetrachlrodibenzofuran (TCDF), the chicken is the most sensitive species, followed by the ring-necked pheasant and Japanese quail. However, when exposed to PeCDF, the sensitivity of the three species is similar. A comparison between the results of the chicken and herring gull supports the second finding.

When exposed to TCDD or TCDF, the chicken is more sensitive than the herring gull, but, when exposed to PeCDF, their sensitivity is similar (Chapter 3). According to my knowledge, this is the first time that it was shown that some avian species have a similar sensitivity than the chicken.

The two first findings highlight the fact that the same DLCs do not behave in the same way in different species. The reasons are not known but several hypotheses, mentioned before, could explain these findings. It is known that the binding affinity is an important determinant for the potency of DLCs. Therefore, we can hypothesize that the binding affinity of PeCDF to the chicken aryl hydrocarbon receptor 1 (AHR1) is similar to that of TCDD, but that the binding affinity of PeCDF to the AHR1 of the ring-necked pheasant, Japanese quail and herring gull is greater than the binding affinity of TCDD. It is interesting to note that the species in which PeCDF is more potent, are considered to have a moderate sensitivity to DLCs (ring-necked pheasant) or a low sensitivity to DLCs (Japanese quail and herring gull), compared to the chicken that has a high sensitivity to DLCs. As mentioned in the previous Chapters, an explanation for this variation in sensitivity is a substitution in the amino acid residues corresponding to Ile324/Ser380 in the chicken. Indeed, the ring-necked pheasant (Ile/Ala), Japanese quail (Val/Ala) and herring gull (Val/Ala) AHR1 are substituted with different amino acid residues that could reduce the binding affinity of TCDD to their AHR1, compared to the binding affinity of TCDD to the chicken AHR1. It would be worthwhile investigating if the binding affinity of PeCDF to the AHR1 of these species would also be affected by the identity of the amino acids or if it would be similar despite the substitution. Numerous wild avian species have the Val/Ala genotype, thus it would be interesting to investigate the effect of PeCDF in these species also. Other explanations, besides binding affinity, can be considered. When the AHR-DLC complex binds to the DNA in the nucleus,

it interacts with co-activators to alter gene expression and these interactions could be species- and ligand-specific. The explanation could also involved physiological factors. For example, in chicken, PeCDF could be metabolized at the same rate as TCDD, while in Japanese quail PeCDF could be metabolized slower than TCDD, leading to a greater potency of PeCDF. However, preliminary results in Japanese quail suggest that there is no change in the potency of TCDD or PeCDF to induce CYP1A between 12 and 48 h, suggesting an absence of metabolism (Hervé *et al.*, manuscripts in preparation). Investigating the effect of time on the potency of DLCs on a longer period and the measurement of metabolites could be of interest. One of the most likely possibility is that what was observed in the present study is a combination of numerous factors, including the ones mentioned above and probably other ones.

The third finding demonstrated that EROD activity, CYP1A4 mRNA and CYP1A5 mRNA expression can be used interchangeably to predict *in ovo* toxicity of DLCs in chicken, ring-necked pheasant and Japanese quail (Chapter 2). Indeed, concentration-response curves of the three endpoints have similar curve slopes and EC50s in chicken, ring-necked pheasant and Japanese quail. It is therefore unlikely that they would lead to great variation in estimates of relative potency or of relative sensitivity. Moreover, the rank order of potency of TCDD, PeCDF and TCDF, is the same for the three endpoints in herring gull: PeCDF > TCDD ≈ TCDF (Chapter 3). Both methods have their particular advantages: depending on time or money constrains, or on sample availability, one can choose the most appropriate method.

Finally, there could be historical importance to the finding that PeCDF is about 20-times more potent than TCDD in herring gull. If this result is confirmed by other *in vitro* and *in ovo* experiments, the way the history of the decline of the bird population in the Great Lakes is told right now could be modified. Specifically – it is possible, that PeCDF could

have contribute as much, if not more, as TCDD to the Great Lake Embryo Mortality, Edema and Deformity Syndrome, which was linked to the poor reproductive success of the fish-eating birds of the Great Lakes in the early 1970s.

This study led to the discovery that PeCDF is a more potent CYP1A inducer than TCDD in certain avian species and that some species can be as sensitive as the chicken to CYP1A induction when exposed to certain DLCs. This is surprising because TCDD is usually considered as the most potent DLCs in all species and the chicken as the most sensitive species to DLCs. These findings suggest that DLCs do not behave the same way in every species and highlight the inaccuracy of the TEF concept for certain avian species. Indeed, PeCDF was classified as equipotent to TCDD in avian species, and assigned a TEF 1.0 by the World Health Organization. This classification was done based on a limited number of studies, mainly carried out on chickens. As mentioned in the previous chapters, while in chicken a TEF of 1.0 for PeCDF is accurate, in ring-necked pheasant, Japanese quail and herring gull, a TEF of 1.0 could underestimate the relative potency of PeCDF. Moreover, if the Val/Ala genotype is found to be one of the determinants for the greater potency of PeCDF in birds, other wild species, with the Val/Ala genotype, could be targeted by this finding. DLCs are still present in the environment today and could potentially affect wildlife. However, intensive clean up of large areas can also influence negatively the ecosystem. Therefore careful evaluation of the risks needs to be done before a decision is taken. Measuring CYP1A induction of DLCs in embryonic cell cultures gives an indication of their potency to cause harmful effects. However, testing every chemical in every bird species is impossible, and extrapolating the results obtained in chicken to all the other avian species can lead to erroneous conclusions. Understanding the molecular mechanisms of DLCs and

testing chemicals in species that are representative of the range of sensitivity found among birds could lead to a more accurate estimation of species sensitivity to DLCs.

Suggestions for future research

Research on molecular mechanism of action is needed to better understand the results of this thesis. Experiments testing the three AHR genotypes for binding affinity and for transactivation, as well as experiments testing the effect of different co-activators with the three different genotypes would provide useful information. Such experiments are being carried out in the Kennedy lab at this moment. Measurement of CYP1A4 and CYP1A5 protein level would also offer additional data that would contribute to understand the variation in maximal activities seen among different species. *In vivo* experiments looking at the species differences in absorption, distribution, metabolism and excretion patterns would provide insights to explain why there are sometimes differences between *in vitro* and *in vivo* data. Looking at inter-individual variability would also be an interesting project.

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Appendix 1: Dose-response curves of EROD activity and maximal activity (efficacy)

The typical dose-response curve of EROD activity is bi-phasic (Figure A.1). Two issues need to be addressed regarding EROD curves: 1) the fall-off in activity at high concentration of inducers and 2) maximal activities (efficacy).

The EROD reaction is competitively inhibited by the inducer (Gooch *et al.*, 1989; Petrulis and Bunce, 1999), which explains that, while CYP1A catalytic activity declines at high inducer concentrations, CYP1A protein and mRNA levels stay elevated (Gooch *et al.*, 1989; Hestermann *et al.*, 2000). This inhibition also explains the different efficacy (represented by the maximal response) among compounds. Indeed, potent inducers (those that bind with high affinity to the AHR) reach their maximal activity, but the weaker inducers (weak binding to the AHR) will inhibit the EROD reaction prior to reaching their maximal activity (Petrulis and Bunce, 1999). This inhibition by the inducer leads to a lower observed maximal activity and an overestimation of the potency is likely (Hahn *et al.*, 1993; Hahn *et al.*, 1996). For example, when maximal EROD activity is lower, as it is for compound 2 (C2), the EC50 is shifted to the left and potency is overestimated (Figure A.1, Table A.1).

When maximal activities are different, the use of the ECthreshold (ECthr) might be more appropriate than the EC50, particularly if it is known the CYP1A protein levels are identical among agonists. ECthr is defined as the concentration of a DLC required to induce a response equivalent to 10% of the maximal response of TCDD. Because the residual concentration of the compound that remains in the hepatocytes at the dose where the ECthr is measured is less than the dose at which EC50 is measured, the competitive inhibition is less likely to have an effect. Therefore, ECthr can be sometimes a better measure of relative potencies among compounds and also sensitivities among species.

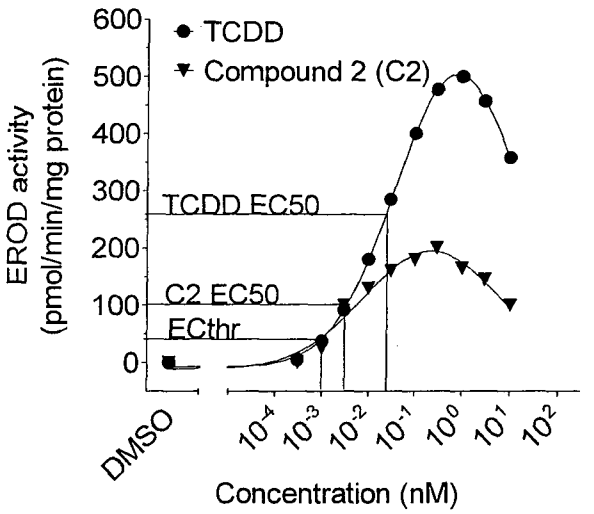

Figure A.1. Hypothetic EROD concentration-response curves for TCDD and compound 2 (C2).

Table A.1. Hypothetic EC50, ECthr, relative potencies (ReP) values, and maximal responses for TCDD and compound 2 (C2).

Compound	EC50	EC50-based ReP	ECthr	Ecthr-based ReP	Maximal activity (pmol/min/mg/protein)
TCDD	0.02	1	0.001	1	500
C2	0.004	5	0.001	1	200