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VARIATION IN THE CYTOCHROME P4501A RESPONSE TO DIOXIN-LIKE COMPOUNDS IN AVIAN SPECIES

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Abstract

Toxicological risk assessment for dioxin-like compounds in avian wildlife is currently limited by a lack of species sensitivity data. This gap in the literature can be partially attributed to difficulties associated with performing *in vivo* dosing studies with wild birds. For this reason, *in vitro* approaches for estimating dioxin sensitivity are required. One such bioassay measures the cytochrome P4501A (CYP1A) response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) as ethoxyresorufin- *O*-deethylase (EROD) activity in cultured embryo hepatocytes. Application of the EROD bioassay to risk assessment in avian wildlife is complicated by the extreme variability of the CYP1A response. The experiments presented in this thesis characterize factors contributing to variation in the CYP1A response to TCDD among individuals, populations, and species. These experiments have the dual purpose of investigating mechanisms underlying variation in dioxin sensitivity in avian species, and improving *in vitro* methods for obtaining species sensitivity estimates.

Basal and TCDD-induced CYP1A mRNA expression was found to be extremely variable in hepatocyte cultures prepared from 55 individual herring gull embryos. Significant differences in basal CYP1A expression between populations suggested that there may be a genetic component to this variation. Possible effects of exposure to contaminants throughout development were investigated using a chicken model. Embryonic exposure to dioxin-like compounds at current environmental levels was determined to be unlikely to contribute to the CYP1A response to TCDD in cultured hepatocytes for all but the most contaminated individuals. Regardless of the source, variation between individuals was shown to be a confounding factor for obtaining reproducible sensitivity estimates from the CYP1A bioassay.

Characterization of mechanisms underlying species differences in the CYP1A response to TCDD led to the development of novel *in vitro* approaches for assessing dioxin sensitivity in birds. A comparison of previously published toxicity data and aryl hydrocarbon receptor (AHR) sequences cloned from 18 avian species revealed that key amino acids in the AHR ligand binding domain predict sensitivity to dioxin-like compounds in diverse avian orders. This finding suggests that a simple genetic test could be used to identify species that are particularly sensitive to dioxin-like compounds within contaminated environments. A second series of experiments led to the development of a novel method for

measuring CYP1A enzymatic activity (EROD) and mRNA expression in the same sample. This technique increases the versatility of the EROD bioassay and maximizes the amount of data that can be obtained from scarce tissues.

Together the results presented in this thesis provide important insight into mechanisms underlying variation in dioxin sensitivity in birds, and enhance our ability to estimate risk to avian wildlife through the use of *in vitro* bioassays.

Résumé

L'évaluation des risques toxicologiques causés par les dioxines chez la faune avienne est limitée par un manque de données de la sensibilité des espèces. Cette lacune peut être partiellement attribuée aux difficultés liées aux études de dosage *in vivo* chez les oiseaux sauvages. Pour cette raison, des approches *in vitro* sont requises pour estimer la sensibilité aux dioxines. Un essai biologique permet en effet de mesurer la réponse du cytochrome P4501A (CYP1A) au 2,3,7,8-tétrachlorodibenzo-*p*-dioxine (TCDD) comme l'activité d'éthoxyresorufin-*O*-deethylase (EROD) retrouvée chez les hépatocytes d'embryons cultivés. L'application de l'essai biologique d'EROD à l'évaluation des risques toxicologiques chez la faune avienne est complexe, car la réponse de CYP1A peut extrêmement variée. Les expériences qui sont présentées dans cette thèse, caractérisent les facteurs qui contribuent à la variation de la réponse de CYP1A à TCDD parmi des individus, des populations, et des espèces. Les deux principaux buts de ces expériences sont la recherche des mécanismes qui contrôlent la variation de la sensibilité des espèces aviennes aux dioxines, ainsi que l'amélioration des méthodes *in vitro* pour l'estimation de la sensibilité des espèces.

L'expression d'ARNm de base de CYP1A, et l'expression induite par TCDD ont été extrêmement variables dans des cultures d'hépatocytes préparées à partir de 55 différents embryons de goélands argentés. Les différences d'expressions de base de CYP1A entre les populations ont suggéré qu'il y a un facteur génétique qui contribue à cette variation. En utilisant le modèle du poulet, ont a étudié les effets de l'exposition aux contaminants pendant le développement de l'embryon. Ce modèle a suggéré que l'exposition des embryons aux niveaux environnementaux de dioxines ne contribue pas à la réponse de CYP1A à TCDD chez les hépatocytes cultivés, sauf pour les individus soumis à un niveau de contamination très élevé. Peu importe la source, la variation entre les individus a été souligné comme étant un facteur confondant pour l'obtention d'estimés de sensibilité reproductibles de l'essai biologique du CYP1A.

La caractérisation des mécanismes liés à la variabilité de la réponse de CYP1A entre les espèces a mené au développement de nouvelles méthodes *in vitro* pour évaluer la sensibilité des oiseaux aux dioxines. Une comparaison entre des données de toxicité

publiées et des séquences du récepteur d'hydrocarbures aryliques (RHA) chez 18 espèces aviennes a permis de constater que les acides aminés principaux dans le domaine obligatoire du ligand d'RHA prévoient la sensibilité des dioxines dans des ordres aviens divers. Ceci suggère qu'un essai génétique simple pourrait être employé pour identifier les espèces qui sont particulièrement sensibles aux dioxines dans les environnements contaminés. Une deuxième série d'expériences a mené au développement d'une nouvelle méthode pour mesurer l'activité enzymatique de CYP1A (EROD) et de l'expression de l'ARNm de CYP1A chez le même échantillon. Cette technique permet d'augmenter la polyvalence de l'essai biologique d'EROD et de maximiser la quantité de données qui peuvent être obtenues à partir des tissus rares.

Les résultats présentés dans cette thèse fournissent des révélations importantes par rapport aux mécanismes de sensibilité aux dioxines chez les oiseaux, et augmentent notre capacité d'estimer le risque chez la faune avienne par l'utilisation des essais biologiques *in vitro*.

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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
5-CFDA, AM	5-carboxyfluorescein diacetate-acetoxymethyl ester
CFIA	Canadian Food Inspection Agency
CI	Chantry Island
Ct	threshold cycle
CWS	Canadian Wildlife Service
CYP1A	cytochrome P4501A
CYP1A4	cytochrome P4501A4
CYP1A5	cytochrome P4501A5
DDT	dichloro-diphenyl-trichloroethane
DMSO	dimethyl sulfoxide
EC50	median effective concentration
EC_{thr}	threshold potency
EROD	ethoxyresorufin-*O*-deethylase activity
HCP	highly carboxylated porphyrin
hrs	hours
KI	Kent Island
LBD	ligand binding domain
LD50	lethal dose for 50% of the population
LOEL	lowest observable effect level
MSI	Middle Sister Island
NOEL	No observable effect level
NR	non-responder
NWRC	National Wildlife Research Centre
OHRI	Ottawa Health Research Institute
PAH	polyaromatic hydrocarbon
PCB	polychlorinated biphenyl
PCB 77	3,3',4,4'-tetrachlorobiphenyl

PCB 105	2,3,3',4,4'-pentachlorobiphenyl
PCB 118	2,3',4,4',5-pentachlorobiphenyl
PCB 126	3,3',4,4',5-pentachlorobiphenyl
PCB 156	2,3,3',4,4',5-hexachlorobiphenyl
PCB 157	2,3,3',4,4',5'-hexachlorobiphenyl
PCB 169	3,3',4,4;,5,5'-hexachlorobiphenyl
Q-PCR	quantitative RT-PCR
RT-PCR	reverse transcriptase-polymerase chain reaction
SNP	single nucleotide polymorphism
$t_{1/2}$	half-life
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachlorodibenzo-furan
TEF	toxic equivalency factor
TEQ	TCDD equivalent
UROX	uroporphyrinogen oxidation
WHO	World Health Organization
XRE	xenobiotic response element

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This thesis is dedicated to the memory of my grandmother, Ursula Abbott.

Chapter 1

Introduction

1.1. General Introduction and Hypothesis

A central goal of wildlife toxicology is the identification of species at risk from exposure to environmental contaminants within polluted ecosystems. In a traditional risk assessment model, this requires chemical data describing the degree of exposure, and toxicity data describing species sensitivity. Chemical analysis of known toxicants in water, sediment, and biota is a routine procedure, but can be prohibitively expensive and does not provide information relating to biological activity. *In vivo* toxicity data are useful for estimating species sensitivity, but these are not available for many ecologically relevant species because dosing studies are difficult to carry out with wild animals. A further confounding factor is that sensitivities to toxicants can vary dramatically between species, making it difficult to extrapolate from one species to another.

A second approach for identifying species at risk from exposure to environmental contaminants is the use of biomarkers and sentinel species. This approach has been extensively applied towards monitoring the effects of dioxin-like compounds on fish-eating birds in the Great Lakes. In particular, cytochrome P4501A (CYP1A) activity has been associated with elevated concentrations of dioxin-like compounds in several avian species known to accumulate high levels of contaminants, including herring gulls, common terns, and double-crested cormorants (Fox 1993). As with traditional approaches to risk assessment, variation in dioxin sensitivity among species, makes interpretation of biomarker data difficult, and extrapolation between species problematic.

The effectiveness of CYP1A induction as a biomarker for dioxin-like compounds in avian wildlife is currently limited by a lack of understanding of mechanisms underlying differential sensitivity. The experiments described in the following chapters attempt to improve risk assessment for wild birds through a better understanding of variation in biomarker responses among species, individuals, and populations. The experiments fall under two overlapping themes:

1) Experimental, environmental, and genetic factors contributing to variation in the CYP1A response to TCDD among individuals, populations, and species were characterized. A better understanding of these factors will improve design of biomarker experiments, and permit more meaningful interpretation of biomarker data (Chapter 3, Chapter 4, Chapter 5, and Chapter 6).

2) Two novel *in vitro* methods for estimating dioxin sensitivity in avian species were developed. Both methods are particularly well suited to the constraints inherent in working with wildlife (Chapter 2 and Chapter 6).

1.2. Dioxin-like Compounds

Dioxin-like compounds are a class of persistent organic pollutants that are extremely toxic to vertebrate species. Effects of exposure include lethality, a skin lesion called chloracne, endocrine disruption, wasting syndrome, immune deficiencies and developmental abnormalities (Poland and Knutson 1982; Pohjanvirta and Tuomisto 1994; Peterson *et al.* 2000; Birnbaum and Tuomisto 2000). The most potent dioxin-like compound is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), sometimes referred to simply as dioxin (Figure 1.1). Other halogenated dibenzodioxins, halogenated dibenzofurans, and certain polychlorinated biphenyls (PCBs) also exhibit dioxin-like properties. Membership in this class of environmental contaminants is defined based on biological outcome rather than common chemical structure, but many dioxin-like chemicals share a planar structure that is a good fit for the ligand binding domain of the aryl hydrocarbon receptor (AHR). For example, non-*ortho* substituted PCBs such as 3,3',4,4',5-pentachlorobiphenyl (PCB 126), and 3,3',4,4'-tetrachlorobiphenyl (PCB 77) bind to the AHR with high affinity, and cause dioxin-like toxicity. The position of chlorine atoms on *ortho* substituted PCBs prevents the molecule from assuming a planar conformation. These congeners are not generally dioxin-like although they can cause toxicity by AHR-independent mechanisms (Seegal *et al.* 1991).

1.2.1. Sources

Low levels of dioxin-like compounds occur naturally, but the most important sources are anthropogenic and the presence of significant levels in the environment is linked to industrialization. Dioxins and dibenzofurans are produced during high temperature incineration of municipal waste, and as unwanted by-products of industrial processes involving chlorine. Another major class of dioxin-like compounds, PCBs, were commercially manufactured, principally as cooling agents, beginning in 1929 (Webster and Commoner 2003). From 1979 onwards, PCBs were no longer produced in North America, and contaminant levels in biota have decreased dramatically following a peak in the 1970s. For example, herring gull eggs collected at Chantry Island, Lake Huron, were contaminated with over 80 μg/g PCBs in 1974, and this figure steadily decreased to less than 10 μg/g by 1998 (Hebert *et al.* 1999b).

1.2.2. Chemical properties

Dioxin-like compounds are semi-volatile and chemically stable. These properties favour persistence in the environment and a wide distribution due to atmospheric distribution far from point sources (Webster and Commoner 2003). Like many other environmental contaminants of concern, dioxin-like compounds are lipophillic and readily accumulate in biota. Lipophillic contaminants tend to biomagnify up the food chain, resulting in elevated concentrations in animals feeding at higher trophic levels. For example, PCB concentrations in herring gulls are over 10 million times higher than concentrations in water, and approximately 100 times higher than concentrations in alewife, a main prey item (Clark *et al.* 1988; Pierotti and Good 1994). These properties, along with a high degree of biological activity and a low capacity for metabolism, contribute to the extreme toxicity of dioxin-like environmental contaminants.

1.2.3. Toxic equivalency factors

Although all dioxin-like compounds are believed to work through a similar mode of action, the degree of toxicity varies significantly among congeners. In 1997 the World Health Organization (WHO) convened a panel of experts to determine literature based toxic equivalency factors (TEFs) for dioxin-like compounds in fish, mammals and birds (Van den Berg *et al.* 1998). This methodology provides a 'common currency' for dioxin-like compounds by expressing concentrations of individual congeners or environmental mixtures in terms of TCDD equivalents (TEQs), or the potency of the mixture relative to TCDD. For example, PCB 126 is approximately 10 times less potent than TCDD in chickens (Powell *et al.* 1996a), and has a TEF of 0.1 (Van den Berg *et al.* 1998). According to the WHO methodology, concentrations of 10 μg/kg PCB126 and 1 μg/kg TCDD are both equivalent to 1 μg/kg TEQ.

1.3. The Dioxin Response Pathway

1.3.1. Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) belongs to a well characterized family of nuclear transcription factors known as basic helix-loop-helix Per Arnt Sim (bHLH-PAS) proteins. Dioxin-like compounds bind the AHR with affinities roughly proportional to the degree of toxicity that they produce (Poland *et al.* 1976). This interaction is thought to be the starting point of most, if not all, of the toxic effects of TCDD and related chemicals. In the absence of ligand, the AHR is associated with chaperone proteins hsp 90, XAP2, and p23 in the cytosol (Figure 1.2). Ligand binding causes the AHR to undergo a conformational change, and translocate to the nucleus of the cell. There, it forms a heterodimer with another bHLH-PAS protein, the AHR nuclear translocator (Arnt). This complex recruits other transcription factors, and binds to xenobiotic response elements (XREs) in the promoter region of dioxin-responsive genes. XREs are found in the regulatory regions of a number of metabolic enzymes including cytochrome P4501A (CYP1A) and CYP1B isoforms

(Whitlock, Jr. 1993; Mimura and Fujii-Kuriyama 2003). Microarray experiments demonstrate that a large number of other genes are also up- or down-regulated in response to exposure to dioxin. The abundance of 310 gene transcripts was altered by at least 2-fold in human hepatoma cells treated with TCDD. Of these, 108 changes were considered to be primary effects, while the occurrence of the remaining 202 changes required protein synthesis (Puga *et al.* 2000).

Multiple lines of evidence suggest that the AHR plays a central role in dioxin toxicity. Polymorphisms in several regions of the AHR, including the ligand binding domain and the transactivation domain, have been associated with altered sensitivity to dioxin-like compounds in rodents (Poland *et al.* 1994; Pohjanvirta *et al.* 1998). Further evidence for the role of the AHR in dioxin toxicity is suggested by AHR-null mice generated by three independent laboratories. Developmental effects of the AHR-null genotype differed, but each group found that AHR -/- mice were highly resistant to toxic and biochemical effects of TCDD (Fernandez-Salguero *et al.* 1995; Schmidt *et al.* 1996; Mimura *et al.* 1997). More recently, mutation of the AHR nuclear localization sequence was also shown to induce resistance to the toxic effects of TCDD in mice (Bunger *et al.* 2003). Similarly, mice genetically engineered to express low levels of Arnt are highly resistant to hepatic toxicity normally induced by dioxin (Walisser *et al.* 2004).

Although the AHR pathway is clearly implicated in dioxin toxicity, precise mechanisms leading to physiological responses have yet to be identified. CYP1A induction is probably the most well described biochemical responses to dioxin exposure, but it is not thought to cause overt toxicity. It has been postulated that toxicity most likely occurs through some of the many alterations in gene transcription effected by ligand-activated AHR (Okey *et al.* 2005).

1.3.2. Cytochrome P450

Cytochrome P450 enzymes are membrane bound monooxygenases capable of transforming lipophilic compounds to more soluble derivatives (Murray and Reidy 1990). There are many different members of the CYP family, and these enzymes work on a wide range of substrates. Cytochrome P450s of families CYP1, CYP2, and CYP3 are responsible

for most of the body's metabolism of xenobiotic compounds (Lewis 2000). Of these, mammalian isoforms CYP1A1, CYP1A2, and CYP1B1, are transcriptionally up-regulated by the activated AHR (Nebert *et al.* 2004).

Dioxin-like compounds and polyaromatic hydrocarbons (PAHs) are the two main classes of environmental contaminants that can induce CYP1A mRNA expression by AHR activation. Members of both classes of compounds are also substrates for the CYP1A enzyme. For some congeners this results in detoxification; CYP1A oxidizes the contaminant to a more polar derivative which is eliminated from the body. Other congeners, in particular highly-chlorinated dioxins, are extremely resistant to metabolism (Hu and Bunce 1999). In such cases, the inducer is not eliminated and it can continue to regulate transcription of dioxin-responsive genes. This inappropriately maintained response may be one reason why congeners that are resistant to metabolism tend to be more potent toxicants. Although CYP1A induction can be associated with generation of reactive oxygen species, carcinogens, and toxic metabolites (Schlezinger *et al.* 2000; Jin *et al.* 2001; Nebert *et al.* 2004), these pathways are not generally thought to be the principal mechanism of dioxin toxicity. On balance, CYP1A induction can be considered a protective response because of its role in detoxification (Okey 1990; Nebert *et al.* 2004).

Aside from its physiological role in detoxification, the CYP1A response to dioxin-like compounds is of interest because it is a useful biomarker for AHR activation, the main control point for dioxin toxicity. Two CYP1A isoforms, CYP1A4 and CYP1A5, are constitutively expressed in chicken liver (Rifkind *et al.* 1994; Gilday *et al.* 1996), and both are induced by TCDD (Mahajan and Rifkind 1999). Chicken CYP1A4 and CYP1A5 are most similar to mammalian CYP1A1 in sequence, but they exhibit a number of important metabolic differences from the mammalian isoforms and were therefore given distinct classifications (Gilday *et al.* 1998). The CYP1A4 enzyme exhibits specificity for ethoxyresorufin-*O*-deethylase (EROD) and aryl hydrocarbon hydroxylase activities (Rifkind *et al.* 1994), and the CYP1A5 isoform, preferentially catalyzes arachadonic acid metabolism and uroporphyrinogen oxidation (UROX). Up-regulation of UROX activity can disrupt the heme biosynthesis pathway, leading to the accumulation of porphyrins in cells (Rifkind *et al.* 1994; Sinclair *et al.* 1997). Concentration-dependent effects of TCDD on each of these CYP1A-related endpoints are reported in the literature (Gilday *et al.* 1998; Sanderson *et al.*

1998; Gorman *et al.* 1998), and they can be used as biomarkers for dioxin-like compounds in environmentally exposed samples.

1.4. Dioxin Toxicity in Avian Species

Toxic effects of dioxin-like compounds have been described extensively in laboratory animals (Pohjanvirta and Tuomisto 1994), with similar effects appearing in wildlife (Gilbertson *et al.* 1991). Effects are extremely diverse, and appear in multiple organs. Moreover, the range of effects as well as the dose at which they first occur can vary among species, cell types, and life stages.

1.4.1. Experimental exposure

Dietary exposure of domestic and captive bred avian species to dioxin-like compounds results in a wide range of toxic outcomes, including mortality, weight loss, edema, hemorrhages, and tissue necrosis (Hoffman *et al.* 1996). Reproductive effects of dietary exposure to dioxin-like compounds are also common. The offspring of chickens exposed to Aroclor 1254 in drinking water exhibited poor hatching success, deformities, and liver abnormalities (Tumasonis *et al.* 1973). Male American kestrels treated with PCBs had decreased sperm numbers (Bird *et al.* 1983) and females laid smaller clutches with reduced hatching success (Fernie *et al.* 2001).

Similar embryotoxic effects were observed from PCBs administered via egg injection. Exposure of avian embryos to PCBs has been shown to cause embryo mortality, shortened and crossed beaks, altered embryo growth, subcutaneous and pericardial edema, developmental malformations, and alterations in liver biochemistry (Brunström 1990; Hoffman *et al.* 1998). Susceptibility to the lethal effects of dioxin-like compounds varies dramatically among avian species with chickens being particularly sensitive. For example, injection of 20 μg/kg PCB 77 into fertilized chicken eggs early in development resulted in 100% mortality, while a 50-fold higher dose, caused no observable effects in herring gull or goose embryos (Brunström 1988). Interestingly, exposure to TCDD does not produce many

of the sublethal effects observed from exposure to PCBs, possibly because embryo mortality appears to be the most sensitive endpoint for this potent congener (Nosek *et al.* 1993; Powell *et al.* 1996a).

1.4.2. Environmental exposure

In the 1960s and early 1970s, population declines occurred in several species of Great Lakes fish-eating birds including bald eagles, herring gulls, double-crested cormorants and common terns. Reproductive failure in these birds has since been linked to exposure to environmental contaminants (Grasman *et al.* 1998; Hebert *et al.* 1999b). While some of the more catastrophic effects, such as egg-shell thinning, are attributed to exposure to the insecticide dichloro-diphenyl-trichloroethane (DDT), dioxin-like compounds are also implicated in this widespread reproductive failure. Toxic outcomes described from experimental exposures to dioxin-like compounds – embryo mortality, edema, beak malformations, altered incubation behaviour – were also observed in environmentally exposed embryos in the Great Lakes and elsewhere (Gilbertson *et al.* 1991; Hoffman *et al.* 1996).

Following increased regulation through the 1970s, concentrations of dioxin-like compounds in Great Lakes biota decreased dramatically, and this coincided with the recovery of fish-eating bird populations (Hebert *et al.* 1999b). Overt toxicity is no longer widespread, but reproductive problems are still apparent in highly contaminated areas. Reduced hatching success and poor fledgling success has been associated with elevated concentrations of dioxin-like compounds in tree swallows nesting in contaminated areas (Custer *et al.* 2003, 2005; Dods *et al.* 2005). European starlings breeding at a Superfund site contaminated with PCBs (Crab Orchard National Wildlife Refuge, IL, USA) exhibited poorer nesting behaviour and lower hatchling survival compared with starlings at a reference site (Arenal *et al.* 2004). PCB contamination of eggs was thought to be one factor contributing to reduced hatching success in ospreys in Delaware Bay, but no effects of contaminants on reproductive success in black-crowned night herons were detected (Rattner *et al.* 2000; Toschik *et al.* 2005). Several other studies have failed to detect a correlation between tissue contaminant levels and poor reproductive success, despite elevated TEQs and

biomarker responses (Elliott *et al.* 1996, 2001; Bishop *et al.* 1999). Detection of adverse effects in avian wildlife is likely related to multiple factors including the degree of contamination, the identity and interactive effects of contaminants present in complex environmental mixtures, the sensitivity of each species to the adverse outcome being measured, and differences in overall sensitivity among species.

1.5. Environmental Assessment of Exposure and Effects

The status of fish-eating birds in the Great Lakes has changed dramatically since the first reproductive problems were detected in the 1970s. Contaminant concentrations in biota have stabilized at significantly lower levels, and overt toxicity is much less common. Because of this, and because of lessons learned from the widespread reproductive failures associated with exposure to anthropogenic contaminants in the 1960s and 1970s, the focus of research has shifted to the detection of more subtle biochemical effects that may be precursors to disease. A current goal of wildlife toxicity is the identification of species at risk from exposure to environmental contaminants before population declines occur.

1.5.1. Biomarkers

One approach towards risk assessment and monitoring of wildlife in contaminated environments is the use of biomarkers and sentinel species. A biomarker has been defined as "a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also of toxic effect" (Peakall and Walker 1994). This can refer to overt toxic outcomes such as mortality and poor reproductive success, or to biochemical effects that may be predictive of these outcomes. A large number of biomarkers have been used for monitoring responses to dioxin-like compounds in avian species, including alterations in mixed function oxidase activity, heme biosynthesis, porphyrin biosynthesis, vitamin A homeostasis, immune and endocrine related endpoints, and DNA integrity (Fox 1993)

1.5.2. EROD activity

The most commonly used biomarker for dioxin-like compounds in avian species is induction of the CYP1A4 enzyme, measured as EROD activity (Figure 1.4). Elevated CYP1A expression is not considered to be an overtly toxic response in itself, but it is indicative of AHR activation, an endpoint that *is* associated with overt toxicity. As detailed in the following sections, EROD activity can be used as a biomarker of exposure to dioxin-like compounds and, under certain circumstances, of toxic effects.

Hepatic EROD activity has been positively correlated with environmental exposure to dioxin-like compounds in a number of avian species including black-crowned night herons, common terns, bald eagles, ospreys, and European starlings (Bosveld *et al.* 1995; Elliott *et al.* 1996, 2001; Rattner *et al.* 2000; Arenal *et al.* 2004), but not in herring gulls, juvenile double-crested cormorants, jungle crows, or glaucous gulls (Henriksen *et al.* 1998; Custer *et al.* 2001; Kennedy *et al.* 2003a; Watanabe *et al.* 2005). Negative findings do not appear to indicate the absence of an AHR-mediated response in these species; concentration-dependent effects of TCDD on EROD activity have been detected in hepatocytes cultured from herring gulls, and double-crested cormorants (Sanderson *et al.* 1998), and CYP1A mRNA expression has been correlated with exposure to dioxin-like compounds in jungle crow liver (Watanabe *et al.* 2005). The effectiveness of EROD activity as a biomarker of exposure may depend on a number of factors including age, diet, the identity of dioxin-like congeners present in environmentally exposed tissues, and species sensitivity to CYP1A induction.

EROD activity has also been used as a biomarker of sensitivity to dioxin-like compounds in avian species. For example, the rank order embryotoxicity of PCB congeners 77, 126, and 169 in chickens, assessed as the lethal dose for 50% of the population (LD50), corresponds to the potency of each congener for an EROD response in embryonic liver (Brunström and Andersson 1988). This association is exploited with an *in vitro* bioassay described in Kennedy *et al.* (1996a). Hepatocyte cultures prepared from avian embryos are used to assess concentration-dependent effects of dioxin-like compounds on EROD activity. The dose of test compound producing a half-maximal response (EC50) in cultured cells can be predictive of *in vivo* toxicity (Kennedy *et al.* 1996a). In accordance with this concept, a

strong relationship between EROD EC50 values assessed in chicken embryo hepatocytes (Kennedy *et al.* 1996a, 1996b) and LD50 values measured via egg injection (Brunström and Andersson 1988; Brunström 1990; Brunström and Halldin 1998), is detected for TCDD and selected non-*ortho*, and mono-*ortho* PCBs ($r^2 = 0.926, p < 0.001$) (Figure 1.5).

A relationship between EROD EC50 and LD50 also appears to exist among avian species. Ring-necked pheasants are approximately 10 times less sensitive than chickens to EROD induction by TCDD in cultured hepatocytes, and are also approximately 10 times less sensitive to the lethal effects of TCDD via egg injection (Nosek *et al.* 1993; Kennedy *et al.* 1996a). The number of species for which both EROD EC50 and LD50 data are available is limited, but a review of egg injection studies including no observable effect levels (NOELs) as well as LD50s suggests that EROD EC50 is predictive of *in ovo* toxicity among diverse species (Figure 1.6). A quantitative assessment of the strength of this relationship will require additional avian LD50 data.

1.5.3. CYP1A mRNA expression

Although CYP1A mRNA expression is a more direct measure of AHR activation than EROD activity, it has been described in few avian species, and is rarely used as a biomarker for dioxin-like compounds in environmentally exposed samples. Watanabe *et al.* (2005) detected a statistically significant relationship between TEQs and CYP1A5 mRNA expression in jungle crow liver. In common cormorant, a statistically significant relationship between TEQs and both CYP1A4 and CYP1A5 mRNA expression was detected in liver tissue (Nakayama *et al.* 2006; Kubota *et al.* 2006). Whereas the EROD assay can be applied to multiple species with little to no modification, techniques for measuring mRNA expression are developed specifically for each species based on sequence information. Nucleotide sequences for chicken, common cormorant, and herring gull CYP1A4 are currently publicly available on the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov), as are sequences for chicken, common cormorant, herring gull, turkey and jungle crow CYP1A5.

1.6. Variation in Dioxin Sensitivity

1.6.1. Variation in dioxin sensitivity among species

Species differences in sensitivity to dioxin-like compounds are well known. An often cited example, is that guinea pigs are approximately 1000-times more sensitive to the lethal effects of embryonic exposure to TCDD than hamsters (Poland and Knutson 1982; Pohjanvirta and Tuomisto 1994). Dioxin sensitivity is also extremely variable among species of birds. A review of egg injection studies providing species sensitivity data is presented in Table 6.1. These data show that ecologically relevant species such as herring gulls, common terns, American kestrels, wood ducks, and Eastern bluebirds are between 25 and 330 times less sensitive to the embryotoxic effects of dioxin-like compounds than chickens, the typical avian model. A similar degree of variation among species is reported for the EROD inducing potency of TCDD in avian embryo hepatocytes (Kennedy *et al.* 1996a, 2003b; Sanderson *et al.* 1998).

1.6.2. Molecular basis for species and strain differences in dioxin sensitivity

The aryl hydrocarbon receptor (AHR) emerges as a key regulatory protein that contributes to differential sensitivity to dioxin-like compounds in several animal models. The AHR was first identified by Poland and Glover, based on differential dioxin sensitivity between two strains of mice (Poland *et al.* 1976). Compared to the non-responsive DBA/2 mouse, the responsive C57BL/6 mouse is approximately 10 times more sensitive to CYP1A induction, lethality, and teratogenicity from exposure to TCDD. This difference was later attributed to a single nucleotide polymorphism in the AHR ligand binding domain which results in a substitution of Val-375 in the non-responsive DBA/2 mouse, for Ala-375 in the responsive C57BL/6 mouse (Poland and Knutson 1982; Poland *et al.* 1994; Ema *et al.* 1994). In another rodent model, sensitivity to dioxin-like compounds is independent of CYP1A induction. A resistant rat strain is more than 1000 times less sensitive to toxic effects of TCDD than a sensitive strain, but induces CYP1A at normal levels. Decreased sensitivity in

the resistant rat has been partially attributed to a deletion in the transactivation domain of the AHR (Pohjanvirta and Tuomisto 1994; Pohjanvirta *et al.* 1998; Tuomisto *et al.* 1999).

Recent work also implicates the AHR in differential sensitivity to dioxin-like compounds among avian species. Karchner *et al.* associated two amino acid differences in the ligand binding domain of the chicken and common tern AHR with a 7-fold difference in TCDD binding affinity. Residues Ile-324, and Ser-380 in the high affinity chicken receptor, are replaced by Val and Ala in the low affinity tern receptor (Karchner *et al.* 2006). This finding may explain the 80-fold difference in CYP1A inducibility in cultured hepatocytes (Lorenzen *et al.* 1997b), and the 260-fold difference in embryolethality of dioxin-like compounds (Hoffman *et al.* 1998) in these two species. Interestingly, Ser-380 in the chicken AHR corresponds to Ala-375 and Val-375 in the responsive and non-responsive mouse strains respectively, suggesting an important role for this amino acid residue in dioxin sensitivity for multiple species.

1.6.3. Acquired resistance

Using biomarkers to identify species at risk from exposure to environmental contaminants may be complicated by the appearance of resistance in populations that have been exposed for multiple generations. For example, populations of killifish (*Fundulus heteroclitus*) living in highly contaminated waters have been shown to be more resistant to the effects of dioxin-like compounds than fish living in clean waters. Killifish collected from polluted Newark Bay and exposed to dioxin-like compounds in the laboratory, were not responsive to CYP1A induction by 8.1 ng/g TCDD, the highest dose tested. In contrast, CYP1A was induced in fish collected from Tuckerton, a clean site, at a dose of 0.8 ng/g (Prince and Cooper 1995b). The Newark Bay fish were also resistant to the lethal effects of TCDD. A dose of 9.9 ng/g TCDD increased lethality 23% over vehicle control in the Tuckerton fish and did not affect lethality in the Newark fish (Prince and Cooper 1995a). Research by other groups has shown that resistance to dioxin-like compounds in this species is heritable and may be associated with single nucleotide polymorphisms (SNPs) in the killifish AHR (Hahn *et al.* 2004). It is unknown whether the phenomenon of acquired resistance is common, but this possibility should be considered when monitoring species in

highly contaminated areas. If a population has successfully inhabited a contaminated area for generations, it may be resistant and therefore not representative of the species as a whole.

1.6.4. Interindividual variation

Numerous studies in humans and laboratory animals suggest that expression and induction of cytochrome P450 enzymes varies extensively among individuals (Lin and Lu 2001). For example, basal levels of CYP1A1 mRNA expression were found to vary by 200-fold in cultured lymphocytes prepared from 32 human donors (Lin *et al.* 2003), and the maximal EROD response to TCDD varied by 103-fold among lymphocytes prepared from 30 human donors (Smart and Daly 2000). Similar degrees of variation in CYP1A expression and induction have been reported in avian wildlife. Hepatic EROD activity varied by 17-fold among 10 adult herring gulls collected at Kent Island (New Brunswick, Canada), a relatively uncontaminated maritime colony (Fox *et al.* 2005).

Contaminant concentrations are one factor contributing to interindividual variation in CYP1A expression and induction, but other factors are also involved. In humans, CYP1A expression has been associated with gender (Smart and Daly 2000; Parkinson *et al.* 2004), and history of cigarette smoking (George *et al.* 1995; Kim *et al.* 2004). Effects of age on cytochrome P450 enzyme levels in humans are controversial and remain unclear (Lin and Lu 2001). In fish, many factors including size, age, nutritional status, and diet have been shown to influence EROD activity (Whyte *et al.* 2000), and these might also be expected to contribute to interindividual variation in birds. Genetic factors also contribute to interindividual variation in the CYP1A response. In humans multiple polymorphisms in the CYP1A1 and AHR genes have been identified, some of which are associated with alterations in CYP1A expression and induction (Kiyohara *et al.* 1996; Smart and Daly 2000; Wong *et al.* 2001; Harper *et al.* 2002; Garte *et al.* 2003).

1.7. Thesis Overview

1.7.1. Rationale

Species sensitivity estimates are an integral part of toxicological risk assessment and yet limited toxicity data are available for avian wildlife. This gap in the literature can be at least partially attributed to the difficulties associated with performing dosing studies with wild birds. Determination of toxicity values via egg injection experiments requires collection of a large number of fertilized eggs, and this is not feasible for many ecologically relevant species. Moreover, it would be counter-productive to remove viable eggs from populations at risk or in decline. An appropriate number of eggs can be collected from less sensitive populations, but in insensitive species the concentration of toxicant required to surpass the LD50 may pose risks to lab personnel. Because of these and other challenges associated with egg injection studies, *in vitro* bioassays for assessing dioxin sensitivity in avian species are required.

The potency of dioxin-like compounds for an EROD response in cultured hepatocytes has been developed as one such bioassay for assessing dioxin sensitivity in avian species. The advantages of this technique are numerous. The requirement for fertilized eggs is lower than for egg injection studies, and a single hepatocyte culture preparation can be dosed with multiple chemicals, and with multiple concentrations of the same chemical. Nevertheless, the EROD bioassay is currently limited by a lack of understanding of factors that cause variation in the CYP1A response to TCDD among individuals and species. These factors may contribute to a lack of reproducibility in EROD EC50 values, and compromise the predictive power of EC50 for *in ovo* sensitivity to dioxin-like compounds (Figure 1.6). In the chapters that follow, experimental, environmental, and genetic factors contributing to variation in the CYP1A response to TCDD among individuals, species, and populations are described. These experiments have the dual purpose of investigating mechanisms underlying variation in dioxin sensitivity in avian species, and improving *in vitro* methods for obtaining species sensitivity estimates.

1.7.2. Avian models

Because it is difficult to carry out mechanistic studies with wild species, a laboratory model was required for initial method development. Chickens are useful in this regard because they are extremely well described in terms of physiology, toxicology, and genetics. Fertilized eggs are available year-round, and these come from fairly uniform populations, eliminating many of the genetic and environmental sources of variation that might be expected to contribute to biomarker responses in wild populations. Among avian species, chickens are particularly sensitive to the toxic effects of dioxin-like compounds, and this trait is useful for investigating basic mechanisms of toxicity. However, the large differences in sensitivity between chickens and ecologically relevant species, makes them a less than ideal model for avian wildlife.

The second model organism used in this series of experiments represents the opposite end of the spectrum of the range of dioxin sensitivities reported for avian species. With a NOEL of 50 μg/kg TEQ, herring gulls are among the least sensitive species tested to date (Figure 1.6). Nevertheless, because of their high trophic position and history of effects related to contaminant exposure, herring gulls are a bioindicator species for environmental contaminants in the Great Lakes.

The Great Lakes herring gull population has been closely studied for over 35 years, and a wealth of data is available concerning feeding habits, population structure, contaminant concentrations in eggs, biochemical responses to contaminants, and general population health (Yauk and Quinn 1999; Hebert *et al.* 1999a, 1999b; Morris *et al.* 2003; Fox *et al.* 2005). Because herring gulls are colonial nesters, often breeding on small islands away from predators, they are easily studied throughout the breeding season. The Canadian Wildlife Service (CWS) visits many of these colonies each year to monitor contaminant concentrations in eggs and archive samples for retrospective analyses. This is useful for biomarker studies as it identifies sites of concern, as well as colonies that are relatively uncontaminated and can be used as control sites.

1.7.3. Cell culture model

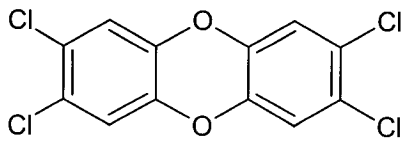
A cell culture model was used to investigate factors contributing to variation in dioxin sensitivity in avian species. This approach was taken primarily because the *in vitro* EROD bioassay is currently the most practical tool available for predicting *in vivo* sensitivity to dioxin-like compounds. A secondary benefit of using a primary embryonic cell culture model for studying variation is that it provides an artificial environment where factors which might contribute to the CYP1A response to TCDD can be precisely controlled and studied. In this way, we have been able to reduce the influence of several factors that have been shown to affect CYP1A expression levels such as age, stress, diet, and nutritional status (see section 1.6.4. Interindividual variation), and highlight others, such as embryonic exposure to contaminants, effects of population of origin, and differential sensitivity among individuals.

1.7.4. Project summary

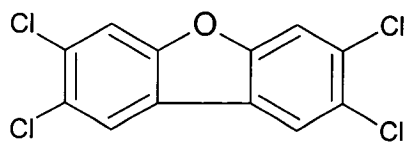
The common objective of the experiments described in this thesis was to investigate mechanisms underlying variation in CYP1A expression and induction in avian species. Each chapter explores the relationship between an experimental factor and the CYP1A response to TCDD in cultured cells. Chapter 2 describes a method for assessing CYP1A4 and CYP1A5 mRNA abundance, and characterizes the relationship between CYP1A induction at the level of enzymatic activity (EROD) and gene expression (CYP1A4 and CYP1A5 mRNA). Chapter 3 compares regulation of CYP1A mRNA expression, induction and stability in chickens and herring gulls, two differentially sensitive species that are commonly studied in avian toxicology. In Chapter 4, effects of embryonic exposure to a potent AHR agonist on CYP1A expression and induction in chicken embryo hepatocytes are investigated. This experiment is presented as a model for how the presence of dioxin-like compounds in eggs collected from the wild might affect CYP1A endpoints measured in cultured cells. Chapter 5 characterizes interindividual variation in the CYP1A response to TCDD in hepatocyte cultures prepared from individual herring gull embryos, and describes environmental and genetic factors contributing to this variation. The final experiment, presented in Chapter 6, identifies three distinct forms of the avian AHR, and describes how

these genotypes relate to sensitivity classifications based on CYP1A induction in cultured cells and *in vivo* toxicity of dioxin-like compounds in avian species

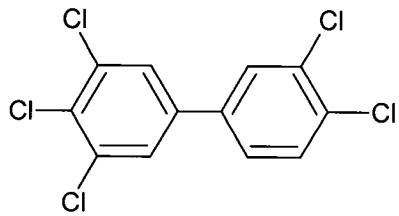
In summary, multiple aspects of the avian CYP1A response to dioxin-like compounds in cultured hepatocytes are described. These include effects of (1) AHR genotype, (2) embryonic exposure to contaminants, (3) species of origin, and (4) population of origin. Technical aspects affecting the CYP1A bioassay, such as effects of RNA quality, pooling samples, and measurement of different biochemical endpoints, are also described. The findings presented in this thesis will contribute to the development of improved *in vitro* methods for assessing dioxin sensitivity in avian species, and will ultimately aid in identification and monitoring of species at risk from dioxin-like compounds in polluted environments.



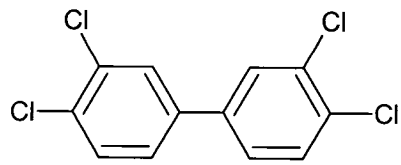
2,3,7,8-tetrachlorodibenzo-*p*-dioxin
(TCDD)



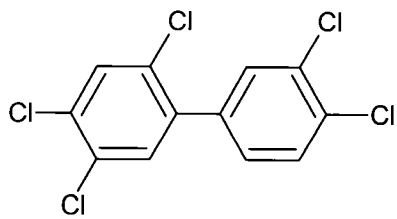
2,3,7,8-tetrachlorodibenzo-furan
(TCDF)



3,3',4,4',5-pentachlorobiphenyl
(PCB 126)



3,3',4,4'-tetrachlorobiphenyl
(PCB 77)



2,3',4,4',5-petachlorobiphenyl
(PCB 118)

Figure 1.1. Chemical structures of representative dioxin-like compounds. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototype dioxin-like compound, and the most potent member of this class of environmental contaminants. Also shown are a dibenzofuran (TCDF), two non-*ortho* substituted polychlorinated biphenyls (PCB 126 and PCB 177), and a mono-*ortho* substituted polychlorinated biphenyl (PCB 118). The potency of these congeners for EROD activity in chicken embryo hepatocytes is TCDD ≈ TCDF > PCB 126 > PCB 77 > PCB 118 (Kennedy *et al.* 1996a).

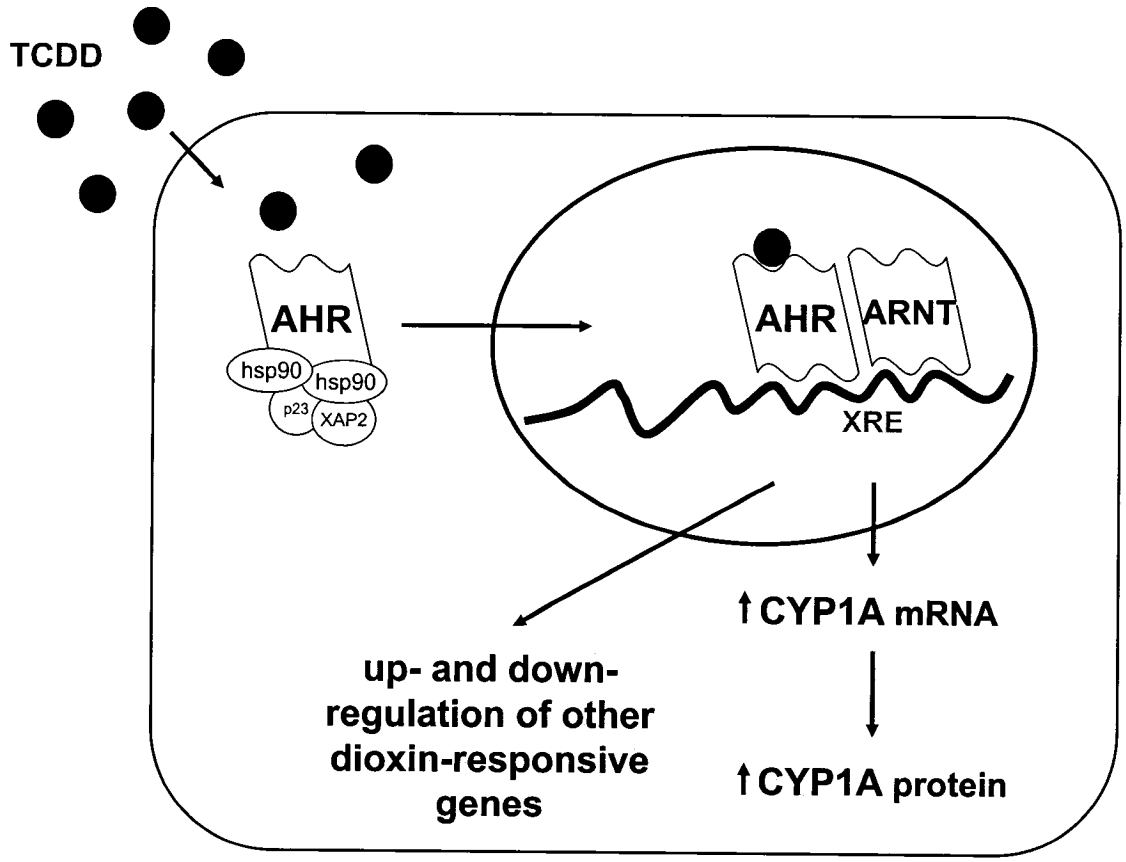


Figure 1.2. Simplified diagram of the dioxin response pathway. Binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related dioxin-like compounds to the aryl hydrocarbon receptor (AHR) induces a conformational change in the receptor which allows it to disassociate from chaperone molecules hsp90, p23, and XAP2, and translocate to the nucleus. There it associates with the aryl hydrocarbon nuclear translocator (Arnt) and binds to xenobiotic response elements (XREs). The AHR/Arnt complex acts as a transcription factor upregulating expression of cytochrome P4501A (CYP1A) isoforms and other dioxin-responsive genes. Toxicity occurs through AHR activation, but may not be directly associated with CYP1A induction (further details presented in text). This figure is adapted from Okey *et al.*, 2005.

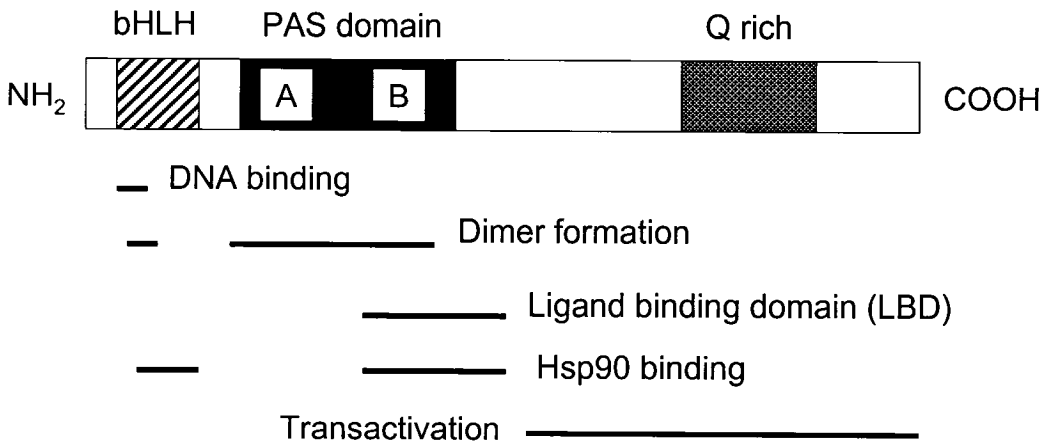


Figure 1.3. The aryl hydrocarbon receptor. Functional domains of the mouse aryl hydrocarbon receptor (AHR) are presented (adapted from Mimura and Fujii-Kuriyama (2003)).

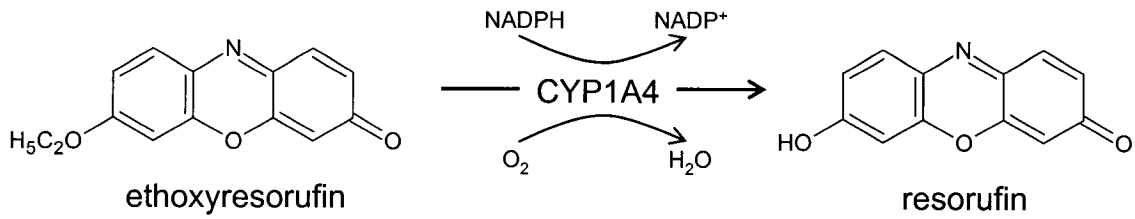


Figure 1.4. The ethoxyresorufin-*O*-deethylase (EROD) reaction. The cytochrome P4501A4 (CYP1A4) enzyme catalyzes the conversion of ethoxyresorufin to resorufin. This reaction is assessed fluorometrically in the EROD bioassay (described in section 2.3.2).

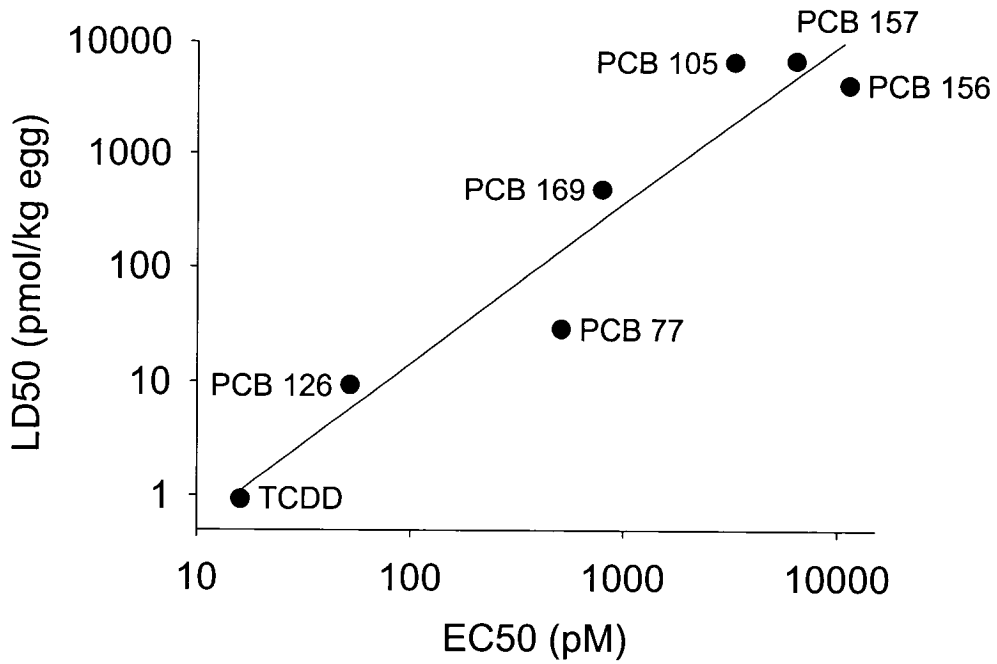


Figure 1.5. Relationship between *in vitro* and *in vivo* measures of potency of selected dioxin-like compounds in chickens. The relationship between *in vitro* EROD induction potency, and *in ovo* embryolethality for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and selected non-*ortho*, or mono-*ortho* polychlorinated biphenyls (PCBs) is illustrated. The raw data in this graph are derived from a literature review of studies providing EROD and LD50 data for chickens. The graphical representation and regression analysis are original. EC50 refers to the dose of each compound that produced a half-maximal EROD response in chicken embryo hepatocytes as presented in Kennedy *et al.* (1996a, 1996b). LD50 refers to the lethal dose for 50% of the population as presented in Brunström and Andersson (1988), Brunström (1990), and Brunström and Halldin (1998). A statistically significant relationship between log-transformed EC50 and LD50 vales was detected ($r^2 = 0.926, p < 0.001$).

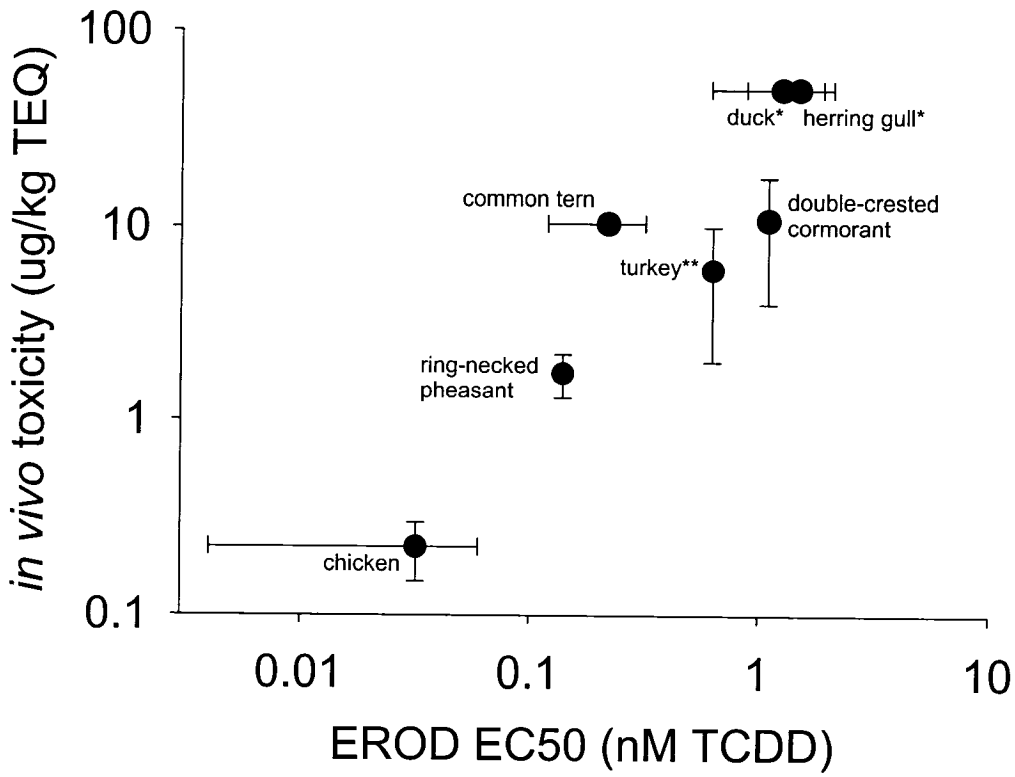


Figure 1.6. Relationship between *in vitro* and *in vivo* measures of sensitivity to dioxin-like compounds in avian species. The data shown in this graph were derived from the literature review of avian studies providing toxicity data presented in Chapter 6. Complete details and citations are found in Table 6.1. Error bars represent the range of values reported for each endpoint, and data points represent the median. The dose of TCDD producing a half-maximal EROD response (EC50) was assessed in avian embryo hepatocytes. *In vivo* toxicity data were taken from studies using various experimental designs, different dosing techniques, and a variety of test compounds. Concentrations of dioxin-like compounds other than TCDD were converted to TEQs using avian specific TEFs (Van den Berg *et al.* 1998). For chicken, ring-necked pheasant, common tern, and double-crested cormorant, toxicity data represent the average LD50 derived from a single experiment, from multiple experiments, or from multiple test compounds (converted to TEQs). For duck and herring gull, the no observable effect level (NOEL) is presented (indicated by *). For turkey, the midway point between the NOEL and the dose causing 100% mortality is presented (indicated by **).

Chapter 2

A method for measuring EROD activity and CYP1A mRNA expression sequentially in cultured chicken embryo hepatocytes

2.1. Abstract

We present a quantitative RT-PCR (Q-PCR) method for measuring cytochrome P4501A (CYP1A) mRNA expression in chicken embryo hepatocyte cultures. The assay can be performed in fresh hepatocytes, or in samples that have previously been analysed for ethoxyresorufin-*O*-deethylase (EROD) activity. Two CYP1A isoforms have been identified in chicken, and both are responsive to induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and other dioxin-like environmental contaminants. The CYP1A4 isoform preferentially catalyses EROD activity, while CYP1A5 mediates porphyrin accumulation. Inducibility of the EROD response *in vitro* has been developed as a bioassay for assessing species sensitivity to the effects of dioxin-like compounds in avian wildlife. Here, we characterize this response at the level of gene expression. Concentration-dependent effects of TCDD on CYP1A4 and CYP1A5 mRNA expression were detected in fresh, and post-EROD hepatocytes. Although the quality of the RNA obtained from post-EROD samples was low, quantification of the CYP1A mRNA response was not compromised. The CYP1A mRNA bioassay may provide more accurate potency estimates for environmental mixtures of contaminants, and has the added advantage of a very low detection limit. When working with hepatocytes cultured from wild or endangered species, our novel sequential approach may help to circumvent the problem of small sample size by maximizing the amount of data obtained from each sample.

2.2. Introduction

The ethoxyresorufin-*O*-deethylase (EROD) assay measures the cytochrome P4501A (CYP1A) response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in cultured embryo hepatocytes. This *in vitro* bioassay has been used to estimate *in vivo* sensitivity to dioxin-like compounds in avian species. The concentration of TCDD causing half-maximal EROD induction in cultured cells (EC50) is predictive of the lethal dose for 50% of the population (LD50) assessed via egg injection (Figure 1.5 & Figure 1.6) (Kennedy *et al.* 1996a). Concentration-dependent effects of TCDD on EROD activity have been described in hepatocytes cultured from many avian species including chicken, turkey, ring-billed gull, herring gull, double-crested cormorant, Forster's tern, common tern and bald eagle (Kennedy *et al.* 1996a, 2003b; Lorenzen *et al.* 1997b; Sanderson *et al.* 1998), but this response has yet to be described at the level of gene expression. The method presented here was developed to assess the possibility of using CYP1A mRNA induction in cultured hepatocytes as a biomarker for dioxin sensitivity in avian species. By measuring EROD activity and CYP1A mRNA expression sequentially in the same sample, we were able to exploit advantages associated with each endpoint. Application of this approach to wild avian species will greatly enhance the flexibility of the EROD bioassay and augment the amount of data that can be obtained from scarce samples.

2.3. Materials and methods

2.3.1. Hepatocyte culture preparation

Fertilized white leghorn chicken (*Gallus domesticus*) eggs obtained from the Canadian Food Inspection Agency (CFIA, Ottawa, ON, Canada) were incubated for 19 days at 37.5°C and 60% humidity in model CK 900 Professional incubator (Curfew, Essex, UK). Embryos were sacrificed one day pre-hatch, and livers were removed. All protocols involving animals were approved by the animal care committee at the National Wildlife Research Centre (NWRC, Ottawa, ON, Canada). Hepatocyte cultures were prepared in 48-well plates by collagenase digestion of a pool of 40 embryo livers as previously described

(Kennedy *et al.* 1993, 2003b) with some modifications. Livers were sliced into small pieces and digested in collagenase (0.5 mg/ml, Sigma, St. Louis, MO, USA) at 37°C in a shaking water bath for approximately 30 min. The digested sample was filtered through four nylon filters (pore size of 20 – 200 μm) and mixed with an equal volume of bovine serum albumin (2%, Sigma). The filtrate was pelleted by centrifugation at 300*g* for 5 min, and resuspended in 25 ml of medium 199 supplemented with sodium bicarbonate (2.24 g/L), penicillin (60 mg/L), streptomycin (100 mg/L), insulin (1 mg/L) and L-thyroxine (1 mg/L) (all reagents supplied by Sigma). The sample was transferred to a tube containing 25 ml of 10% sucrose (2.5 M, EM Science, Darmstadt, Germany) in Percoll (Amersham Biosciences, Uppsala, Germany) solution. After 10 min of centrifugation at 50*g*, the hepatocytes formed a layer at the top of the Percoll/sucrose and were transferred to a 15 ml tube with a sterile pipette. Cells were mixed with 10 ml of DNAse (20 μg/ml, Roche, Mannheim, Germany) and shaken gently until clumps of DNA were no longer visible (5 min), then rinsed several times with medium. Pelleted hepatocytes were resuspended in cell culture medium 199 (Sigma) at a concentration of 1 part cells to 20 parts medium. Cells were plated in 48-well plates by adding 25 μl of resuspended cells and 500 μl medium to each well, and were incubated at 37°C, 5% CO_2 for 20 hours prior to dosing.

Stock solutions of TCDD (kindly donated by Dr. JJ Ryan, Health Canada, Ottawa, Canada) for dosing hepatocytes were prepared in dimethyl sulfoxide (DMSO). Cell culture wells containing hepatocytes and 500 μL of cell culture medium were dosed with solvent control (DMSO), or TCDD stock solutions in triplicate. Each stock solution was added directly to the cell culture medium at a volume of 2.5 μL, resulting in final well concentrations ranging from 0.001 nM to 100 nM TCDD. After 24 hours of exposure to test compounds, medium was removed and cells were flash frozen by burying the cell culture plates in powdered dry ice.

2.3.2. EROD assay

Catalytic activity of the CYP1A4 enzyme was measured in TCDD-dosed chicken embryo hepatocytes via the EROD assay as described in Kennedy *et al.* (1995). All solutions used for this assay were prepared in sodium phosphate buffer (Sigma, 0.05 M, pH

8.0 at 37°C). Cell culture plates were removed from the freezer and allowed to thaw at room temperature for 10 minutes. While cells thawed, resorufin (Sigma) and BSA (Sigma) standards were added to the first 12 wells of the plate. These wells do not contain cells, and are used to establish resorufin and protein standard curves (see Kennedy *et al.* (1995)) for details). All cell wells received 50 µL of 7-ethoxyresorufin working solution (Sigma, final concentration 5 µM) and sodium phosphate buffer to a final volume of 175 µL. After a 15 minute incubation at 37°C, the enzymatic reaction was started by adding 25 µL NADPH (1.2 mg/mL in sodium phosphate buffer, Sigma) to each well. Exactly 7 minutes later, the reaction was stopped with the addition of 150 µL of cold acetonitrile containing fluorescamine (0.15 mg/mL, Sigma). Cells were incubated for 15 minutes at room temperature, after which fluorescence values for resorufin and total proteins were read on a fluorescence plate reader (CytoFluor 2350, Millipore, Bedford, MA, USA). Resorufin was read with a 530 nm excitation filter (25 nm bandwidth) and a 590 nm emission filter (35 nm bandwidth). Protein was read with a 400 nm exitation filter (35 nm bandwidth) and a 460 nm emission filter (40 nm bandwidth).

2.3.3. RNA isolation and cDNA synthesis

RNA was isolated from hepatocytes that had previously been analysed for EROD activity (post-EROD) and from hepatocytes that were not exposed to EROD assay conditions (control) using TRIZOL reagent (Invitrogen Canada, Burlington, Ontario, Canada). Control plates were removed from the freezer and left to thaw at room temperature for 10 minutes prior to RNA isolation. Trizol reagent was added directly to each well at a volume of 200 µL. Triplicate samples of identically dosed wells were pooled, and RNA isolation was carried out according to manufacturer's instructions. RNA isolation from post-EROD samples was begun immediately after completion of the EROD assay. For each TCDD or solvent control dose used, the contents of three identically dosed wells (consisting of hepatocytes and various reagents used for EROD analysis) were transferred to a 1.5 mL tube. Samples were pelleted by centrifugation at 10 000 rpm for 1 min, the supernatant was removed, and cells were rinsed with phosphate-buffered saline/EDTA (pH 7.4) solution. Samples were then incubated in 500 µL of TRIZOL reagent for 5 minutes at room

temperature. This softened the pellet and allowed for homogenisation of the sample by repeated pipetting. RNA isolation was carried as described for control hepatocytes.

RNA concentration was measured in control and post-EROD samples with Ribogreen reagent (Molecular probes, Invitrogen) according to manufacturer's instructions. This fluorescence-based assay allows for quantification of RNA at concentrations as low as 1 ng/mL. RNA quality was assessed visually on a formaldehyde denaturing gel. Control and post-EROD RNA were loaded at 2 μg/lane. The post-EROD sample was precipitated using two volumes of absolute ethanol and 1/10 volume of 3M sodium acetate (Sigma) and concentrated by a factor of 10 before loading.

Control and post-EROD RNA samples were reconstituted in 50μL of RNAse free water and DNAse treated with DNA-*free* reagent (Ambion, Austin, TX, USA). Template RNA was reverse transcribed to cDNA with SuperSCRIPT II and random hexamer primers (Invitrogen). A template volume of 10 μL was used in each reaction because we have found that diluting RNA to standardize input RNA concentration results in higher variance in gene expression data. This may be due to measurement error in the RNA quantification or to variable dilution of PCR inhibitors present in the samples. The cDNA samples were diluted 1:20 in diethylpyrocarbonate-treated water and aliquoted into strip tubes at a volume of 5 μL. The samples were stored at –80°C prior to analysis of gene expression.

2.3.4. Quantitative RT-PCR – Assay conditions

A multiplex quantitative RT-PCR (Q-PCR) assay was developed using dual-labelled fluorescent probes (Biosearch, Novato, CA). Primer and probe sets were designed based on GenBank sequences for chicken CYP1A4 (X99453), CYP1A5 (X99454), and the normalizer gene, beta-actin (L08165) (Table 2.1). Primers (Invitrogen) were designed with similar annealing temperatures (60°C) to permit amplification of all three targets in one reaction tube. Probe annealing temperatures were 10°C higher. The concentration of beta-actin primers was limited in the master mix because beta-actin was considerably more abundant than CYP1A4 or CYP1A5 in our samples. Without primer limitation, early amplification of the beta-actin transcript exhausts essential reagents and compromises amplification of more rare transcripts. Each 25 μL reaction contained primers and probes, 1X PCR buffer, 5 mM

MgCl2, 0.8 mM dNTPs, 0.08 v/v glycerol, 0.0125 U Surestart Taq DNA polymerase, 60 nM reference dye (Rox), and 5 μL of diluted cDNA. Brilliant core reagent kits (Stratagene, La Jolla, CA) were used to make this master mix, but master mix kits can also be used. In the case of the latter, it is advisable to supplement the master mix with 0.8 mM dNTPs per reaction.

The three target genes were amplified in a single tube using a MX4000 Q-PCR instrument (Stratagene). The thermal profile was as follows: 10 min at 95°C, followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. Data collection was at the end of the 60°C segment.

2.3.5. Quantitative RT-PCR – Analysis

Amplification plots were generated for the two genes of interest (CYP1A4, CYP1A5) multiplexed with the normalizer gene (beta-actin). The role of the normalizer is to correct for variation in template loading. Stratagene Mx4000 software was used to determine the cycle number where each amplification plot crossed a threshold representing the first detectable fluorescence value above baseline noise. This threshold cycle (Ct) is inversely proportional to initial transcript quantity. To validate the assay, relative standard curves were generated from a 1:2 dilution series of cDNA by plotting Ct values against relative cDNA concentration. Fold induction was then calculated from Ct values using the 2^{-ddCt} method described in Livak and Schmittgen (2001). This method calculates fold changes in gene expression based on transcript abundance in a treated sample relative to a control sample. Relative abundance of each gene of interest is also expressed relative to normalizer gene abundance. To assess fold changes in CYP1A expression between TCDD and DMSO-treated cells, we defined ddCt as $(Ct_{CYP1A} - Ct_{beta\text{-}actin})_{TCDD} - (Ct_{CYP1A} - Ct_{beta\text{-}actin})_{DMSO}$

Control and post-EROD cDNA samples were analysed in three separate Q-PCR runs. Each run included a complete set of samples isolated from one control plate, a complete set of samples isolated from one post-EROD plate, and appropriate negative controls. Within each run, all samples were analysed in triplicate.

2.3.6. Data analysis

CYP1A mRNA expression data were fit to a 4 parameter logistic equation (GraphPad Prism software 3.0, San Diego, USA). This equation incorporates hillslope, EC50 and maximal response values as parameters: Y = (maximum – baseline)/(1 + 10^((logEC50-X)*Hillslope)). EROD data were fit to the modified Gaussian curve described in Kennedy *et al.* (1993). To compare hillslope values between EROD and CYP1A mRNA dose-response curves, we also fit EROD data to the 4 parameter logistic model. Since we only wanted to model the increasing phase of the EROD response, data points above the concentration of TCDD that caused a maximal response (1 nM) were not included in this curve fit.

Curves were fit to CYP1A mRNA expression and EROD activity data originating from each cell culture plate separately. Curve parameters are presented as the mean value for 3 plates ± standard deviation. Data were analyzed using one-way ANOVA with Bonferonni tests. A p-value of less than 0.05 was considered statistically significant in all cases.

2.4. Results

2.4.1. Q-PCR standard curves

Relative standard curves were generated for CYP1A4, CYP1A5, and beta-actin by plotting Ct values against relative cDNA concentration of a 1:2 dilution series of cDNA (Figure 2.1). Data were fit to a linear regression model with r^2 values of 1.000 for CYP1A4 and beta-actin, and 0.999 for CYP1A5. Standard curves for all three genes were linear and nearly parallel (slopes within 0.1) over a dynamic range of at least 15 cycles. Parallel standard curves indicate that the reaction efficiencies for each amplicon are similar, and that the target genes can be multiplexed.

2.4.2. RNA integrity

Exposure to EROD assay conditions and reagents decreased the integrity of RNA isolated from cultured hepatocytes (Figure 2.2). The quantity of RNA obtained from each well was on average approximately 8-fold higher for control plates than for post-EROD plates (1.27 and 0.15 μg/well, respectively) (Figure 2.2.A). This corresponded to a 6.4-cycle difference in the average beta-actin Ct values for control (Ct = 18.7) and post-EROD (Ct = 25.1) samples. RNA quality was also compromised. Ribosomal bands were clearly visible when 2 μg of control sample was run on a denaturing gel (Figure 2.2.B). Post-EROD RNA was not visible on the gel, so samples were pooled, and concentrated by a factor of 10 to obtain 2 μg of RNA. The concentrated sample was badly degraded, appearing as a faint smear. We also assessed the rate of degradation of CYP1A4 and CYP1A5 mRNA relative to beta-actin mRNA by comparing average dCt values in control and post-EROD DMSO-treated samples. The difference in Ct values between each gene of interest and the normalizer ($dCt = Ct_{CYP1A} - Ct_{beta\text{-}actin}$) was significantly higher in post-EROD samples, indicating that beta-actin mRNA degraded more slowly than either CYP1A transcript (Figure 2.2.C).

2.4.3. CYP1A mRNA induction in control and post-EROD hepatocytes

Although RNA isolated from post-EROD samples was low in concentration and poor in quality, quantification of the CYP1A mRNA response to TCDD in these samples was not generally affected by exposure to EROD assay conditions (Figure 2.3). Moreover, the shapes of post-EROD curves, as indicated by EC50, slope parameters, and maximal fold induction values, were not significantly different from control curves.

2.4.4. Concentration-dependent effects of TCDD on EROD activity and CYP1A mRNA expression

EROD activity in chicken embryo hepatocytes increased with TCDD concentration to a maximum response at 1 nM, and then decreased at higher concentrations of TCDD. Concentration-dependent effects of TCDD on CYP1A4 and CYP1A5 mRNA expression were also observed. In contrast to the EROD curves, the CYP1A mRNA response reached a plateau at concentrations of TCDD above 1 nM (Figure 2.4). Exposure to TCDD maximally induced CYP1A4 mRNA, CYP1A5 mRNA, and EROD activity by 58.4-, 29.9-, and 17.9-fold respectively. Significant differences in maximal fold-induction values were detected between all three endpoints ($p < 0.05$) (Table 2.2). With data expressed as percent maximal response, CYP1A4 and CYP1A5 mRNA dose-response curves were nearly superimposable and very similar to the EROD curve (Figure 2.4). Differences in EC50 and hillslope parameters for CYP1A4, CYP1A5 and EROD dose-response curves were not detected (Table 2.2).

2.5. Discussion

2.5.1. Quantification of gene expression from degraded RNA

Unlike DNA which is relatively stable, RNA is susceptible to degradation. The hydroxy group on the 2' carbon allows RNA to be easily hydrolysed, and it is sensitive to degradation by endogenous ribonucleases. RNA isolated from post-EROD plates was of poor quality and approximately 8 times lower in concentration than control RNA (Figure 2.2). Possible causes of degradation of the post-EROD RNA sample include exposure to 37.5°C, contact with EROD assay reagents including acetonitrile, and extra time on the bench while the EROD assay was performed. Despite low RNA quality, fold induction values and curve parameters derived from post-EROD samples were not significantly different from control values (Figure 2.3). A number of factors may have contributed to our ability to accurately quantify CYP1A mRNA fold induction in post-EROD samples.

1. We used a sensitive method of detection, and both CYP1A isoforms are relatively abundant in DMSO-treated samples (average Ct < 25 for both genes). Rare transcripts with high Ct values in intact samples would likely be below the detection limit in degraded samples.

2. Multiplexed CYP1A4, CYP1A5, and beta-actin Ct values were highly predictive of relative initial cDNA concentration over several orders of magnitude (Figure 2.1). Standard curves were parallel and linear for at least 15 cycles suggesting that increases in gene expression of up to 30,000-fold can be reliably detected using this assay. This large linear dynamic range was needed to cover the entire range of Ct values produced by intact and degraded RNA samples.

3. Primer and probe sets were designed to produce amplicons less than 100 base pairs in length (Table 2.1). Shorter amplicons tend to produce lower Ct values in degraded samples because of the reduced likelihood of an RNA break occurring between primer sites. The consistency of dCt values ($Ct_{target} - Ct_{normalizer}$) between intact and

degraded RNA samples has also been shown to improve with reduced amplicon length (Antonov *et al.* 2005).

4. Random hexamer primers were used for reverse transcription. This may produce a better result than oligo-(dT) primers if the amplicon is closer to the 5' end of the gene. Any RNA break that is 3' to the amplicon will separate it from the poly-A tail, and significantly reduce the amplicon abundance in oligo-d(T) primed cDNA.

It is generally held that RNA used in Q-PCR assays must be of excellent quality (Bustin and Nolan 2004), but this may be more of a guiding principal than an absolute necessity. Several groups report consistent results for gene expression measured from intact and degraded RNA. These studies found that although Ct values increase with decreasing RNA quality, normalization compensates for this change allowing for accurate quantification of gene expression by Q-PCR in degraded samples (Schoor *et al.* 2003; Antonov *et al.* 2005; Fleige and Pfaffl 2006).

It may be possible to use the Q-PCR method presented here to measure CYP1A gene expression in a number of types of degraded samples. Biological samples from wildlife are often difficult to collect, especially from threatened or endangered species. It would be tremendously useful to be able to measure gene expression in field-collected samples that were not immediately frozen, or placed in RNA stabilization buffers. Many archived samples collected from carcasses hours or days after death would be available for analysis. Homogenized samples would also be of interest. These approaches might work, but should be undertaken with caution. Perez-Novo *et al.* (2005) have reported that RNA transcripts degrade at different rates, and we observed a similar phenomenon. The dCt value ($Ct_{CYP1A} - Ct_{beta\text{-}actin}$) in DMSO-treated hepatocytes was significantly larger in degraded samples than in intact samples, suggesting that beta-actin degraded more slowly than either target gene (Figure 2.2.C). This was not problematic for our study because all of our data were expressed in terms of fold-induction, and treated and control samples were likely to be equally degraded. What these data do suggest is that normalization does not compensate for degradation in all cases. For example, it would not be advisable to compare gene expression

in tissue samples collected on different days, from different sites, or by two different methods, if the degree of degradation was not known. As others have pointed out, this highlights the need for a reliable way to measure RNA quality that does not require large quantities of sample (Miller *et al.* 2004; Imbeaud *et al.* 2005).

2.5.2. Evaluation of the EROD/CYP1A mRNA sequential approach

EROD activity has been assessed in liver tissue and cultured hepatocytes from many different avian species. These studies represent an important source of exposure and sensitivity data for risk assessment in birds. We expanded upon these data by characterizing the CYP1A response to TCDD at the level of gene expression.

Concentration-dependent effects of TCDD on CYP1A mRNA expression and EROD activity were similar. No significant differences were detected in EC50, or hillslope values for EROD, CYP1A4, and CYP1A5 dose-response curves (Table 2.2). Coinduction of CYP1A4 and CYP1A5 responses by an AHR agonist has been reported previously. Treatment of chicken embryo hepatocytes with hexachlorobenzene resulted in superimposable EROD and UROX dose-response curves (Sinclair *et al.* 1997). The relative potency of TCDD for EROD and CYP1A mRNA responses is likely dependent on timing of exposure. Bastien *et al.* (1997) report a time-dependent increase in EROD EC50s for chicken embryo hepatocytes exposed to TCDD for 24, 48, or 72 hours.

Several differences between CYP1A mRNA and EROD dose-response curves were apparent, and these differences point to benefits of our sequential approach. Both endpoints increased with TCDD concentration to a maximal response at 1 nM TCDD. The CYP1A mRNA response then reached a plateau, while the EROD response was attenuated with further increases in TCDD concentration (Figure 2.4.). The bell-shaped EROD dose-response curve is well-characterized, and is thought to result from competitive inhibition of the CYP1A4 enzyme. At high concentrations of inducer, dioxin-like compounds compete with 7-ethoxyresorufin for the CYP1A4 active site and inhibit enzymatic activity. Competitive inhibition can also decrease the maximal EROD response, shifting EC50 values, and compromising sensitivity estimates derived from EROD data. This does not

occur for potent congeners like TCDD, but when inhibition is much stronger than binding affinity, an overestimate of sensitivity is likely (Petrulis and Bunce 1999). CYP1A mRNA dose-response curves, which are not affected by competitive inhibition, may provide more accurate estimates of potency for weak AHR agonists, and for environmental extracts containing a mixture of inducers and inhibitors.

Differences in the magnitude of CYP1A responses to TCDD were also apparent. The most responsive endpoint was CYP1A4 mRNA, followed by CYP1A5 mRNA, and then EROD activity (Table 2.2). With equivalent EC50s, the magnitude of the response to inducing agent may be a key factor determining relative sensitivities of CYP1A mRNA expression and EROD activity as biomarkers. Few studies have measured both endpoints in environmentally exposed tissues, but CYP1A mRNA expression shows promise as a sensitive biomarker of exposure. Tissue concentrations of dioxin-like compounds were correlated with CYP1A5 mRNA expression, but not with EROD activity in jungle crow liver (Watanabe *et al.* 2005). This finding may be partially related to the fact that the CYP1A5 isoform is preferentially induced in some avian species (Chapter 3). Routine use of CYP1A mRNA expression as a biomarker in wild birds would depend on the development of multi-species Q-PCR primer and probe sets. Limited sequence information exists for CYP1A isoforms in birds, but alignments of the sequences that are available indicate regions of high homology. For example, the 64 base pair region that is amplified by Q-PCR primers designed for herring gull CYP1A5 (Chapter 3), differs from chicken, turkey and jungle crow sequences by 4, 5, and 6 nucleotides respectively.

Perhaps the most important advantage of the Q-PCR method is that it measures mRNA expression with a very high degree of sensitivity. When culturing hepatocytes from scarce samples or unfamiliar species, the amount of starting material does not always allow for an ideal cell density. We did not determine the lowest number of cells required for detection of CYP1A mRNA expression, but we hypothesize that it is very low, and much lower than the cell density required for detection of EROD activity. For example, we observed dose-dependent effects of TCDD on CYP1A4 and CYP1A5 mRNA expression in hepatocytes cultured from individual herring gull embryos, but failed to detect an EROD response (Chapter 5). This result appeared to be related to low cell density. The ability to measure CYP1A mRNA expression when EROD activity is not detectable is very valuable,

especially if samples originate from a sensitive population or a labour-intensive field collection. The EROD/CYP1A mRNA sequential approach may sometimes be useful as a 'plan B' when EROD activity is not detected. RNA could be isolated from post-EROD samples, and archived until species appropriate Q-PCR assays were developed. Since the quantity of RNA isolated from one cell culture plate is sufficient for many Q-PCR reactions, expression of other genes of interest could also be measured at a later date.

CYP1A mRNA expression shows promise as a biomarker for exposure to and hazard from dioxin-like compounds in avian species. The method is very sensitive and may provide better potency estimates for weak AHR agonists or environmental mixtures. The EROD bioassay is preferable in terms of cost, labour, and ease of application to multiple species. The EROD/CYP1A mRNA sequential approach presented here measures gene expression and enzymatic activity in the same sample, exploiting the advantages of each technique, and maximizing the amount of data that can be obtained from scarce tissues.

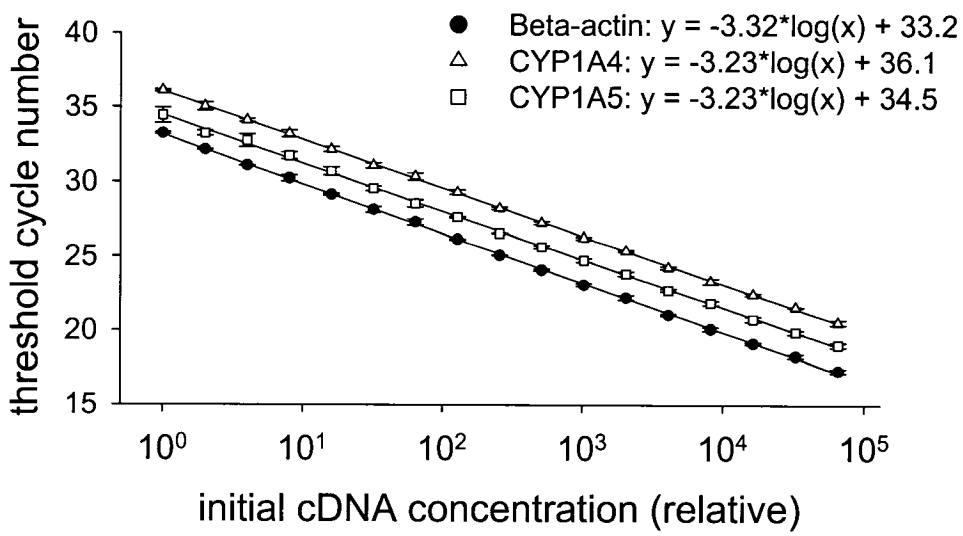


Figure 2.1. Relative standard curves for detection of CYP1A4, CYP1A5 and beta-actin transcripts by quantitative RT-PCR. The standard curves were created from a 1:2 serial dilution of a concentrated cDNA sample. Relative initial cDNA concentration is plotted against threshold cycle number (Ct). Each data point represents the average of three replicate analyses, and bars represent standard error. Ct values are highly correlated with the initial cDNA concentration for all three genes. Equations for the regressions are shown on the graph. The r^2 values were 1.000 for CYP1A4 and beta-actin, and 0.999 for CYP1A5.

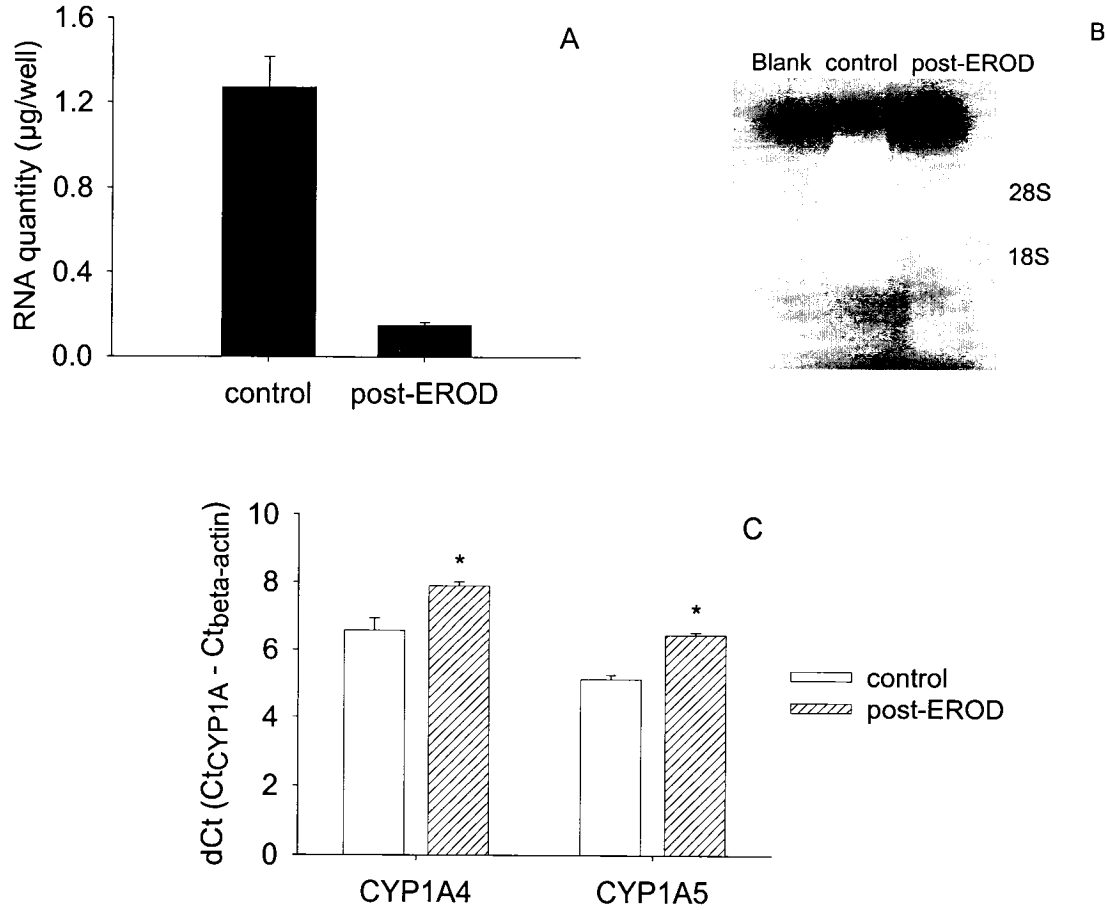


Figure 2.2. Integrity of RNA isolated from control and post-EROD samples. (A) Quantity of RNA isolated from control and post-EROD samples. Bars represent the average quantity of RNA per well ± standard error (n = 33). (B) Quality of RNA isolated from control and post-EROD samples. RNA was loaded into a formaldehyde denaturing gel at 2 µg/lane. Ribosomal bands (28S and 18S) are clearly visible in the control sample. Post-EROD RNA was concentrated prior to electrophoresis in order to permit visualization of the sample. It appears as a faint smear in the right lane. (C) Difference in Ct values (dCt) between CYP1A isoforms and the normalizer gene, beta-actin. Bars represent the average dCt value in DMSO-treated samples from 3 control, or 3 post-EROD plates ± standard error. For both CYP1A4 and CYP1A5, dCt values were significantly elevated in post-EROD samples, suggesting that beta-actin mRNA transcripts were degraded more slowly than CYP1A4 or CYP1A5. Asterisks indicate that the dCt value was significantly different from the control value in post-EROD samples ($p < 0.05$).

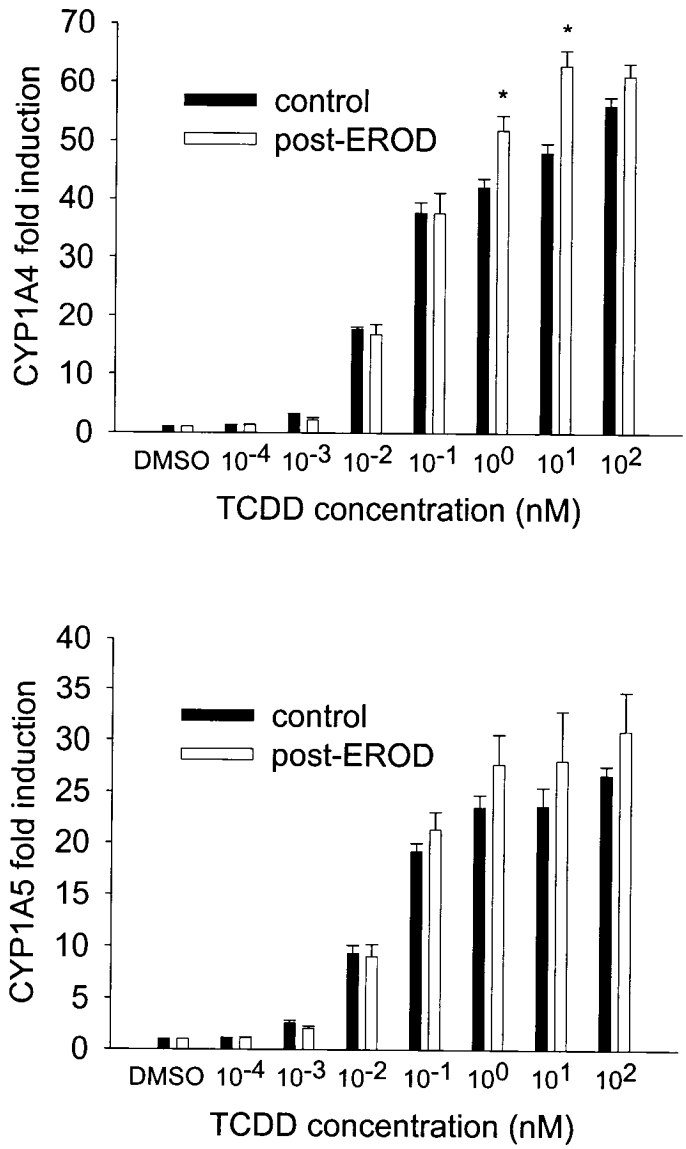


Figure 2.3. Concentration-dependent effects of TCDD on CYP1A mRNA expression in control and post-EROD chicken embryo hepatocyte cultures. Bars represent mean fold-induction measured on three replicate cell culture plates. Error bars represent standard error between plates. Control samples (black bars) were thawed immediately prior to RNA isolation. Post-EROD samples (white bars) were thawed at room temperature and then analyzed for EROD activity prior to RNA isolation. Asterisks indicate that a significant difference in fold-induction values between control and post-EROD samples was detected at a particular concentration of TCDD ($p < 0.05$).

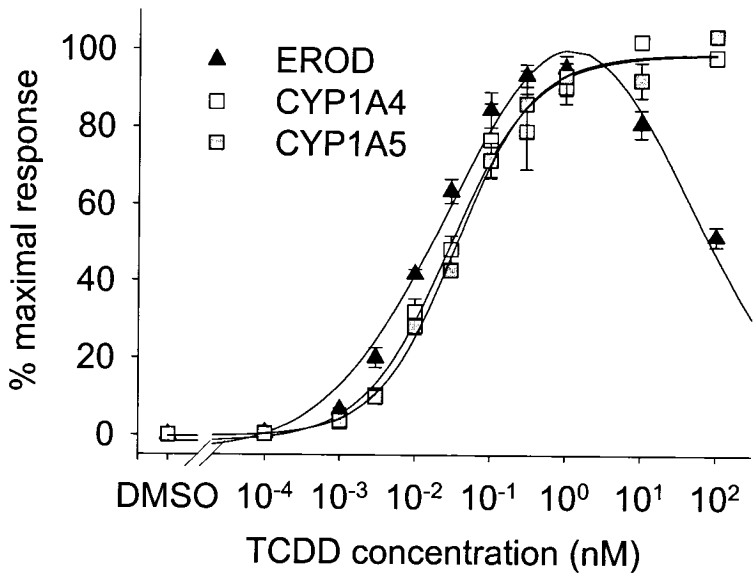


Figure 2.4. Concentration-dependent effects of TCDD on CYP1A4 mRNA expression, CYP1A5 mRNA expression, and EROD activity in chicken embryo hepatocytes. For comparative purposes, data are expressed as percent maximal response. The CYP1A mRNA response was measured in post-EROD hepatocytes. Each data point represents the mean response caused by a particular dose on three replicate cell culture plates. Bars represent standard error of the mean. Curve fit parameters for these data are presented in Table 2.2.

Table 2.1. Chicken CYP1A4, CYP1A5, and beta-actin primer and probe sequences, dyes, and amplicon sizes. Final primer and probe concentrations used in the PCR master mix are presented in parentheses following each sequence.

Chicken CYP1A4	
Dye/quencher	FAM/BHQ-1
Amplicon size	89 bp
Forward primer	5'-TAAGGACGTCAATGCTCGTTTC-3' (300 nM)
Reverse primer	5'-CGTCCCGAATGTGCTCCTTAT-3' (300 nM)
Probe[a]	5'-TGCCTTCGTACAGAAAATTGTCCAGAAC-3' (200 nM)

Chicken CYP1A5	
Dye/quencher	CY5/BHQ-3
Amplicon size	85 bp
Forward primer	5'-ACAGCTGTGGAAGAGCACTACCA-3' (300 nM)
Reverse primer	5'-TCTCCACGCACTGCTCGAT-3' (300 nM)
Probe[a]	5'-CCGAGACGTCACCGACTCCCTCA-3' (200 nM)

Chicken Beta-actin	
Dye/quencher	HEX/BHQ-1
Amplicon size	76 bp
Forward primer	5'-AAATTGTGCGTGACATCAAGGA-3' (50 nM)
Reverse primer	5'-GAGGCAGCTGTGGCCATCT-3' (50 nM)
Probe[a]	5'-TGCTACGTCGCACTGGATTTCGAGC-3' (50 nM)

[a]probes anneal to same strand as forward primers

Table 2.2. Curve fit parameters for concentration-dependent effects of TCDD on EROD activity and CYP1A mRNA expression in chicken embryo hepatocytes. EROD data were fit to a modified Gaussian curve, and CYP1A mRNA expression data were fit to a 4 parameter logistic curve. EROD data were also fit to a 4 parameter logistic curve in order to determine hillslope values. No significant differences in EC50 (p = 0.075) or hillslope (p = 0.412) were detected between endpoints. Significant differences were detected between EROD, CYP1A4 mRNA, and CYP1A5 mRNA maximal fold induction values ($p < 0.05$).

Endpoint	EC_{50} (nM)[a]	Hillslope[a]	Maximum Fold induction[a]
EROD	0.015 ± 0.002	0.918 ± 0.198	17.9 ± 1.8*
CYP1A4 mRNA	0.023 ± 0.005	0.770 ± 0.138	58.4 ± 4.4**
CYP1A5 mRNA	0.038 ± 0.006	0.863 ± 0.266	29.9 ± 6.9***

[a]mean of value derived from three replicate cell culture plates ± standard deviation
*Asterisks indicate significant differences between endpoints ($p < 0.05$)

Chapter 3

Expression, induction, and stability of CYP1A4 and CYP1A5 mRNA in chicken and herring gull embryo hepatocytes

3.1. Abstract

To investigate mechanisms underlying variability in the effectiveness of ethoxyresorufin-*O*-deethylase (EROD) activity as a biomarker for exposure to dioxin-like compounds in avian models, we characterized inter-species differences in isoform-specific cytochrome P4501A (CYP1A) mRNA expression, induction and stability in chickens and herring gulls. In avian species, the CYP1A4 isoform preferentially catalyzes EROD activity, and the CYP1A5 isoform mediates uroporphyrinogen oxidation (UROX). Exposure to 100 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) significantly increased CYP1A4 and CYP1A5 mRNA expression in chicken and herring gull embryo hepatocyte cultures. Chicken CYP1A4 and CYP1A5 were induced by 61-fold and 25-fold respectively. The herring gull isoforms were induced by 2.2- and 4.3-fold respectively. In both species, the isoform that was preferentially induced exhibited lower constitutive expression. Half-lives of chicken CYP1A4, chicken CYP1A5, and herring gull CYP1A5 mRNA ranged from 5.0 to 7.0 hours in cultured hepatocytes. The half-life of herring gull CYP1A4 mRNA was 2.5 hours. Our findings indicate that expression, induction, and stability of CYP1A4 and CYP1A5 mRNA are differentially regulated in chickens and herring gulls. In particular, CYP1A4 is preferentially induced in chickens, while CYP1A5 is preferentially induced in herring gulls. We propose that CYP1A5 mRNA expression may be a more sensitive biomarker than EROD activity in some avian species.

3.2. Introduction

Identifying species that are at risk from exposure to environmental contaminants is a central goal of wildlife toxicology. Biomarkers can help with this task by providing discrete measures of exposure and hazard in species for which little toxicity data is available. A commonly used biomarker for exposure to dioxin-like compounds in wild birds is induction of cytochrome P4501A (CYP1A), generally measured as the ethoxyresorufin-*O*-deethylase (EROD) response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Rattner *et al.* 1989).

EROD activity has been used as a biomarker of exposure to dioxin-like compounds in avian species, with varying degrees of success. A significant correlation between hepatic EROD activity and polychlorinated biphenyls (PCBs) and/or TCDD equivalents (TEQs) has been detected in a number of species, including bald eagle, black-crowned night heron, common tern, European starling, and osprey (Bosveld *et al.* 1995; Elliott *et al.* 1996, 2001; Rattner *et al.* 2000; Arenal *et al.* 2004). No relationship between hepatic EROD activity and PCB concentration was found in herring gulls or jungle crows, and a negative relationship was detected in glaucous gulls (Henriksen *et al.* 1998; Kennedy *et al.* 2003a; Watanabe *et al.* 2005). Some variation in effectiveness of EROD as a biomarker may depend on the age of individuals, or the identity of the dioxin-like compounds being measured. Hepatic EROD activity was correlated with PCB concentration in pipping double-crested cormorant embryos, but not in 10 day-old chicks (Custer *et al.* 2001). In black-headed gulls, EROD activity was correlated with TEQs, but not with total PCBs (Fossi *et al.* 1986; Yamashita *et al.* 1992).

Another biomarker for exposure to dioxin-like compounds in wild birds is the accumulation of highly carboxylated porphyrins (HCPs) (Casini *et al.* 2003). Hepatic concentrations of PCBs have been correlated with HCP levels in herring gulls, bald eagles, and tree swallows, but not in glaucous gulls or spectacled eiders (Elliott *et al.* 1996; Kennedy *et al.* 1998; Bishop *et al.* 1999; Henriksen *et al.* 2000; Trust *et al.* 2000). The strength of correlation between contaminant concentrations and EROD activity or porphyrin accumulation may depend on several factors, including the sensitivity and specificity of the response. Lorenzen *et al.* (1997a) report that porphyrin accumulation is first observed at doses of non-*ortho* substituted PCBs that maximally induce EROD activity in chicken

embryo hepatocytes. Many *ortho*-substituted PCBs are highly porphyrinogenic but do not produce an EROD response in cultured cells.

The effectiveness of EROD as a biomarker for environmental exposure to dioxin-like compounds may also be partially related to the sensitivity and magnitude of the CYP1A response in each species. Concentration-dependent effects of dioxin-like compounds on EROD activity have been observed in hepatocytes cultured from chicken, turkey, ring-necked pheasant, herring gull, ring-billed gull, bald eagle, double-crested cormorant, Forster's tern, and common tern. Although each of these species is responsive to EROD induction by TCDD, the magnitude of the response and the dose at which it first appears varies considerably (Kennedy *et al.* 1996a, 2003b; Lorenzen *et al.* 1997b; Sanderson *et al.* 1998). Compared to chickens, colonial fish-eating birds are relatively insensitive to EROD induction. Exposure to TCDD maximally induced EROD activity 15-fold in chicken embryo hepatocytes, but only 2.4- to 3-fold in hepatocytes cultured from herring gull, ring-billed gull, double-crested cormorant, or Forster's tern. The dose of TCDD causing a half-maximal EROD response (EC50) is also variable among species. For example, the EC50 was 18 – 208 times lower in chicken than in the other species tested (Sanderson *et al.* 1998).

Understanding species variation in responses to environmental contaminants is important for effective interpretation of biomarker data. Based on EC50 values, chickens are 35-80 times more sensitive to EROD induction by TCDD than common terns or herring gulls (Kennedy *et al.* 1996a; Lorenzen *et al.* 1997b; Sanderson *et al.* 1998). Recent work suggests that differential sensitivity to EROD induction in these species may be associated with the genetic sequence of the aryl hydrocarbon receptor (AHR). Two amino acid differences in the ligand binding domain of chicken and common tern AHR confer 7-fold higher TCDD binding affinity to the chicken receptor. The tern receptor is also less effective at transcriptional activation in cultured cells (Karchner *et al.* 2006). Functional aspects of the herring gull AHR (GenBank accession no. DQ371287) are not yet characterized, but it is identical to the tern receptor at the key amino acid sites identified by Karchner *et al.* (2006). These findings suggest that AHR-mediated regulation of CYP1A4 mRNA expression may play an important role in species variation in the EROD response to dioxin-like compounds.

In the present study we characterize expression, induction, and stability of CYP1A4 and CYP1A5 mRNA in the domestic chicken and herring gull, two species commonly used

in avian toxicology. Characterization of the CYP1A response at the level of gene expression in these differentially sensitive species will provide a better understanding of interspecies variation in the EROD response to TCDD, and help to clarify interpretation of biomarker data in environmentally exposed birds.

3.3. Materials and methods

3.3.1. Preparation of cultured hepatocytes

Fertilized, unincubated white leghorn chicken (*Gallus domesticus*) eggs were obtained from the Canadian Food Inspection Agency (CFIA, Ottawa, ON, Canada). Unincubated herring gull (*Larus argentatus*) eggs were collected from one-egg nests on Chantry Island (Port Elgin, Lake Huron, ON, Canada) in April of 2005. Eggs of both species were incubated at 37.5°C and 60% humidity. Embryos were sacrificed by decapitation on embryonic day 19 for chicken, and embryonic day 26 for herring gull (one day pre-hatch), as approved by the animal care committee at the National Wildlife Research Centre (NWRC, Ottawa, ON, Canada). Hepatocytes were isolated by collagenase digestion and filtration according to the method described in Kennedy *et al.* (1993) and updated in Chapter 2. Cell pellets were weighed and suspended in 20 mL of medium 199 (Sigma, St. Louis, MO, USA) per 1 g of hepatoctyes. Samples were plated in 48-well cell culture plates by adding 25 µL of the cell suspension to 500 µL of complete medium. For chickens, three batches of hepatocytes were prepared on three separate days from pools of 28, 35, or 33 embryonic livers. For herring gulls, one batch of hepatocytes was prepared from a pool of 18 embryonic livers. Chicken and herring gull embryo hepatocytes were incubated for 20 hours at 37.5°C and 5% CO_2 prior to dosing.

3.3.2. Dosing of cultured hepatocytes

A concentrated TCDD stock solution was made by dissolving TCDD (donated by Dr. J. Ryan, Health Canada, Ottawa, ON, Canada) in dimethyl sulfoxide (DMSO). Wells containing hepatocytes and 500 µL of cell culture medium were dosed with 2.5 µL of DMSO (solvent control), or 2.5 µL of diluted TCDD stock solution. The final concentration of TCDD in each well was 100 nM, a concentration that has been shown to be above the dose to elicit a maximal CYP1A response in chickens and herring gulls (Kennedy *et al.* 1996a). Solvent control and TCDD-treated hepatocytes were prepared in triplicate wells on 6 separate cell culture plates. After 19 hours of incubation in the presence of test compound, the medium in each well was replaced with 500 µL of fresh medium spiked with actinomycin D (Sigma) at 4 µg/mL. Actinomycin D is a transcriptional inhibitor. Hepatocytes were sampled immediately before the addition of actinomycin D (time 0), and 3, 6, 9, 12, and 24 hours later. At each time point, medium was removed leaving behind a monolayer of hepatocytes attached to the bottom of each well. Plates were immediately frozen in powdered dry ice and stored at –80°C.

3.3.3. RNA isolation and cDNA synthesis

RNA was isolated from chicken and herring gull embyro hepatocytes using the Qiagen RNeasy 96 kit (Qiagen, Mississauga, ON) according to manufacturer's instructions with one slight modification: a 50% ethanol solution was used for RNA isolation instead of the recommended 70% ethanol solution because we found that this produced higher RNA yields. RNA samples were DNAse treated with DNA-*free* (Ambion, Austin, TX, USA) and were found to be free of genomic DNA as verified by Q-PCR. RNA was reverse transcribed to cDNA as described in Chapter 2.

3.3.4. Quantitative RT-PCR assay

Chicken CYP1A4, CYP1A5, and beta-actin mRNA expression were measured using the quantitative RT-PCR (Q-PCR) multiplex assay described in Chapter 2. A similar assay

was developed for herring gull target genes. Primer and probe sets were designed based on GenBank sequences for herring gull CYP1A4 (GenBank accession no. AY233271), CYP1A5 (GenBank accession no. AY220876), and beta-actin (GenBank accession no. AY045724). Each 25 μL Q-PCR reaction mixture contained primers (Invitrogen) and probes (Biosearch, Novato, CA), 1X PCR buffer, 5 mM MgCl2, 0.8 mM dNTPs, 0.08 v/v glycerol, 0.0125 U Surestart Taq DNA polymerase, 60 nM reference dye (Rox) (Stratagene, La Jolla, CA, USA), and 5 μL of diluted cDNA. Primer and probe concentrations and sequences are presented in Table 3.1. The three target genes were amplified in a single tube on an Mx4000 instrument (Stratagene). The temperature profile was 10 min at 95°C, followed by 40 cycles of 95°C for 30 seconds and 60°C for 1 minute. Data were collected at the end of the 60°C segment.

3.3.5. Data analysis – Q-PCR

CYP1A4 and CYP1A5 fold induction was calculated from threshold cycle (Ct) values using the 2^{-ddCt} method of data analysis as described in Chapter 2. Each target gene was calibrated to a normalizer gene, beta-actin. One-way analysis of variance and Bonferonni *t* tests were used to determine if fold induction values in TCDD-treated hepatocytes were significantly different from solvent control values ($p < 0.05$).

Relative constitutive expression of the two CYP1A isoforms was assessed based on Ct values for each gene in untreated cells. The difference in Ct values between the two genes (dCt) was expressed as $Ct_{CYP1A4} - Ct_{CYP1A5}$ for each species. A positive dCt value indicated that CYP1A5 was more abundant than CYP1A4, while a negative value indicated that CYP1A4 was more abundant. Direct comparison of Ct values between target genes is somewhat problematic because Ct values are dependent on the efficiency of the PCR reaction and on how the threshold fluorescence is set. We minimized these problems by ensuring that standard curves of each target gene were parallel indicating similar efficiencies, and by multiplexing the two target genes in one reaction.

3.3.6. Data analysis – mRNA stability

Messenger RNA decay was tracked over time by measuring transcript quantity at discrete time points over a 24 hour period after the addition of actinomycin D to the cell culture medium. The first data point was defined as time 0 with 100% mRNA remaining. The fraction of mRNA remaining at each time point thereafter was calculated by the formula 2^{-dCt} where $dCt = (Ct_{time\ x} - Ct_{time\ 0})$. No normalizer was used in this case because all mRNA transcripts, including beta-actin, are affected by actinomycin D treatment. With no internal control gene, quality of data generated by the 2^{-dCt} method depends on consistent sample loading (Livak and Schmittgen 2001).

For chickens, the first data point (time 0, 100% mRNA abundance) corresponds to the sample that was taken immediately prior to the addition of actinomycin D. For herring gulls, the first data point (time 0, 100% mRNA abundance) corresponds to the sample that was taken 3 hours after the addition of actinomycin D, and the remaining time points are adjusted accordingly. The analysis was done this way because the abundance of beta-actin transcript was higher in the sample taken 3 hours after addition of actinomycin D than in the pre-actinomycin D sample. Because mRNA decay is essentially a stochastic process (Ross 1995), the decay constant should not depend on the amount of starting material and the starting point of the data set is not expected to alter decay curve parameters.

Exponential decay curves ($y = ae^{-kdt}$) were fit to CYP1A4 and CYP1A5 mRNA decay data. A half-life was calculated from constants derived from each curve. For chickens, curves were fit to data derived from each of three separate cell culture preparations. Half-lives presented in Table 3.2 represent the average of values calculated for each curve separately. Differences in half-lives between treatment groups were tested for significance using one-way analysis of variance with Bonferonni tests ($p < 0.05$). For herring gulls, only one cell culture experiment was performed, so no statistical analysis was undertaken.

3.4. Results

3.4.1. Relative induction of CYP1A4 and CYP1A5 mRNA

Chicken CYP1A4 mRNA expression was induced 61-fold over solvent control values in 100 nM TCDD-treated cultured embryo hepatocytes, whereas chicken CYP1A5 mRNA expression was induced 25-fold. Herring gull embryo hepatocytes were less responsive to CYP1A induction by 100 nM TCDD. The herring gull CYP1A4 and CYP1A5 isoforms were induced 2.2-, and 4.3-fold over control values respectively (Figure 3.1).

3.4.2. Relative expression of CYP1A4 and CYP1A5 mRNA

A comparison of CYP1A4 and CYP1A5 Ct values in DMSO-treated hepatocytes provided an indication of relative constitutive expression of the two CYP1A isoforms. With dCt defined as $Ct_{CYP1A4} - Ct_{CYP1A5}$, a positive value indicates that CYP1A5 is the more abundant isoform and a negative value indicates that CYP1A4 is the more abundant isoform. The dCt values for chicken and herring gull were 2.0 and –4.2 respectively (Figure 3.2).

3.4.3. mRNA stability

Exponential decay curves were fit to the CYP1A4 and CYP1A5 mRNA data with r^2 values ranging from 0.928 to 0.999 (Figure 3.3). The decay constants derived from these curves were used to calculate half-lives for each transcript (Table 3.2). The two chicken CYP1A isoforms had similar mRNA stabilities. Half-lives of chicken CYP1A4 and CYP1A5 mRNA were 5.2 ± 1.7 and 5.0 ± 1.2 hours, respectively. Half-lives measured in 100 nM-treated hepatocytes were elevated over control values, but significant effects of treatment were not detected ($p > 0.05$). The stability of herring gull CYP1A5 mRNA was similar to the chicken isoforms with a half-life of 7.0 hours. Herring gull CYP1A4 was the least stable mRNA analyzed with a half-life of 2.5 hours.

3.5. Discussion

The magnitude of the EROD response to dioxin-like compounds is known to vary considerably among avian species. For example, exposure to TCDD maximally increased EROD activity in chicken and herring gull embryo hepatocytes by 15- and 2.8-fold respectively (Sanderson *et al.* 1998). We observed a similar pattern of induction at the mRNA level. Exposure to 100 nM TCDD up-regulated CYP1A4 and CYP1A5 mRNA expression in both species tested, and the increase was larger for chicken than for herring gull (Figure 3.1).

Transcript stability is one of many factors that can affect pre- and post-induction levels of mRNA expression (Kanzawa *et al.* 2004). We wondered if species differences in CYP1A expression and induction were related to differential regulation of mRNA half-life by TCDD. There is some evidence that the large induction of mouse CYP1A mRNA by TCDD is maintained by stabilization of the CYP1A transcript (Kimura *et al.* 1986). Increases in mRNA half-life associated with the presence of inducers have also been reported for other transcriptionally controlled genes where large fold inductions are observed. For example, estrogen up-regulates transcription of the avian yolk precursor vitellogenin, and also stabilizes the vitellogenin mRNA transcript (Shapiro *et al.* 1989).

Half-lives of chicken CYP1A4 and CYP1A5 mRNA in DMSO-treated cells from three separate cell culture preparations were approximately 5 hours (Table 3.2). Mahajan *et al.* (1999) have previously reported that the two chicken CYP1A isoforms have similar mRNA stabilities. Due to egg availability (herring gull eggs can be collected only once a year), stabilities of herring gull CYP1A mRNA isoforms were measured in a single preparation of cultured hepatocytes. This was not ideal, because as observed with the chicken cultures, there was a high degree of variation between replicate experiments (Figure 3.3). Taking standard deviations of the chicken data into consideration, half-lives of herring gull CYP1A5, chicken CYP1A4 and chicken CYP1A5 were similar. Herring gull CYP1A4 mRNA was less stable than any of the other isoforms in both DMSO- and 100 nM TCDD-treated hepatocytes. The low responsiveness of herring gull CYP1A4 to induction by TCDD may be related to its relatively short mRNA half-life. Measurement of transcription rates of CYP1A isoforms in DMSO and TCDD-treated cells would be required to further

characterize the relationship between mRNA stability and the magnitude of the CYP1A response to TCDD. We did not find evidence that mRNA half-life is regulated by TCDD in chicken or herring gull embryo hepatocytes. Half-lives of CYP1A4 and CYP1A5 mRNA were approximately 2 hours longer in TCDD-treated cells, but this difference was not statistically significant (Table 3.2).

Species differences in which of the two CYP1A isoforms was preferentially expressed and induced were observed. Chicken CYP1A4 was induced by 61.3-fold, and chicken CYP1A5 was induced by 25.0-fold in embryo hepatocytes treated with 100 nM TCDD (Figure 3.1). Preferential induction of the CYP1A4 isoform was also found in liver tissue isolated from PCB 126-treated chicken embryos (Chapter 4). In herring gulls, the CYP1A5 isoform was preferentially induced. Exposure to 100 nM TCDD increased herring gull CYP1A4 and CYP1A5 mRNA expression by 2.2-, and 4.3-fold respectively (Figure 3.1). Relative constitutive abundance of the two isoforms was estimated from differences in CYP1A4 and CYP1A5 Ct values in DMSO-treated cells. Based on the available data, we hypothesize that in both species, the isoform that is preferentially induced also exhibits lower constitutive expression (CYP1A4 for chicken, CYP1A5 for herring gull). This hypothesis is supported by nuclear run-on assays performed in nuclei isolated from control and TCDD-treated chicken embryo livers (Mahajan and Rifkind 1999). Constitutive transcription rates were higher for CYP1A5, but the fold increase in transcription was larger for CYP1A4.

The effectiveness of each CYP1A isoform as a biomarker for environmental exposure to dioxin-like compounds may be related to both the magnitude of the response and the dose at which it first occurs in cultured cells. In chickens, EC50 values for the two isoforms are similar. When scaled to account for differences in maximal response, CYP1A4, CYP1A5, and EROD dose-response curves from TCDD-treated chicken embryo hepatocytes were nearly identical (Chapter 2). With equivalent EC50s, the magnitude of the response to dioxin-like compounds becomes the more important parameter for determining biomarker sensitivity of CYP1A isoforms within a species.

We have shown that CYP1A4, the isoform that is most responsive to induction by TCDD in chickens, is least responsive to induction in herring gulls (Figure 3.1). Our findings suggest that for some species, CYP1A5 mRNA expression may be a more sensitive

biomarker for exposure to dioxin-like compounds than CYP1A4 mRNA expression or EROD activity. Correlative biomarker studies with field-collected samples support this hypothesis. TEQs were positively correlated with CYP1A5 mRNA expression, but not with EROD activity in jungle crow liver (Watanabe *et al.* 2005). In adult herring gulls, liver concentrations of PCBs were associated with accumulation of HCPs (mediated by CYP1A5), but not with EROD activity (catalyzed by CYP1A4) (Kennedy *et al.* 1998, 2003). Preferential induction of CYP1A5 mRNA may be one of several factors contributing to this result. Also relevant is the identity of contaminants present in the tissues of the birds, and the specificity of these compounds for an EROD or porphyrin response. For example, PCB 118, one of two PCB congeners that most contributed to elevated HCPs in herring gull liver, does not induce EROD activity in herring gull embryo hepatocytes up to concentrations of 3000 nM (Kennedy *et al.* 1996a,1998). The mixture of contaminants present in environmentally exposed tissues and species sensitivity to particular classes of contaminants may contribute to inter-species variation in effectiveness of CYP1A-related biomarkers. It is not known if particular classes of dioxin-like compounds exhibit preferential specificity for induction of the CYP1A4 or CYP1A5 isoform, but this would be important information for a complete characterization of CYP1A mRNA expression as a biomarker.

We conclude that species differences in expression, induction, and stability of CYP1A isoforms are apparent, and should be considered when choosing appropriate biomarkers for exposure to environmental contaminants in wild avian species. The Q-PCR method for measuring CYP1A5 mRNA expression presented here shows promise as a sensitive biomarker for dioxin-like compounds in herring gulls and other colonial fish-eating birds.

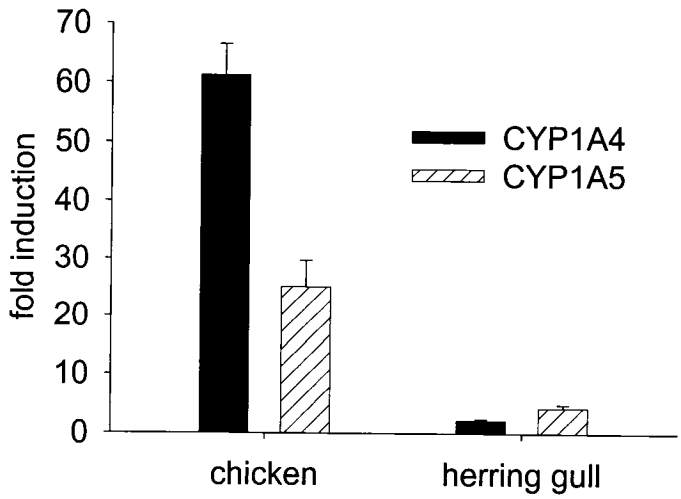


Figure 3.1. Comparison of the CYP1A response to TCDD in chicken and herring gull embryo hepatocytes. Cultured embryo hepatocytes from each species were treated with 100 nM TCDD, or dimethyl sulfoxide (solvent control). Bars represent the fold increase in CYP1A4 or CYP1A5 mRNA expression in treated cells relative to control cells. For chickens, each bar represents mean fold induction for three separate cell culture preparations ± standard error. For herring gulls, each bar represents mean fold induction of four replicate analyses of the same sample ± standard error. Fold increases in CYP1A4 and CYP1A5 expression were statistically significant for both chickens and herring gulls ($p<0.05$). Differences between CYP1A4 and CYP1A5 fold induction were also statistically significant for both species ($p<0.05$).

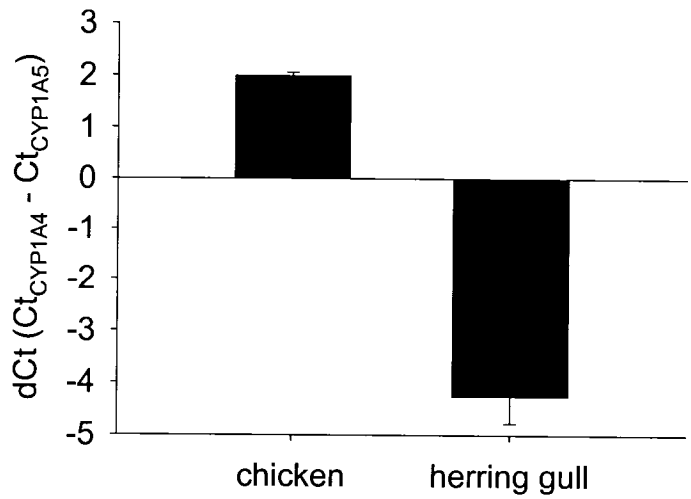


Figure 3.2. Relative constitutive expression of CYP1A4 and CYP1A5 mRNA in chicken and herring gull embryo hepatocytes. The relationship between CYP1A4 and CYP1A5 expression levels is described by the difference in threshold cycle (Ct) values in uninduced samples. With dCt defined as $Ct_{CYP1A4} - Ct_{CYP1A5}$, a positive dCt value suggests that the CYP1A5 isoform is more abundant, and a negative dCt value suggests that the CYP1A4 isoform is more abundant. For chickens, bars represent the average dCt value in DMSO-treated hepatocytes from three separate cell culture preparations ± standard error. For herring gulls, bars represent the average dCt value in four replicate analyses of DMSO-treated hepatocytes from one cell culture preparation ± standard error.

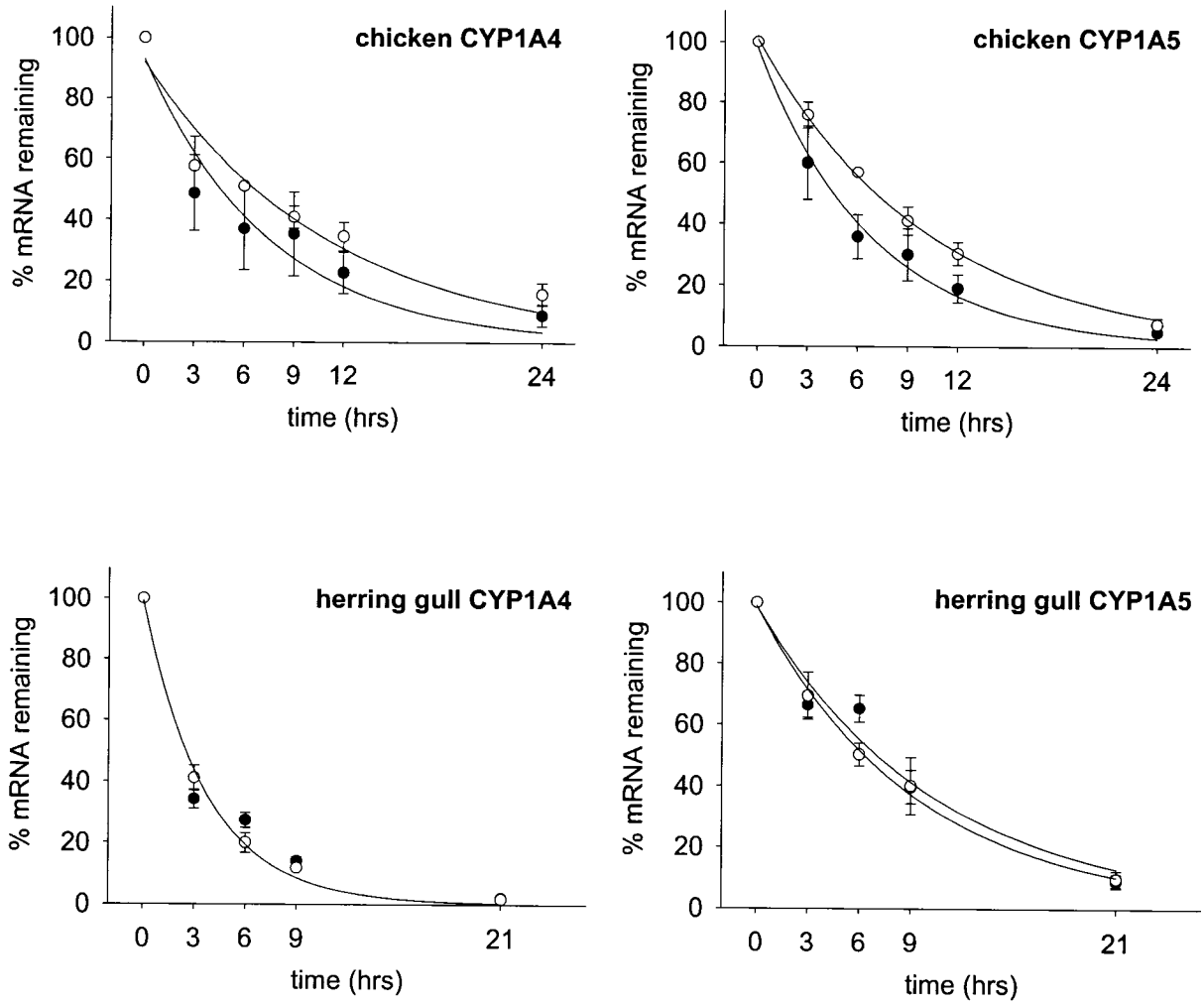


Figure 3.3. Decay of chicken and herring gull CYP1A mRNA in transcriptionally inhibited embryo hepatocyte cultures. Hepatocytes were treated with DMSO (•), or 100 nM TCDD (○) for 19 hours prior to exposure to actinomycin D. Messenger RNA levels were monitored at discrete time points over the next 24 hours. The amount of mRNA remaining at each time point is expressed as a percentage of the sample with the lowest beta-actin Ct value. For chickens, this was the pre-actinomycin D sample. For herring gulls, the sample taken three hours after addition of actinomycin D had the lowest beta-actin Ct, so this time point was labelled as time 0 and other time points were adjusted accordingly. Chicken data points represent mean values from three separate cell culture preparations ± standard error. Herring gull data points represent mean values of four replicate measurements of the same samples ± standard error. Exponential decay curves were fit to the data with r^2 values ranging from 0.928 to 0.999. For herring gull CYP1A4, only one curve is visible because the curve fits were nearly identical in DMSO- and 100 nM TCDD-treated samples.

Table 3.1. Herring gull CYP1A4, CYP1A5, and beta-actin primer and probe sequences, dyes, and amplicon sizes. Final primer and probe concentrations used in the PCR master mix are presented in brackets following each sequence. Chicken Q-PCR primer and probe sequences used in this study are presented in Table 2.1.

Herring gull CYP1A4	
Dye/quencher	Quasar 670/BHQ-2
Amplicon size	72 bp
Forward primer	5'-GATGTCACGGACTCGTTGAT-3' (600 nM)
Reverse primer	5'-GGAGAGTGGGACATAGGTATCC-3' (50 nM)
Probe[a]	5'-CCCTACACTATTCTCCTGGCAGTGCTCA -3' (200 nM)

Herring gull CYP1A5	
Dye/quencher	FAM/BHQ-1
Amplicon size	64 bp
Forward primer	5'-TGAATGACCTCTTTGGAGCA-3' (300 nM)
Reverse primer	5'-TACATGAGGCACCAGGACAG-3' (300 nM)
Probe[b]	5'-GCTTTGACACCGTGACAACTGGC -3' (200 nM)

Herring gull beta-actin	
Dye/quencher	HEX/BHQ-1
Amplicon size	70 bp
Forward primer	5'-TGGGTATGGAGTCCTGTGGTA-3' (300 nM)
Reverse primer	5'-CGGATATCCACATCGCACTT-3' (60 nM)
Probe[b]	5'-CCATGAAACCACCTTCAACTCCATCA-3' (200 nM)

[a]probe anneals to same strand as reverse primer
[b]probe anneals to same strand as forward primer

Table 3.2. CYP1A mRNA half-life in DMSO- and 100 nM TCDD-treated chicken and herring gull embryo hepatocytes. Half-lives were calculated from decay curves presented in Figure 3.3.

Gene	Species	$t_{1/2}$ DMSO (hrs)[a]	$t_{1/2}$ 100 nM TCDD (hrs)[a]
CYP1A4	Chicken	5.2 ± 1.7	7.0 ± 0.5
CYP1A5	Chicken	5.0 ± 1.2	7.0 ± 0.5
CYP1A4	Herring gull	2.5	2.5
CYP1A5	Herring gull	7.0	6.3

[a]For chickens, values represent the mean half-life ($t_{1/2}$) derived from three separate cell culture experiments ± standard deviation. No statistical differences were detected by one-way ANOVA ($p<0.05$). For herring gull, one cell culture experiment was performed and no statistical analysis was undertaken.

Chapter 4

Effects of embryonic exposure to PCB 126 on the CYP1A response to TCDD in chicken embryo hepatocytes[1]

4.1. Abstract

Concentrations of dioxin-like compounds in avian eggs can vary substantially between individuals, species, and collection sites. Although cytochrome P4501A (CYP1A) inducibility in hepatocyte cultures is commonly used to predict species sensitivity to environmental contaminants, it is not known how exposure to dioxin-like compounds during embryonic development might alter this biomarker response. To investigate this question, we injected vehicle, 0.4, 0.8, or 1.6 μg/kg 3,3',4,4',5-pentachlorobiphenyl (PCB 126) into the air cell of fertilized chicken eggs prior to incubation, and measured CYP1A endpoints in cultured hepatocytes of day 19 embryos from each treatment group. The CYP1A response to PCB 126 was also assessed in whole liver tissue. Embryonic exposure to the most environmentally relevant treatment, 0.4 μg/kg PCB 126, increased CYP1A4 mRNA expression 29-fold over control values in whole liver tissue, but only twofold over control values in cultured hepatocytes. The CYP1A response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was not altered in hepatocytes cultured from 0.4 μg/kg treated embryos, but at 0.8 and 1.6 μg/kg PCB 126, several concentration-dependent effects were observed. Embryos exposed to higher concentrations of PCB 126 *in ovo* were more responsive to CYP1A mRNA induction by TCDD *in vitro*. These findings suggest that exposure to environmental levels of dioxin-like compounds during incubation is not likely to alter species sensitivity estimates derived from *in vitro* CYP1A data.

[1] Adapted from: Head,J.A., O'Brien,J., and Kennedy,S.W. 2006. Environ Toxicol Chem **25**: [in press]

4.2. Introduction

Toxicological risk assessment for avian wildlife requires accurate estimates of species sensitivity to environmental contaminants. The *in vitro* ethoxyresorufin-*O*-deethylase (EROD) bioassay has been used to assess sensitivity to dioxin-like compounds in many avian species, including herring gull, double-crested cormorant, common tern, and bald eagle (Lorenzen *et al.* 1997b; Sanderson *et al.* 1998; Kennedy *et al.* 2003b). Previous authors have speculated that the presence of contaminants in eggs of wild birds might affect the cytochrome P4501A (CYP1A) response to dioxin-like compounds in cultured tissue or cells (Lorenzen *et al.* 1997b; Brunström and Halldin 1998). Complex mixtures of contaminants maternally deposited in the yolk can vary considerably between species, individuals, and collection sites in terms of concentration and composition. Effects of embryonic contaminant load on *in vitro* CYP1A endpoints are currently unknown and may affect sensitivity estimates derived from cultured embryo hepatocyte bioassays.

Previous studies have shown that EROD activity is enhanced in liver tissue of embryos exposed to dioxin-like compounds *in ovo*. For example, a 12- or 50-fold increase in EROD activity was observed in liver microsomes isolated from day 19 embryos injected with 1.6 μg/kg 3,3',4,4',5-pentachlorobiphenyl (PCB 126) prior to incubation (Jin *et al.* 2001; Katynski *et al.* 2004). We were interested in whether this upregulation would persist in cultured hepatocytes, and alter sensitivity estimates based on the CYP1A response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD).

To investigate this question, fertilized chicken eggs were injected with PCB 126 prior to incubation and CYP1A4 mRNA expression, CYP1A5 mRNA expression, and CYP1A4 enzymatic activity (EROD) were measured in hepatocytes cultured from control and PCB 126-treated embryos. Cells were also dosed with TCDD *in vitro* to examine the effect of *in ovo* exposure to PCB 126 on the CYP1A dose-response curves that are commonly used to predict species sensitivity. This experimental model allowed us to address the question of how variation in contaminant concentration in wild bird eggs might alter the CYP1A response to TCDD in cultured hepatocytes and confound sensitivity estimates.

4.3. Materials and Methods

4.3.1. Chemicals

Stocks for egg injection were prepared by dissolving PCB 126 in charcoal-filtered, sterilized vegetable oil at concentrations of 2, 4, and 8 μg/ml. The PCB 126 (AccuStandard, New Haven, CT, USA) was initially dissolved in dichloromethane, which was later evaporated under a gentle stream of nitrogen. Stock solutions of TCDD (kindly provided by J.J. Ryan, Health Canada, Ottawa, ON) and PCB 126 for dosing hepatocytes were prepared in dimethylsulfoxide.

4.3.2. Egg injection

The egg injection protocol used for this study was approved by the animal care committee at the National Wildlife Research Centre (NWRC, Ottawa, ON). White leghorn chicken (*Gallus domesticus*) eggs obtained from the Canadian Food Inspection Agency (CFIA, Ottawa, ON) were weighed and the air cell was marked in pencil. Two small holes were drilled through the shell above the air cell using a dental drill (X35 micromotor system, Buffalo Dental Manufacturing, Syosset, NY, USA). A PCB 126 working solution or sterile vegetable oil was injected into one hole using an automatic pipette, while the membrane of the second hole was held open with a sterile pipette tip. The injection volume was approximately 10 μl and was adjusted to the egg mass. Eggs were divided into five treatment groups as follows: untreated (no injection – 15 eggs), vehicle (sterile vegetable oil – 15 eggs), 0.4 μg/kg PCB 126 (25 eggs), 0.8 μg/kg PCB 126 (25 eggs), and 1.6 μg/kg PCB 126 (25 eggs). The holes were sealed with melted paraffin wax shortly after injection and eggs sat at room temperature overnight with the air cell pointing up. The eggs were placed in an incubator (Curfew CK 900 professional, Essex, UK) the next morning, and kept for 19 d at 37.5°C, 65% humidity. Eggs were candled periodically throughout development and removed from incubators if they were unfertilized or contained dead embryos. An approximate developmental stage was determined for all dead embryos.

4.3.3. Tissue preparation

Embryos were sacrificed by decapitation at day 19 of development. Livers were removed, weighed, and placed in cold Krebs-Ringer buffer. The left lobe from 2 or 3 individuals in each treatment group was flash frozen in liquid nitrogen and used for microsome isolation. Approximately 20 mg of each of these samples was removed for analysis of CYP1A mRNA expression in whole liver tissue. The remaining liver lobes were pooled according to treatment group and used to prepare cultured hepatocytes. Pools for each treatment group contained tissue from the following numbers of individuals: untreated (9), vehicle (8), 0.4 μg/kg PCB 126 (14), 0.8 μg/kg PCB 126 (5), 1.6 μg/kg PCB 126 (4).

4.3.4. Hepatocyte culture

Cultured hepatocytes were prepared in 48-well plates by collagenase digestion of pooled liver samples as described in Chapter 2. Plates of hepatocytes were incubated in 5% CO_2 at 37.5°C for 20 h, then dosed with TCDD and/or PCB 126 and incubated for a further 24 h (Figures 4.1 and 4.2). Working solutions were added to the cell culture medium at a volume of 2.5 μl per well, resulting in TCDD concentrations ranging from 0.0001 to 100 nM. For the co-exposure experiment, both TCDD and PCB 126 were added directly to the cell culture medium. Well concentrations of PCB 126 were 0.0006, 0.006, or 0.06 nM. A solvent control (DMSO) was included on all plates, and this well was used to compare expression and activity levels in cultured hepatocytes and whole liver samples. After 24 h of exposure, cell culture plates were removed from the incubator, flash-frozen in powdered dry ice (Praxair, Ottawa, ON, Canada), and stored at -80°C until the time of analysis.

4.3.5. Cell viability

The 5-carboxyfluorescein diacetate-acetoxymethyl ester (5-CFDA, AM) assay was used to assess viability of cells exposed to TCDD and/or PCB 126. This fluorescence

based method tests for intracellular esterase activity and indicates the presence of intact cells. Cell viability was assessed in three different concentrations of hepatocytes from each PCB 126 treatment group as well as hepatocytes treated with the following: reagent alcohol (negative control), TCDD alone, PCB 126 alone, or combinations of PCB 126 and TCDD. The cell viability culture plate was removed from the incubator after 24 h of exposure to test compounds. Complete medium spiked with 5-CFDA, AM (Molecular Probes, Invitrogen Canada, Burlington, ON) was aliquoted into each well at a concentration of 4 μM. After 30 min in the incubator at 37°C, fluorescence values in each well were read on a fluorescent plate reader (excitation: 485 nm, emission: 530 nm).

4.3.6. Microsome isolation and EROD assays

Microsomes were isolated from homogenized whole liver tissue by ultracentrifugation. Nuclei and mitochondria were removed by centrifugation of the homogenate at 9 000*g* for 15 min. The supernatant (S9 fraction) was then centrifuged at 10 000*g* for 60 min. The resulting pellet was dissolved in phosphate buffer (0.1 M, pH 7.4, Sigma), and stored at -80°C until analysis. All centrifugation steps were carried out at 4°C and the sample was kept on ice throughout the procedure.

Assessment of EROD activity in microsomes and cultured hepatocytes was performed according to previously detailed methods (Kennedy *et al.* 1993; Kennedy and Jones 1994). Briefly, formation of the fluorescent product resorufin was measured in 48-well plates containing microsomes or cultured hepatocytes, β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt, and the substrate 7-ethoxyresorufin. Values were fitted to a standard curve and normalized to the amount of total protein measured in each well. The EROD dose-response data were fitted to a modified Gaussian curve according to the equations outlined in Kennedy *et al.* (1993).

4.3.7. RNA isolation and quantification

Total RNA was isolated from slices of whole liver tissue with TRIZOL reagent (Invitrogen Canada, Burlington, ON) and from cultured hepatocytes with the Qiagen

RNeasy 96 kit (Qiagen, Mississauga, ON, Canada). Genomic DNA was detected in RNA isolated by both methods, so samples were DNAse-treated with DNA-free reagent (Ambion, Austin, TX, USA). This treatment produced RNA that was free from contamination as verified by quantitative RT-PCR. Relative CYP1A4 and CYP1A5 mRNA abundance was measured in a multiplex quantitative RT-PCR (Q-PCR) assay as described in Chapter 2.

4.3.8. Data analysis

The lethal dose for 50% of individuals (LD50) for PCB 126 administered by egg injection was calculated by linear interpolation between the data points above and below 50% mortality. For endpoints measured in cultured hepatocytes, treatment group means were compared using one-way analysis of variance and Tukey tests. In all cases, differences were considered statistically significant if p values were less than 0.05. Statistical analysis was not undertaken for the EROD and CYP1A mRNA expression data in microsomes or whole liver tissue because of the small sample size.

4.4. Results

4.4.1. Toxicological effects

Embryo mortality - Injection of PCB 126 into the air cell of unincubated chicken eggs caused embryo mortality at all doses tested. An LD50 of 0.6 μg/kg was estimated by linear interpolation between the 0.4 μg/kg and 0.8 μg/kg data points (Figure 4.3). Since it is common for a certain number of eggs to be unfertilized, and these were present in approximately equal proportion in all treatment groups, eggs that showed no visible development of the embryo were not included in the LD50 analysis.

Liver abnormalities - Of the three PCB 126 doses injected, two were above the LD50. Livers of surviving individuals from these treatment groups were severely discoloured.

The discoloration was particularly pronounced in embryos receiving 1.6 µg/kg PCB 126, and the dark green colour persisted until cells were cultured. No significant differences in liver mass between treatment groups were observed. The average liver mass for the 40 individuals used in this study was 0.52 ± 0.05 g.

4.4.2. Viability of cultured hepatocytes - Cell viability was measured as intracellular hydrolysis of 5-CFDA, AM to a fluorescent derivative. Fluorescence values were stable across all treatment groups and significantly elevated over background values. The cells were also observed under a light microscope. Cells from all treatment groups appeared normal, and no blebbing was observed.

4.4.3. Comparison between whole liver and cultured hepatocytes

EROD activity – Treatment with PCB 126 caused a dose-dependent increase in EROD activity in microsomes isolated from embryonic liver (Figure 4.4.A). The increase was large (EROD activity was 110-fold above control values in microsomes isolated from 1.6 µg/kg PCB 126-treated embryos), and followed an upward trend with dose. Statistical analysis for endpoints measured in microsomes and whole liver tissue was not undertaken because the sample size was small (2-3 livers in each treatment group). Dose-dependent increases in EROD activity were detected in hepatocytes cultured from pools of untreated, vehicle-treated, and PCB 126-treated embryo livers ($p < 0.05$). The response in cultured cells was notably diminished with respect to the response in microsomes isolated from whole liver (Figure 4.4.A).

CYP1A4 and CYP1A5 mRNA expression - Relative CYP1A4 and CYP1A5 mRNA expression increased in a dose-dependent manner in whole liver from PCB 126-treated individuals. In cultured hepatocytes, statistically significant induction of both CYP1A mRNA isoforms was detected ($p < 0.05$). The increase in cultured cells was substantially less than the increase measured in whole liver (Figs. 2B and C), but because of the small number of whole liver samples, this finding was not tested for statistical significance. A strong linear relationship between CYP1A4 enzymatic activity (EROD) and mRNA

expression was detected in whole liver (r^2=0.97) and cultured hepatocytes (r^2= 0.91) ($p <$ 0.05).

4.4.4. CYP1A response to TCDD in cultured hepatocytes

EROD activity – Exposure to PCB 126 during incubation had a minimal effect on the EROD response to TCDD in cultured hepatocytes. Baseline EROD activity increased in a PCB 126-dependent manner across treatment groups (Figure 4.4.A, and Figure 4.5), but activity at low doses of TCDD was only elevated in cells cultured from 1.6 µg/kg PCB 126-treated embryos (Figure 4.5.A). The maximal TCDD-dependent EROD response was variable and particularly high in the 0.4 µg/kg PCB 126 treatment group. Variation in maximal EROD activity was not associated with PCB 126 treatment. EC50s in hepatocytes cultured from 0.4 and 0.8 µg/kg PCB 126-treated embryos were significantly higher than control values. The EC50 in hepatocytes cultured from the highest treatment group, 1.6 µg/kg PCB 126 was not different from the control value (Table 4.1).

CYP1A4 and CYP1A5 mRNA expression – Exposure to PCB 126 during incubation enhanced CYP1A mRNA induction by TCDD in cultured hepatocytes. In contrast to the EROD data, this increase was observed at all TCDD doses tested; both baseline and maximal CYP1A expression increased with PCB 126 treatment, and the dose-response curves were essentially shifted upwards (Figure 4.6.A). Additionally, the magnitude of the CYP1A mRNA response increased in a PCB 126-dependent manner. Embryos that were exposed to higher levels of PCB 126 *in ovo* were more responsive to CYP1A induction by TCDD in cultured hepatocytes. Induction, measured as baseline expression subtracted from maximal expression, was significantly higher in hepatocytes from the 0.8 and 1.6 µg/kg PCB 126 treatment groups ($p < 0.05$) (Figure 4.7). When hepatocytes were co-exposed to TCDD and PCB 126 *in vitro*, a different pattern of expression emerged. Levels of CYP1A mRNA were elevated in PCB 126 treated hepatocytes at low doses of TCDD, but the maximal response was not affected (Figure 4.6.B).

4.5. Discussion

4.5.1. Toxicological effects

Injection of PCB 126 into the air cell of unincubated chicken eggs was lethal to 50% of individuals at a dose of approximately 0.6 μg/kg (Figure 4.3). Similar LD50 values have been previously reported. Hoffman *et al.* (1998) report an LD50 of 0.4 μg/kg for PCB 126 injected into the air cell on embryonic day 4, and *studies* using yolk injection techniques found LD50s ranging from 0.6 to 3.0 μg/kg (Powell *et al.* 1996b; Zhao *et al.* 1997). No deaths were observed among untreated embryos in the present study, but mortality was 17% for embryos injected with corn oil vehicle. The high mortality associated with the vehicle group suggests that we may have underestimated the LD50. Other endpoints presented in this paper do not differ between untreated and vehicle-treated groups.

Effects of treatment were also apparent from the colour and texture of embryonic livers exposed to 0.8 and 1.6 μg/kg PCB 126. Despite this, a routine cell viability assay (5-CFDA, AM) indicated that cells from treated tissues were intact, with normal intracellular esterase activity. Furthermore, hepatocytes from PCB-126 treated individuals were responsive to CYP1A induction by TCDD, indicating that they were transcriptionally active.

4.5.2. Comparison between whole liver and cultured hepatocytes

Several groups have reported elevated EROD activity in embryonic liver exposed to dioxin-like compounds *in ovo* (Brunström 1990; Katynski *et al.* 2004), but it was not known if this effect would persist in hepatocytes cultured from contaminated tissue. For comparative purposes, CYP1A data from whole liver and cultured hepatocytes were expressed relative to the untreated sample for each endpoint and sample type. In all cases, the PCB 126 induced CYP1A response was considerably larger in whole liver than in cultured cells (Figure 4.4). For example, EROD activity was induced 57-fold over control

values in whole liver from 0.8 μg/kg PCB 126-treated embryos and only twofold in cultured hepatocytes.

Our results indicate that although *in ovo* exposure to PCB 126 has a dramatic effect on CYP1A induction in embryonic liver, the effect is diminished in cultured hepatocytes. The relative reduction in CYP1A response may be related to sample preparation. Levels of PCB 126 are maintained in embryonic liver by continuous absorption of contaminant from the yolk sac as the embryo develops (de Roode and van den Brink 2002). For tissue analysis, the left lobe of the liver was removed shortly after sacrifice and immediately frozen in liquid nitrogen. Levels of EROD activity and CYP1A mRNA expression measured in samples harvested in this way are representative of hepatic activity in day 19 embryonic liver. In contrast, hepatocyte preparation involved digestion of the tissue, filtering, and 44 h of incubation before culture plates were flash frozen. Down-regulation of the CYP1A response could occur through loss of PCB 126 during hepatocyte isolation or because the 44 h incubation period gives cells time to adapt to a new environment with no continuous source of exposure to PCB. Data from a polyaromatic hydrocarbon egg injection experiment are consistent with this explanation. Davis *et al.* (1997) found that EROD activity in hepatocytes cultured from ß-naphthoflavone injected chicken embryos was initially elevated, but steadily decreased with time in culture. After 50 hours in culture, EROD activity was similar in hepatocytes cultured from ß-naphthoflavone treated and untreated embryos.

4.5.3. CYP1A response to TCDD in cultured hepatocytes

In ovo exposure to PCB 126 - Dioxin-like compounds present in cultured hepatocytes during the course of the exposure period included PCB 126 (residual levels from *in ovo* exposure) and/or TCDD (*in vitro* exposure) (see Figure 4.1 for exposure protocol). The EROD response to both of these test compounds has been shown to persist for at least 48 h in cultured chicken hepatocytes (Bastien *et al.* 1997). After 20 h of incubation in the presence of residual levels of PCB 126, and a further 24 h exposure to TCDD, one might expect to see a combined CYP1A response that is greater than the response elicited by either of these two compounds individually. For CYP1A mRNA dose-response curves,

this is what we observed. Expression of CYP1A mRNA was enhanced by *in ovo* PCB 126 treatment in TCDD-dosed hepatocytes at all concentrations tested (Figure 4.6.A). In contrast, the EROD response to TCDD was relatively unaffected by PCB 126 treatment *in ovo*. A concentration-dependent effect on baseline EROD activity was detected across treatment groups (Figures 4.4.A and 4.5), but induction at low doses of TCDD was only enhanced by the most concentrated PCB 126 treatment. For example, EROD activity at 0.001 nM TCDD was elevated in hepatocytes cultured from 1.6 μg/kg PCB 126-treated embryos, but was not different from control values in hepatocytes cultured from 0.4 or 0.8 μg/kg PCB 126-treated embryos (Figure 4.5). At higher concentrations of TCDD, we detected variation in the EROD response, but this variability was not associated with *in ovo* PCB 126 treatment. Inconsistent values for maximal EROD activity have been noted previously (Sanderson *et al.* 1998), and are currently unexplained. Because maximal EROD activity was not associated with maximal CYP1A4 mRNA expression or *in ovo* PCB 126 exposure, we conclude that variation in the EROD maximum is a property of the enzyme assay itself and not a biological response to PCB 126 treatment. Variation in the EROD maximal response may be a result of measurement error.

These data suggest that embryonic exposure to PCB 126 regulates CYP1A mRNA expression and enzymatic activity in different ways. *In ovo* PCB 126 treatment enhanced CYP1A mRNA induction by TCDD at all doses tested, but EROD activity was only increased by low doses of TCDD. This finding may reflect a balance between the ability of the inducer to activate CYP1A4 mRNA transcription and to inhibit EROD activity. Many CYP1A inducers, including PCBs and TCDD, competitively inhibit activity of CYP1A enzymes (Petrulis and Bunce 1999). At low doses of TCDD, the presence of residual levels of PCB 126 in cells is associated with elevated CYP1A4 mRNA expression and EROD activity. At higher doses, competitive inhibition may counterbalance the effects of up-regulation of CYP1A mRNA by PCB 126.

The results described above contrast with experiments where hepatocyte cultures were exposed to mixtures of dioxin-like compounds *in vitro*. Petrulis *et al.* (2001) report that maximal EROD induction by TCDD was reduced when primary rat hepatocytes were co-exposed to a partial AHR agonist, PCB 77. The authors suggest that at saturating concentrations of TCDD, a less potent inducer can compete for AHR binding sites and

lower the apparent EROD maximum. We did not find evidence for competition between PCB 126 and TCDD for AHR binding in the present study (the highest CYP1A mRNA expression occurred at maximal doses of TCDD and PCB 126 [Figure 4.6.A]). This may be due to the fact that both test compounds are strong AHR agonists, but it could also be related to the dosing method. Hepatocytes in both studies were exposed to a mixture of PCB and TCDD *in vitro*, but in the present study, cells were also exposed to PCB 126 throughout incubation. Embryonic up-regulation of dioxin-responsive genes may persist in cultured cells and contribute to some of the effects outlined above. For example, AHR up-regulation in PCB 126-treated tissue would minimize competition for binding sites.

Our findings suggest two possible mechanisms for effects of PCB 126 injection on CYP1A endpoints in cultured hepatocytes. The CYP1A response to TCDD may be altered through direct exposure to residual levels of PCB 126 in cells or through cellular changes associated with altered liver function during development. Evidence for this second mechanism can be found in the CYP1A mRNA dose-response curve data (Figure 4.6.A). Not only is maximal TCDD-dependent expression of CYP1A mRNA higher in hepatocytes cultured from PCB 126-treated embryos, the magnitude of the CYP1A response (baseline expression subtracted from maximal expression) increases with PCB 126 treatment as well (Figure 4.7). Hepatocytes that were pre-exposed to PCB 126 *in ovo* are more responsive to induction by TCDD *in vitro*. To test the hypothesis that this effect was associated with embryonic exposure to PCB 126, and is not simply a synergistic effect of exposure to a mixture of two strong AHR agonists, we carried out the cell culture experiment using a second dosing method.

In vitro exposure to PCB 126 – Hepatocytes cultured from untreated embryos were dosed with a full range of TCDD stocks to create CYP1A mRNA dose-response curves. Cells were also exposed to PCB 126, but this time instead of treating the embryos *in ovo*, we added PCB 126 directly to the hepatoycte culture medium (Figure 4.2). Co-exposure to PCB 126 *in vitro* increased CYP1A mRNA expression at low doses of TCDD but did not affect maximal expression (Figure 4.6.B). The elevated baseline and constant maximal response meant that CYP1A mRNA induction by TCDD (or the height of the curve) actually decreased with PCB 126 treatment. This finding leads us to believe that the

enhanced sensitivity to CYP1A mRNA induction that we observed in hepatocytes cultured from PCB 126-treated embryos occurs by mechanisms other than straightforward exposure to residual levels of PCB 126. Embryonic exposure to contaminants could cause a number of cellular changes in the developing liver that might influence *in vitro* endpoints. One hypothesis that warrants further investigation is that AHR mRNA levels may be upregulated in hepatocytes cultured from embryos exposed to PCB 126 *in ovo*. High levels of AHR could reduce competition between compounds for binding sites and might also account for the heightened sensitivity of pre-exposed hepatocytes to CYP1A induction by TCDD.

4.5.4. Implications

Environmental relevance of PCB 126 treatments - We hoped to make this chicken model relevant to wild avian species by choosing PCB 126 treatments that simulate potency of embryonic exposure to CYP1A inducers in the environment. This task was complicated by the fact that wild embryos are exposed to a mixture of contaminants *in ovo*. Avian specific toxic equivalency factors (TEFs) (Kennedy and Jones 1994) help with this problem by allowing us to estimate the potency of environmental mixtures in terms of TCDD equivalents (TEQs), or the potency of the mixture relative to TCDD. Levels of dioxin-like compounds ranged from 0.07 to 1.0 µg/kg TEQs in herring gull eggs collected from Great Lakes colonies in 2001 and 2002. These TEQ concentrations were calculated from contaminant data for dioxin, furan, and non-*ortho* PCB congeners (Jermyn-Gee *et al.* 2005) using avian specific toxic equivalency factors (TEFs) (Kennedy and Jones 1994). The large majority of studies referenced in the derivation of these TEFs were done in chicken. Given that the avian TEF for PCB 126 is 0.1 (Kennedy and Jones 1994), our lowest PCB 126 treatment (0.4 µg/kg) is approximately one tenth of the average TEQs currently found in eggs of Great Lakes colonial fish-eating birds.

For our findings to be biologically relevant to wild species we also need to consider the fact that our model organism, the chicken, is far more sensitive to dioxin-like compounds than other avian species studied to date. The EROD-inducing potency of TCDD is approximately 34 and 18 times higher in chicken embryo hepatocytes than in

cells cultured from herring gull and double-crested cormorant respectively (Sanderson *et al.* 1998). Given these order of magnitude differences in species sensitivity, we must assume that any effects seen with 0.4 μg/kg PCB 126 treatment in chicken would only be observed at a higher concentration of inducer in less sensitive species. We propose that while the 0.4 μg/kg treatment is an underestimate of actual TEQs in environmental samples, it is probably a conservative overestimate of the potency of CYP1A inducers currently present in eggs of wild avian species studied to date.

The two highest PCB 126 treatments caused embryo mortality in more than 70% of individuals tested, and clearly surpass current levels of dioxin-like compounds in the environment. Effects observed at 0.8 and 1.6 μg/kg PCB 126 are mainly of interest in terms of mechanism of action, although they may also sometimes be applicable to environmental samples. This could occur if a species is particularly sensitive to the effects of dioxin-like compounds or if certain individuals are exposed to abnormally high contaminant levels.

Sensitivity estimates - In vitro EROD dose-response curves are useful for assessing species sensitivity to dioxin-like compounds and the key parameter for this type of application is the EC50. Because we often work with wild species, we are not always aware of the identity or concentration of CYP1A inducers in the egg. We were therefore interested in how prior exposure to a strong AHR agonist during incubation might alter *in vitro* EROD dose-response curve parameters, particularly the EC50.

Exposure to PCB 126 during incubation was associated with concentration-dependent changes to several *in vitro* CYP1A endpoints, but EC50s were not consistently altered by treatment (Table 4.1). Because the actual concentration of inducer in hepatocytes cultured from PCB 126-treated embryos is probably higher than the apparent concentration on the *x*-axis, embryonic exposure to PCB 126 might be expected to decrease the EC50 by shifting EROD dose-response curves to the left. Alterations in either the apparent baseline or the apparent maximal response could also shift the EC50 if they resulted in a change in the height of the dose-response curve. In hepatocytes cultured from 1.6 μg/kg PCB 126-treated embryos, baseline EROD activity was elevated while the maximal response to TCDD remained unchanged. The entire curve was also slightly

shifted to the left (Figure 4.5.C). These PCB 126-associated alterations in the dose-response curve had opposite effects on the EC50, resulting in an EC50 that was not significantly different from the control value (Table 4.1).

In hepatocytes cultured from 0.4 and 0.8 μg/kg PCB 126-treated embryos, EC50s were significantly higher than control values, but this increase does not appear to be related to PCB 126 treatment. At 0.4 μg/kg PCB 126 the elevated EC50 is due to a particularly high maximal EROD response. It has been previously reported that the maximal EROD response to TCDD can vary between cell culture preparations (Sanderson *et al.* 1998). Because we have seen this type of variation before, and because maximal EROD activity was not correlated with PCB 126 treatment or CYP1A mRNA expression in this study, we conclude that the high maximal EROD response, and elevated EC50 were not caused by PCB 126 treatment. In hepatocytes cultured from 0.8 μg/kg PCB 126-treated embryos, the EC50 was also higher than control values, but the reason behind this shift is not clear. Baseline EROD activity is slightly elevated, but this does not entirely account for the change. The 0.003 nM TCDD data point is considerably lower than the corresponding point in control cells and may be partially responsible for shifting the curve slightly to the right. With this point removed, the EC50 falls within the range of values that we normally see for hepatocytes cultured from untreated chicken embryos.

At environmentally relevant levels of PCB 126, we observed little impact on biomarker endpoints in cultured hepatocytes. Baseline CYP1A mRNA expression and EROD activity were slightly elevated over control values in hepatocytes cultured from 0.4 μg/kg PCB 126-treated embryos, but dose-response curves were otherwise unaltered by PCB 126 treatment. Based on this result, and the finding that EC50s were not directly affected by *in ovo* exposure to PCB 126, it seems unlikely that environmental levels of dioxin-like compounds in avian eggs would alter sensitivity estimates derived from EROD data.

4.5.5. Conclusions

The impetus to this work was to provide an avian model for how exposure to dioxin-like compounds during incubation might alter the CYP1A response to TCDD in cultured embryo hepatocytes. We found that effects of *in ovo* exposure to environmental levels of inducer on *in vitro* CYP1A endpoints are minimal, mostly because the response to the test compound is greatly reduced in cultured cells. The EC50, an *in vitro* measure of sensitivity, was not altered by PCB 126 treatment in this study.

An intriguing effect observed at higher PCB 126 treatments warrants further investigation. Pre-exposure to PCB 126 *in ovo* increased sensitivity to induction by TCDD in cultured hepatocytes. We hypothesize that this priming effect is associated with altered transcriptional activity in tissue of PCB 126-treated embryos.

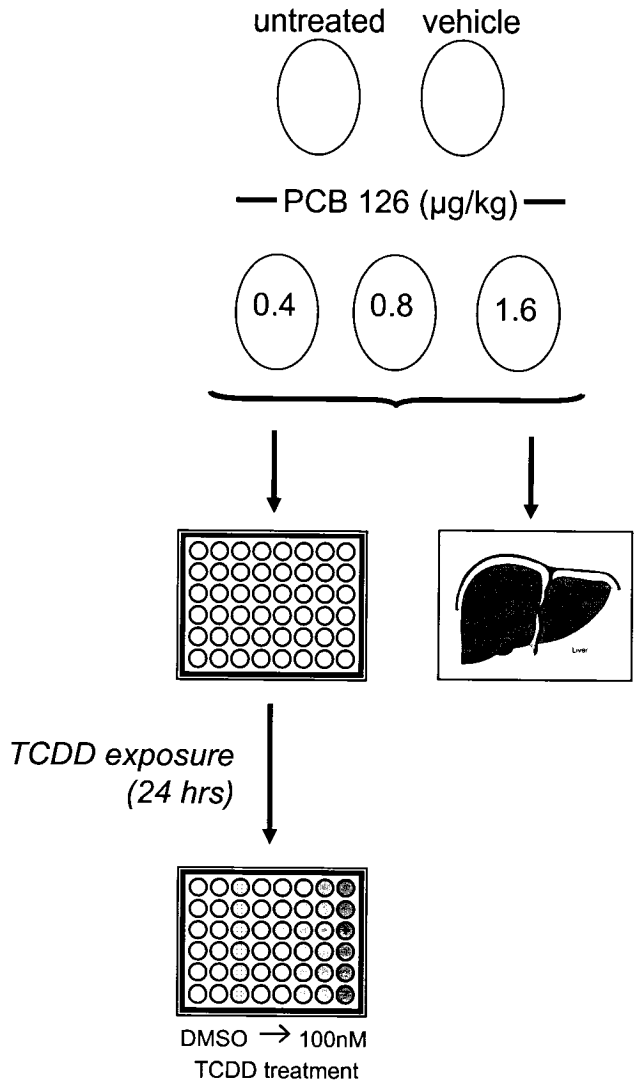


Figure 4.1. Schematic of the protocol used for *in vivo* PCB 126 exposure. As detailed in section 4.3.2, unincubated chicken eggs were injected with corn oil vehicle, or PCB 126 dissolved in corn oil vehicle at concentrations of 0.4, 0.8, and 1.6 µg/kg egg. After 19 days of incubation, embryos were sacrificed and livers were removed. A subsection was taken for analysis of EROD activity and CYP1A mRNA expression in whole liver tissue. The remaining livers were pooled according to treatment group and used to prepare cultured hepatocytes in 48-well plates. Hepatocyte cultures prepared from each PCB 126 treatment group were dosed with a range of concentrations of TCDD.

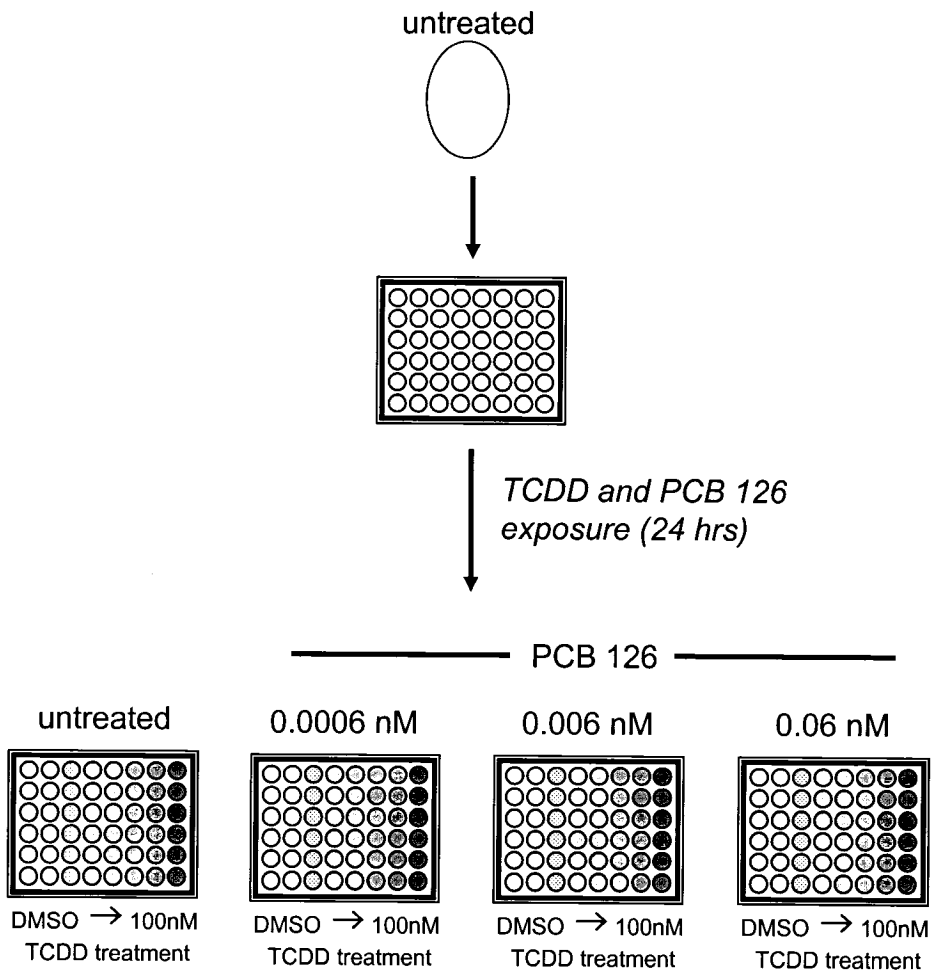


Figure 4.2. Schematic of the protocol used for *in vitro* PCB 126 exposure. Livers of 19-day chicken embryos were pooled and used to prepare cultured hepatocytes in 48-well cell culture plates. Hepatocytes were dosed with a range of concentrations of TCDD. Hepatocytes were also dosed with PCB 126 at concentrations of 0.0006, 0.006, or 0.06 nM.

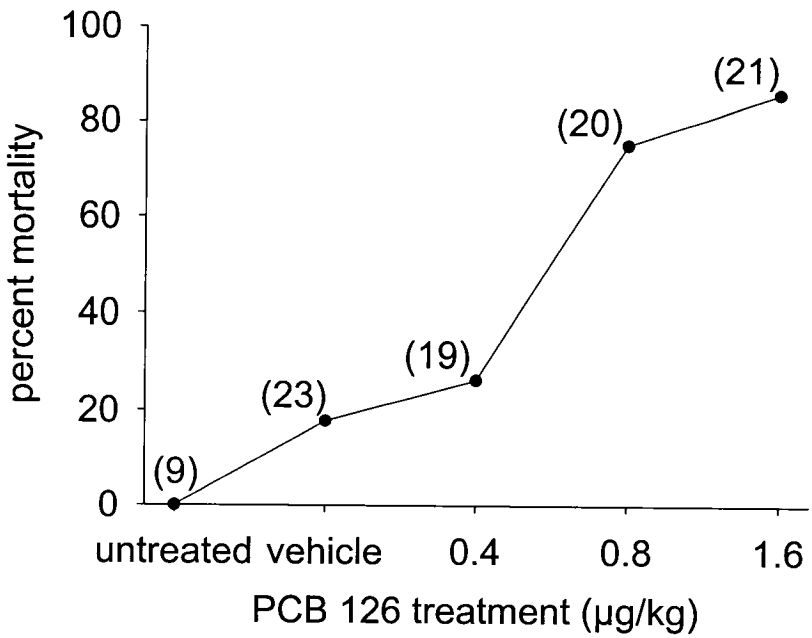
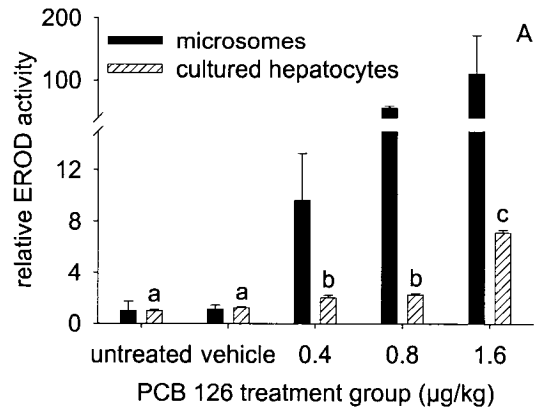
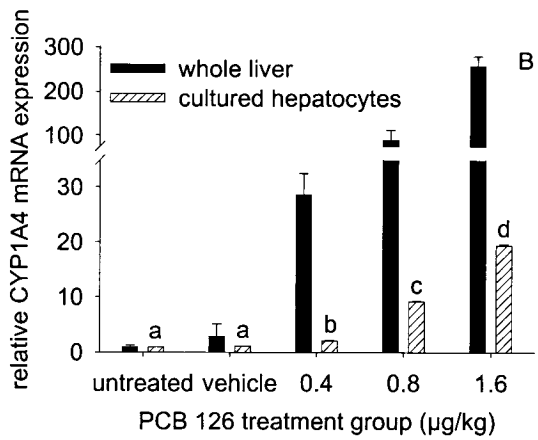


Figure 4.3. Embryo mortality associated with injection of PCB 126 into the air cell of chicken eggs prior to incubation. Numbers in parentheses represent the number of fertilized eggs in each treatment group. The lethal dose for 50% of individuals (LD50) was 0.6 µg/kg. This value was obtained by linear interpolation between the 0.4 µg/kg and 0.8 µg/kg data points.

Figure 4.4. Relative CYP1A induction in whole liver and cultured hepatocytes from control and PCB 126-treated embryos (see next page). Black bars represent EROD activity in microsomes isolated from whole liver (A), or CYP1A mRNA expression in whole liver (B,C). Hatched bars represent EROD activity (A) or CYP1A mRNA expression (B,C) in cultured hepatocytes. Results are expressed as fold-induction relative to the CYP1A response in the untreated sample for each endpoint and sample type. The error bars included in this figure represent standard error among 2-3 individual livers (black bars), standard error among three wells of hepatocytes (hatched bars; A), or standard error among three replicate analyses of a pool of three wells of hepatocytes (hatched bars; B,C). Letters above hatched bars represent statistically significant differences between treatment group means ($p < 0.05$).


A
microsomes
cultured hepatocytes
relative EROD activity
200
100
12
8
4
0
a
a
b
b
c
untreated vehicle
0.4
0.8
1.6
PCB 126 treatment group (µg/kg)


B
whole liver
cultured hepatocytes
relative CYP1A4 mRNA expression
300
200
100
30
20
10
0
a
a
b
c
d
untreated vehicle
0.4
0.8
1.6
PCB 126 treatment group (µg/kg)

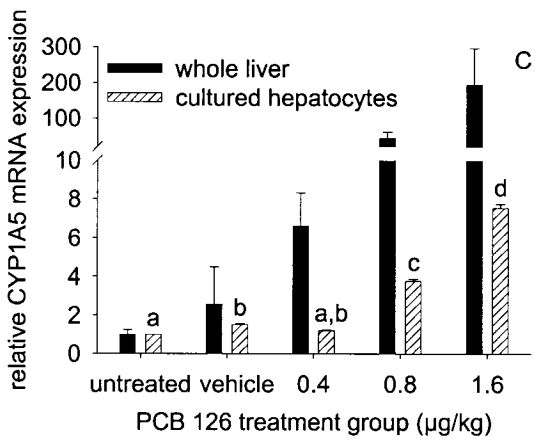
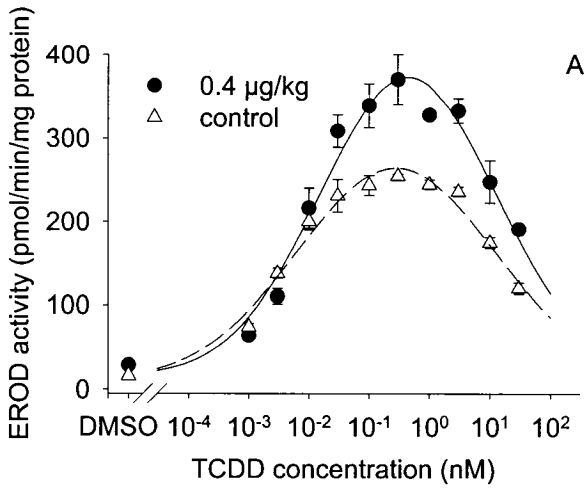
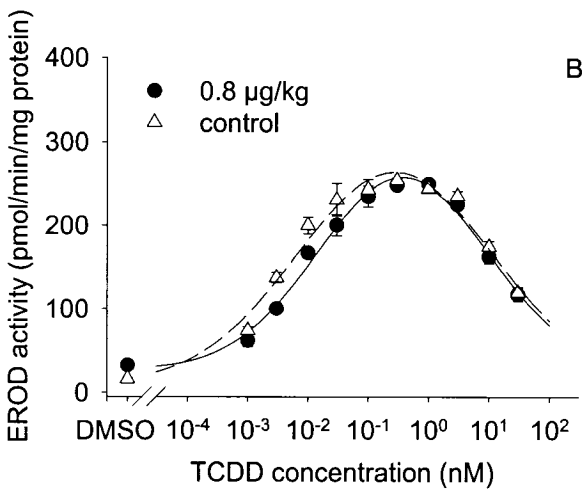
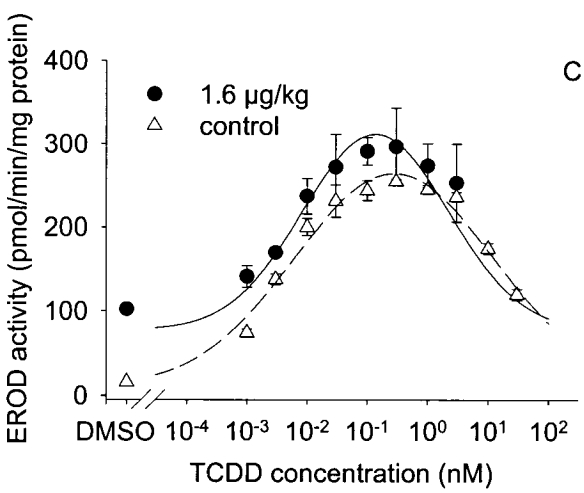

C
whole liver
cultured hepatocytes
relative CYP1A5 mRNA expression
300
200
100
10
8
6
4
2
0
a
b
a,b
c
d
untreated vehicle
0.4
0.8
1.6
PCB 126 treatment group (µg/kg)

Figure 4.5. Concentration-dependent effect of TCDD on EROD activity in hepatocytes cultured from control and PCB 126-treated embryos (see next page). Closed circles represent mean EROD activity analyzed in triplicate wells from two (0.8 and 1.6 μg/kg PCB 126) or three (0.4 μg/kg PCB 126) separate cell culture plates. For figure clarity, untreated and vehicle-treated data are presented as an average and are repeated on each graph. Open triangles, labelled 'control', represent mean EROD activity analyzed in triplicate wells from three untreated and three vehicle-treated cell culture plates. Bars represent standard error among plates. EROD activity in DMSO treated hepatocytes increased in a PCB 126-dependent manner in across all treatment groups (also shown in Figure 2A; hatched bars), but activity at the lowest dose, 0.001 nM TCDD, was only elevated at 1.6 μg/kg PCB 126 treatment. As described in the text, the maximal TCDD-dependent EROD response was variable between treatment groups, but this variation does not appear to be related to PCB 126 treatment.


A
0.4 µg/kg
control
EROD activity (pmol/min/mg protein)
400
300
200
100
0
DMSO 10-4 10-3 10-2 10-1 100 101 102
TCDD concentration (nM)


B
0.8 µg/kg
control
EROD activity (pmol/min/mg protein)
400
300
200
100
0
DMSO 10-4 10-3 10-2 10-1 100 101 102
TCDD concentration (nM)


C
1.6 µg/kg
control
EROD activity (pmol/min/mg protein)
400
300
200
100
0
DMSO 10-4 10-3 10-2 10-1 100 101 102
TCDD concentration (nM)

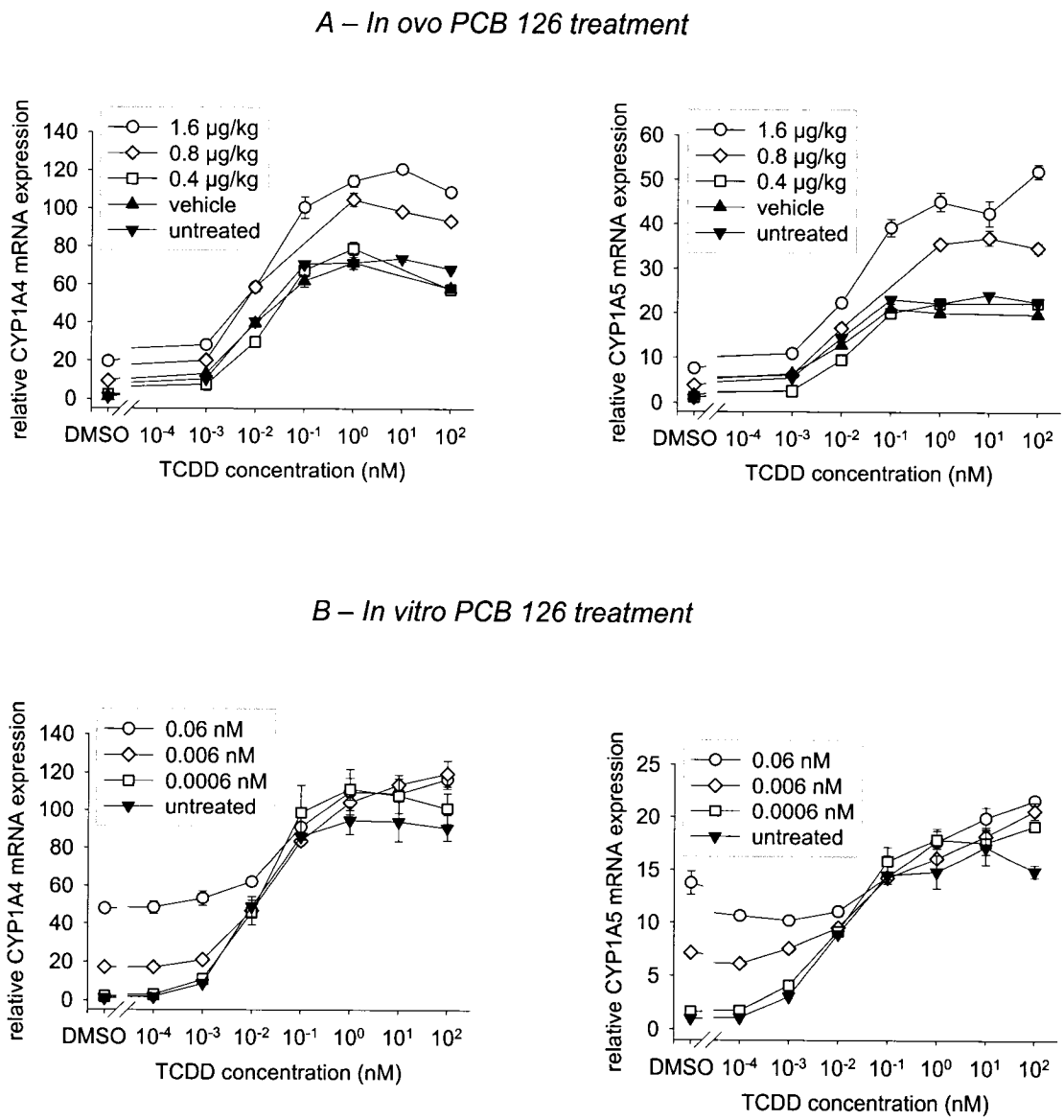


Figure 4.6. Concentration-dependent effect of TCDD on CYP1A4 and CYP1A5 mRNA expression in embryo hepatocytes cultured from control and PCB 126-treated embryos. Each point represents CYP1A mRNA expression normalized to beta-actin and expressed relative to the solvent control (DMSO) sample. Hepatocytes from three wells on each cell culture plate were pooled and analyzed in triplicate. Error bars represent the standard error of these three replicate analyses and are indicative of instrument variability. In addition to TCDD, cells were exposed to various concentrations of PCB 126 by two different methods; injection into the air cell of the unincubated egg (A), or direct dosing of the cultured medium (B). Injection of 1.6 µg/kg PCB 126 produced a similar level of EROD activity in DMSO-treated cells as direct exposure to 0.006 nM PCB 126 (data not shown)

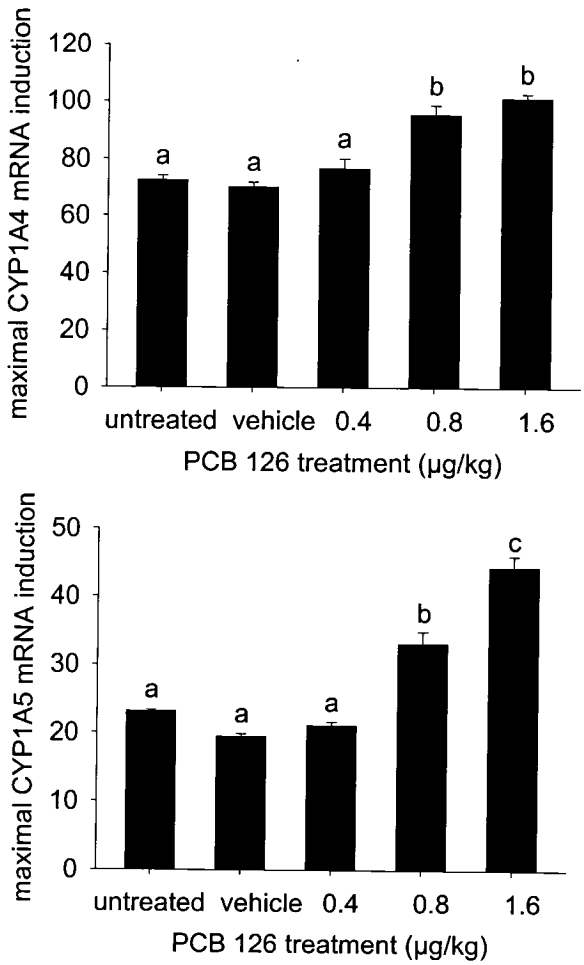


Figure 4.7. Maximal CYP1A4 and CYP1A5 mRNA induction by TCDD in hepatocytes cultured from control and PCB 126-treated embryos. Data were calculated by subtracting baseline expression from maximal expression for each of the dose-response curves in Figure 4.6.A. Error bars represent standard error. Letters a, b, and c indicate significant differences between treatment groups ($p < 0.05$).

Table 4.1. EROD inducing potency of TCDD in chicken embryo hepatocytes from control and PCB 126-treated embryos. Data are presented as the mean dose causing a half-maximal EROD response (EC50) ± standardard deviation. Numbers in parentheses represent the number of dose-response curves (cell culture plates) analyzed for each treatment group.

PCB 126 treatment	EC50 (nM TCDD)
untreated (3)	0.0029 ± 0.0006
vehicle (3)	0.0037 ± 0.0003
control [a] (6)	0.0033 ± 0.0006
0.4 µg/kg (3)	0.0088 ± 0.0018*
0.8 µg/kg (2)	0.0083 ± 0.0007*
1.6 µg/kg (2)	0.0055 ± 0.0000

[a]control refers to the mean of untreated and vehicle-treated data (as represented in figure 3)
*significantly different from untreated and vehicle-treated values ($p < 0.05$)

Chapter 5

Factors contributing to interindividual variation in the CYP1A response to TCDD in herring gull embryo hepatocytes

5.1. Abstract

Concentration-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on cytochrome p4501A (CYP1A) mRNA induction were measured in hepatocyte cultures prepared from 55 individual herring gull embryos. A large degree of variation was observed among samples. For example, TCDD did not significantly induce CYP1A4 mRNA expression above baseline values in 41% of hepatocyte culture preparations, whereas CYP1A4 was induced by 57-fold in the most responsive sample. Environmental and genetic factors contributing to the observed variation were investigated. Embryonic exposure to dioxin-like compounds was below the threshold value that would be expected to alter CYP1A mRNA expression in cultured hepatocytes for all but one individual. Interestingly, hepatocytes cultured from this individual were also the most sensitive to CYP1A mRNA induction by TCDD. Significant differences in CYP1A basal expression were detected in hepatocytes cultured from Great Lakes or Atlantic herring gulls, and these may be related to genetic differences between the two populations. Our findings suggest that the potency of TCDD for a CYP1A response in pooled hepatocyte cultures may be strongly influenced by particularly responsive samples. Characterization of factors contributing to interindividual variation in the CYP1A response to TCDD in cultured cells suggested several considerations for bioassay design which may improve the reproducibility of sensitivity estimates based on this *in vitro* bioassay for dioxin sensitivity.

5.2. Introduction

Concentration-dependent effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on cytochrome P4501A (CYP1A) induction have been observed in hepatocytes cultured from many domestic and wild avian species (Kennedy *et al.* 1996a; Sanderson *et al.* 1998). A bioassay measuring CYP1A4 and CYP1A5 induction at the level of mRNA expression has been described (Chapters 2 and 3), but the CYP1A response is more commonly assessed as ethoxyresorufin-*O*-deethylase (EROD) activity. A strong linear relationship was observed between the EROD inducing potency of polychlorinated biphenyl (PCB) congeners in chicken embryo hepatocytes (assessed as EC50), and the LD50 of the same congeners injected into chicken eggs early in development ($r^2 = 0.926$, $p < 0.001$) (Figure 1.5). A similar relationship may exist across species, but a lack of LD50 data precludes a quantitative assessment of the strength of this correlation. In general, avian species that are less sensitive to the lethal effects of dioxin-like compounds *in ovo*, are also less sensitive to EROD induction *in vitro* (Kennedy *et al.* 1996a). More rigorous validation of *in vitro* approaches for estimating sensitivity to dioxin-like compounds in avian species will require additional LD50 data.

Toxicity data for dioxin-like compounds in avian species are scarce, but the values that have been published are in general agreement. A review of the literature indicates the LD50 for TCDD in chickens varies by less than 2-fold between four labs, despite differences in injection method, dosing scheme, and the identity of the vehicle (Powell *et al.* 1996a). *In vitro* measures of species sensitivity appear to be less consistent. The EC50 for EROD induction by TCDD in chicken embryo hepatocytes varied by 15-fold (from 0.004 to 0.06 nM TCDD) among labs (Bosveld 1995; Kennedy *et al.* 1996a; Sanderson *et al.* 1998), and by 4-fold (from 0.004 to 0.016 nM TCDD) in two experiments from the same lab (Kennedy *et al.* 1996a; Lorenzen *et al.* 1997b).[2] This lack of reproducibility appears to be related to differences between cell culture preparations rather than measurement error associated with the EROD assay itself. Variance in EROD EC50 values between replicate cell culture plates within experiments is quite low (Chapter 2).

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[2] EC50 values varied by 5-fold (from 0.003 to 0.015 nM TCDD) in Chapters 2 and 4 of this thesis.

Further validation of EROD EC50 as a predictor of dioxin sensitivity may require a deeper understanding of sources of variation in this biomarker response. A large degree of variation in the EROD response to dioxin-like compounds has been observed in hepatocytes cultured from individual avian embryos (Davis *et al.* 1997; Sanderson *et al.* 1998). For example, EROD EC50 values ranged from 0.1 to 2 nM TCDD (20-fold) in individual hepatocyte cultures prepared from 19 chickens, and two samples did not respond to concentrations of TCDD up to 100 nM (Sanderson *et al.* 1998)[3]. These data suggest that the lack of reproducibility in EROD EC50 values may be partially related to biological variation in sensitivity to CYP1A induction among individuals. For wild avian species, additional factors related to genetic and environmental heterogeneity would also be expected to contribute to variation in the CYP1A response to TCDD.

In the current study we used herring gulls as a model for studying factors that might contribute to variation in avian dioxin sensitivity estimates based on EROD EC50 values. Concentration-dependent effects of TCDD on CYP1A4 and CYP1A5 mRNA expression were assessed in hepatocyte cultures prepared from 55 individual herring gull embryos. Fertilized eggs were collected from three separate breeding colonies representing a gradient of exposure to environmental contaminants. Two of these colonies, Chantry and Middle Sister Islands, are in the Great Lakes. The third colony, Kent Island, belongs to a geographically and reproductively isolated Altantic population (Gross 1940; Yauk and Quinn 1999). This study design allowed us to assess the impact of population of origin, and embryonic exposure to contaminants on CYP1A mRNA expression and induction in cultured cells. A better understanding of sources of variation in the CYP1A response to TCDD in cultured cells may improve reproducibility and accuracy of dioxin sensitivity estimates based on this *in vitro* bioassay.

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[3] These EC50 values cannot be directly compared to the values in the preceding paragraph because a different vehicle was used (Sanderson et al. 1998).

5.3. Materials and Methods

5.3.1. Sample collection and preparation of cultured hepatocytes

Unincubated herring gull eggs were collected from one egg nests on Chantry Island (Lake Huron, ON, Canada), Middle Sister Island (Western Lake Erie, ON, Canada) and Kent Island (Bay of Fundy, NB, Canada) in the spring of 2003. Twenty-five eggs were collected from each colony. The two Great Lakes colonies, Chantry and Middle Sister Islands, were visited on April 23 and 24 of 2003. Eggs collected at Middle Sister Island were set on April 25, and eggs collected at Chantry Island were kept at room temperature and set three days later. The Atlantic colony, Kent Island, was visited on May 29, and eggs were set on May 31, 2003. Herring gull eggs were incubated for 26 days (until one day pre-hatch) at 37.5°C and 60% humidity in model CK 900 Professional incubators (Curfew, Essex, UK).

Herring gull embryos were sacrificed by decapitation on day 26 of development according to a protocol approved by the animal care committee at the National Wildlife Research Centre (NWRC, Ottawa, ON, Canada). Yolk sacs were placed in chemically cleaned glass jars and frozen at -20°C. The sex of each individual was determined by visual inspection of the gonads. Hepatocyte cultures were prepared from embryonic livers according to the method for pooled livers outlined in Chapter 2 with slight modifications to account for the smaller sample size. Each liver was processed individually, and care was taken to prevent cross-contamination of samples. The volume of collagenase (Sigma, St. Louis, MO, USA) used to digest each embryonic liver was approximately 7 mL, and other reagent volumes were adjusted accordingly.

Samples were processed on separate days depending on the colony of origin. The yield of hepatocytes varied between individuals, but was consistent between colonies. Hepatocytes were resuspended in complete medium according to pellet weight, at a ratio of 1 part cells to 32 parts medium. Cells were plated by adding 25 μL of this cell suspension, to wells containing 500 μL of medium. Each embryonic liver yielded enough cells to fill at least one full 48-well plate. The 48 wells of hepatocytes prepared from each individual were used as follows: 8 wells were used for mRNA analysis, 4 wells were used to assess cell viability, and 36 wells were used for EROD analysis. Due to technical problems EROD data

were obtained for only 3 individuals, and these data are to be presented elsewhere. Remaining hepatocytes isolated from Kent Island individuals were pooled and plated at 1x and 2x normal volumes. This pool was used to assess the effects of cell density on CYP1A mRNA expression. Hepatocytes were incubated for exactly 20 hours at 37°C and 5% CO_2 prior to dosing.

5.3.2. Treatment of hepatocytes

Hepatocytes were treated with TCDD dissolved in dimethyl sulfoxide (DMSO), as described in Chapter 2. For each hepatocyte culture preparation, 8 wells of hepatocytes were treated with solvent control or TCDD stocks of various concentrations. The resulting well concentrations ranged from 0.1 to 100 nM TCDD. Pooled hepatocytes at 1x and 2x normal cell density were treated with DMSO and 30 nM TCDD in duplicate. Cell culture plates were removed from the incubator after 24 hours of exposure to test compounds. The medium was removed from each well and cells were flash-frozen by burying the cell culture plate in powdered dry ice. Plates of cells were stored at -80°C until the samples were analyzed.

5.3.3. Cell viability

Cell viability was tested via the 5-carboxyfluorescein diacetate-acetoxymethyl ester (5-CFDA, AM, Molecular Probes, Invitrogen Canada, Burlington, ON) assay as described in Chapter 2.

5.3.4. RNA isolation and cDNA synthesis

Total RNA was isolated from cultured hepatocytes using the Qiagen RNeasy 96 kit with DNAse treatment as described in Chapter 3. RNA quantification and reverse transcription was carried out according to methods presented in Chapter 2.

5.3.5. Quantitative RT-PCR

Relative abundance of CYP1A4, CYP1A5, and beta-actin mRNA was measured by quantitative RT-PCR (Q-PCR) according to the method outlined in Chapter 3. Fold induction values in TCDD-treated samples relative to solvent control samples were calculated using the 2^{-ddCt} method of data analysis (Livak and Schmittgen 2001) with beta-actin as a normalizer as described in Chapter 2. Samples were analyzed in triplicate, and a no template control was included in each run. A relative standard curve constructed from a dilution series of concentrated cDNA was used to assess relative basal expression of CYP1A mRNA. For this analysis DMSO-treated samples for each individual were analyzed in triplicate alongside a triplicate standard curve.

5.3.6. Preparation of embryo yolk sac extracts

Halogenated aromatic hydrocarbons were extracted from embryo yolk sacs of 5 individuals from each colony. Individuals were chosen to represent a range of CYP1A mRNA expression and fold induction values. In particular, the most responsive individual from each colony was included. Frozen yolk sacs from selected individuals were thawed for 20 minutes at room temperature then homogenized. All glassware was chemically cleaned with acetone and hexane to prevent contamination of the sample. Extracts were prepared according to the method of Norstrom *et al.* (1986). Briefly, 5g of homogenized sample was dried with anhydrous sodium sulfate, rinsed with 1:1 dichlormethane/hexane, and cleaned up using gel permeation chromatography. Moisture and lipid content of each extract was determined. A 100 mg equivalent of the initial sample was reconstituted in hexane for chemical analysis of PCB residues. The remaining sample was dissolved in an appropriate volume of DMSO according to lipid content. The resulting concentration of each extract was 10 mg lipid/μL DMSO. Contaminants expected to be present in this extract would include dioxins, dibenzofurans, PCBs, chlorinated pesticides and other structurally related nonpolar molecules.

Extracts prepared in this manner were not completely free of lipids, and this prevented us from treating hepatocytes with two of the 15 extracts. For future experiments it

would be advisable to run the samples through a fluorosil column prior to reconstitution. This would remove excess lipids and make the extracts easier to work with.

5.3.7. Potency determination for embryo yolk sac extracts

Extract solutions for treating cells were prepared by serial dilution of concentrated embryo yolk sac extract in DMSO. Dilutions ranged from 1/10 to 1/100 000 of the original 10 mg lipid/μL DMSO extract. Chicken embryo hepatocyte cultures were prepared from a pool of 33 individuals in 48-well plates according to methods described in Chapter 2. Triplicate wells of hepatocytes were treated with 2.5 μL of extract dilution or 2.5 μL of TCDD per 500 μL of cell culture medium. Each of the 13 extracts and TCDD was tested on a separate 48-well plate. Measurement of EROD activity was carried out as previously described (Chapter 2).

5.3.8. Chemical analysis

Extracts prepared from 100 mg equivalents of yolk sac homogenate were analyzed for organochlorine compounds by high-resolution gas chromatography with an electron-capture detector as described in Norstrom *et al.* (1988). This work was done by the Laboratory Services Division of the National Wildlife Research Centre (Ottawa, ON, Canada). Concentrations of 69 *ortho* substituted PCB congeners are reported as ΣPCBs in μg/kg wet weight.

5.3.9. Data analysis

Extract potency values

The increasing phase of EROD data produced by treating chicken embryo hepatocyte cultures with yolk sac extracts were fit to 4 parameter logistic curves using Sigmaplot 8.0 software (Systat Software, Point Richmond, CA). The potency of each extract was evaluated

as the lowest concentration that produced a 2-fold increase in EROD activity compared to the DMSO dose. This value was then converted to TCDD equivalents (TEQs) by comparing it to the dose of TCDD that caused an equivalent response.

TCDD potency in individual hepatocyte cultures

It was not possible to estimate EC50 values for individual hepatocyte culture preparations because we were not able to consistently fit curves to the CYP1A4 and CYP1A5 mRNA fold induction data. Instead, we obtained a semi-quantitative estimate of the threshold potency (ECthr) of TCDD in each hepatocyte culture preparation. The ECthr was calculated by identifying the first dose of TCDD that caused a significant increase in CYP1A mRNA expression that was greater than 2-fold.

Composite dose-response curves

It was not possible to fit dose-response curves to CYP1A mRNA fold induction data measured in individual hepatocyte culture preparations, but the average response to TCDD at each colony could be modelled with a 4 parameter logistic equation. We also analyzed composite dose-response curves representing the mean fold induction values measured in hepatocyte cultures prepared from 1) all Great Lakes individuals, 2) all Great Lakes individuals excluding non-responders, and 3) all Great Lakes individuals excluding two particularly responsive samples. EC50 values were calculated from curve fit parameters using Sigmaplot 8.0 software (Systat Software, Point Richmond, CA).

Statistics

Colony differences in CYP1A mRNA basal expression and maximal fold induction were tested for significance using one-way ANOVA and Bonferonni tests. A p-value of less than 0.05 was considered statistically significant. Pearson correlation analysis was used to examine associations between ΣPCBs, TEQs, and CYP1A gene expression values. All statistical analyses were performed using Sigmastat 2.03 software (Systat Software).

5.4. Results

5.4.1. Cell viability and density

Cell viability was measured as intracellular hydrolysis of 5-CFDA, AM to a fluorescent derivative. Fluorescence values were consistent with volume of cells plated for all hepatocyte culture preparations, indicating that the cells were viable. Hepatocytes observed under a light microscope appeared normal and no blebbing was observed. No significant differences in CYP1A mRNA expression or induction were detected in herring gull embryo hepatocytes plated at 25 and 50 μL.

5.4.2. Basal CYP1A mRNA expression

Expression of CYP1A4 and CYP1A5 mRNA was detected in DMSO-treated hepatocyte cultures prepared from each individual used in this study. In 52/55 individuals CYP1A4 was the more abundant isoform. A significant relationship between basal CYP1A4 and CYP1A5 mRNA abundance was detected ($r^2 = 0.48, p < 0.001$). A large degree of variation in basal CYP1A mRNA expression was observed among individual hepatocyte culture preparations (Figure 5.1). Basal expression over the entire data set varied by 20-fold for CYP1A4, and 126-fold for CYP1A5. Variation was less within colonies, ranging from 5-fold to 26-fold (Table 5.1). Statistically significant differences in mean basal expression among colonies were detected. CYP1A4 mRNA expression in DMSO-treated hepatocytes was 2.5-fold higher in Kent Island samples than in samples collected at Chantry or Middle Sister islands ($p < 0.05$). CYP1A5 mRNA expression was 3.1- and 3.6-fold higher at Kent Island than at Chantry and Middle Sister Islands respectively. No significant differences in basal expression values were observed between the two Great Lakes colonies (Table 5.1).

5.4.3. Effects of TCDD on CYP1A mRNA expression

Exposure to TCDD significantly induced CYP1A mRNA expression in most, but not all of the hepatocyte cultures preparations. The proportion of non-responsive samples was

particularly high at Kent Island (Table 5.2). CYP1A5 was preferentially induced in 80% of the 36/55 hepatocyte culture preparations that were responsive to induction of both isoforms. Log transformed CYP1A4 and CYP1A5 fold induction values were correlated with an r^2 of 0.75 ($p < 0.001$).

Among responsive cell culture preparations, a large degree of variation in maximal fold induction values was observed (Figure 5.2). Coefficients of variation for the entire data set were 1.7 for CYP1A4 and 1.0 for CYP1A5 (Table 5.2). Cultured hepatocytes prepared from at least one individual per colony were identified as being particularly sensitive to CYP1A induction by TCDD. For example, median CYP1A4 fold induction values at Kent, Chantry and Middle Sister islands were 1.7, 3.8, and 1.9 respectively, while most elevated fold induction values were 14.7, 27.5, and 56.6 respectively (Table 5.2).

5.4.4. TCDD potency estimates

Concentration-dependent effects of TCDD on CYP1A mRNA expression were detected in many, but not all of the responsive hepatocyte culture preparations. Some dose-response curves did not reach a concentration-dependent maximal response, and others did not consistently increase with dose. As a semi-quantitative estimate of the potency of TCDD for CYP1A induction in individual hepatocyte culture preparations we determined ECthr values, as described in the methods section. The ECthr for individual cell culture preparations ranged from 0.1 to 100 nM TCDD for CYP1A4, and from 0.1 to 30 nM TCDD for CYP1A5. Median ECthr values for CYP1A4 were 1, 3, and 3 nM TCDD for Kent, Chantry, and Middle Sister Islands respectively. Median ECthrs for CYP1A5 were 1, 1, and 3 nM TCDD respectively (Figure 5.3).

Mean fold induction values for each colony were fit with 4 parameter logistic curves. The CYP1A4 EC50 values derived from these composite curve fits were 0.38, 0.64, and 1.1 nM TCDD for Kent, Chantry and Middle Sister Islands respectively. CYP1A5 EC50s were slightly higher, with values of 0.55, 0.86, and 1.7 nM TCDD respectively. For all Great Lakes individuals, CYP1A4 and CYP1A5 EC50 values were 0.76, and 1.21 nM TCDD respectively.

5.4.5. ΣPCB concentrations in embryonic yolk sacs

Concentrations of *ortho* substituted PCBs were determined in yolk sacs of five individuals from each colony. The rank order of average ΣPCB concentrations in yolk sacs from the three colonies was Kent < Chantry < Middle Sister. Mean ΣPCB concentrations were significantly higher in yolk sacs from Middle Sister Island individuals than at either of the two other colonies ($p < 0.05$) (Figure 5.4).

5.4.6. Potency of embryonic yolk sac extracts in chicken embryo hepatocytes

TEQs in embryonic yolk sacs were estimated based on the potency of yolk sac extracts for induction of EROD activity in chicken embryo hepatocytes. Of the 15 yolk sac extracts analyzed for ΣPCBs, 13 were tested in this manner. Of these, one extract failed to produce an EROD response in chicken embryo hepatocytes at any concentration tested. Yolk sac TEQs for the remaining 12 samples ranged from 0.007 to 1.6 ppb wet weight or 0.05 to 10.1 ppb lipid weight. Colony average TEQs were lowest at Kent Island, followed by Chantry Island, then Middle Sister Island. No significant differences were detected between colonies.

5.4.7. Correlations

Log transformed yolk sac ΣPCB concentrations were correlated with TEQs with an r^2 value of 0.565 ($p < 0.05$) (Figure 5.5). Other comparisons of ΣPCBs, TEQs, basal CYP1A expression, and CYP1A fold induction did not yield statistically significant correlations. No significant effects of sex on expression or induction of CYP1A mRNA were detected.

5.5. Discussion

5.5.1. Variation in CYP1A mRNA expression and induction

A large degree of variation in CYP1A mRNA expression was observed in hepatocyte cultures prepared from individual herring gull embryos. Basal expression among 55 hepatocyte cultures varied by 20-fold for CYP1A4 and by 125-fold for CYP1A5 (Table 5.1). This amount of variation is not unusual for cytochrome P450 enzymes, and has been documented extensively in humans (Lin and Lu 2001). For example, basal levels of CYP1A1 mRNA expression were found to vary by 200-fold in cultured lymphocytes prepared from 32 human donors (Lin *et al.* 2003). Concentration-dependent effects of TCDD on CYP1A mRNA expression were variable between hepatocyte culture preparations in terms of both the maximal response (Figure 5.2), and ECthr values (Figure 5.3). This type of variation has also been reported for induction of EROD activity in wild birds (Davis *et al.* 1997; Sanderson *et al.* 1998). In each of these studies, a proportion of hepatocyte preparations from each population did not induce CYP1A upon exposure to test compounds. Davis *et al.* (1997) report that 18% of hepatocyte cultures prepared from individual double-crested cormorants did not appreciably induce EROD activity in response to ß-naphthoflavone. Sanderson *et al.* (1998) observed that the proportion of non-responsive hepatocyte cultures prepared from individual embryos of 4 avian species ranged from 8 to 17%. The overall percentages of non-responsive hepatocyte cultures in our study were somewhat higher at 41% for CYP1A4 and 20% for CYP1A5.

The studies of Sanderson and Davis, and numerous others in humans suggest that CYP1A expression and induction can vary extensively between individuals. The degree of variation that we observed in hepatocyte cultures prepared from individual herring gull embryos was large, but not unusually so. In the following sections, we describe potential sources of variation in the CYP1A biomarker response in our samples. We then speculate on how the observed variation might affect use of CYP1A mRNA induction as a biomarker for susceptibility to dioxin-like environmental contaminants in avian species.

5.5.2. Sources of variation in basal and TCDD-induced CYP1A mRNA expression

Experimental sources of variation

Some of the observed variation in CYP1A mRNA expression and induction was likely due to experimental error inherent in the study design. We have previously shown that replicate plates from the same hepatocyte culture preparation produce similar results, as do replicate measurements of the same sample (Chapter 2). Most of the experimental variation would therefore be expected to originate from differences between hepatocyte culture preparations. Several lines of evidence suggest that while experimental error certainly contributes to variation in CYP1A mRNA expression between individual hepatocyte preparations, it is unlikely to be its main cause. Davis et al observed a good correlation between EROD activity measured in whole liver tissue and DMSO-treated hepatocytes cultured from the same individual (Davis *et al.* 1997). Sanderson *et al.* (1998) observed that variation in EROD EC50 was considerably lower among hepatocyte cultures prepared from pools of two or three livers than among hepatocyte cultures prepared from individuals. The authors also noted that non-responsive hepatocyte cultures had only been observed in samples prepared from individuals, and never from pools. Nevertheless, because constraints in experimental design prevent replicate analyses, it is important to note that the observed variation is at least partially related to technical factors rather than to biological differences between individuals.

Environmental sources of variation

Several authors have suggested that maternal deposition of environmental contaminants in eggs might alter CYP1A endpoints in hepatocytes cultured from exposed embryos (Davis *et al.* 1997; Lorenzen *et al.* 1997b; Head *et al.* 2006). Herring gull egg collection sites were chosen to represent a gradient of embryonic contaminant exposure (Kent < Chantry < Middle Sister), but no corresponding trends in CYP1A mRNA expression or induction were observed. Yolk sac ΣPCB concentrations were measured for a subset of individuals to verify if embryonic exposure to contaminants was correlated with *in vitro*

CYP1A endpoints on an individual basis. No relationship was observed between ΣPCB concentrations and basal or maximally induced CYP1A mRNA expression. Herring gulls are not particularly sensitive to induction of EROD activity by PCBs (Kennedy *et al.* 1996a), and are likely to be contaminated with other more potent dioxin-like compounds. To estimate the potency of the entire mixture of contaminants, we also measured yolk sac TEQs. This was done by assessing the EROD inducing potency of yolk sac extracts in chicken embryo hepatocytes, and comparing these values to the EROD inducing potency of TCDD. Yolk sac TEQs derived in this manner were correlated with ΣPCB concentrations with an r^2 of 0.565 ($p = 0.002$) (Figure 5.5). No relationship between yolk sac TEQs and CYP1A mRNA expression or induction was observed.

The absence of correlation between CYP1A mRNA abundance in cultured hepatocytes and yolk sac ΣPCBs or TEQs may be related to the relatively low concentrations of CYP1A inducers found in herring gull eggs. The findings presented in Chapter 4 indicate that *in ovo* exposure to dioxin-like compounds at current environmental levels would be expected to have a minimal impact on CYP1A mRNA expression in cultured cells. Injection of PCB 126 into the air cell of chicken eggs at an environmentally relevant concentration of 0.4 μg/kg minimally increased CYP1A4 mRNA expression in cultured hepatocytes, and had no effect on CYP1A5 mRNA expression or maximal fold induction of either isoform. At 0.8 μg/kg, increases in expression of CYP1A4 and CYP1A5 mRNA were observed, and the magnitude of the response to TCDD was significantly increased (Chapter 4). Using this result, and data from the literature, we estimated the lowest concentration of embryonic liver TEQs that would be expected to alter CYP1A mRNA endpoints in cultured herring gull embryo hepatocytes. This threshold concentration was calculated using an avian toxic equivalency factor (TEF) of 0.1 for PCB 126 (Van den Berg *et al.* 1998), a factor of 6.4 to describes the relationship between injected PCB 126 and lipid weight based liver concentrations of PCB 126 in 19-day chick embryos (Katynski 2001), and a species sensitivity factor of 20 to account for differences in CYP1A inducibility in herring gull and chicken hepatocyte cultures (Sanderson *et al.* 1998). The 20-fold correction factor refers to the difference in EC50 values between the most sensitive herring gull, and an average chicken.

Based on this calculation, we predict that the lowest embryonic exposure to dioxin-like compounds that would be expected to alter CYP1A endpoints in herring gull embryo hepatocyte cultures would be between 5.1 and 10.2 μg/kg TEQ lipid weight. These values correspond to the NOEL (no observable effect level - 0.4 μg/kg) and LOEL (lowest observable effect level - 0.8 μg/kg) for injection of PCB 126 into chicken eggs. We did not measure liver TEQs in the current study, but we did obtain estimates of yolk sac TEQs for a subset of individuals. An assumption of equivalence between liver and yolk sac lipid weight TEQs was made based on the observation that dioxin-like compounds are expected to partition equally between embryo and yolk sac lipids (Drouillard *et al.* 2003).

TEQ concentrations in yolk sacs from only one of the 12 individuals for which we determined extract potencies were above the 5.1 μg/kg TEQ lipid weight NOEL (Figure 5.6). This individual, labelled 'M13', was collected from Middle Sister Island. Interestingly, of all 55 individuals used in this study, hepatocytes cultured from M13 were the most responsive to CYP1A mRNA fold induction by TCDD. Basal CYP1A4 and CYP1A5 mRNA expression values were also high, at 1.4-, and 16.9-fold above median values for the Middle Sister Island colony. We conclude that although CYP1A endpoints in hepatocytes cultured from the majority of individuals in this study were probably unaffected by maternal deposition of contaminants, the particularly high CYP1A fold induction value in M13 hepatocyte cultures may have been associated with elevated embryonic TEQs. We previously hypothesized that increased sensitivity to induction of CYP1A isoforms in hepatocytes cultured from embryos exposed to inducers *in ovo* may be related to embryonic upregulation of the AHR (Chapter 4). A comparison of AHR mRNA abundance in hepatocytes cultured from individual M13 and the other individuals used in this study would be of interest.

Genetic sources of variation

We also considered the contribution of genetic factors to the observed variation in CYP1A mRNA expression and induction. Without genetic sequence or relatedness data for the animals used in this study, it was not possible to assess the influence of genetics on individual variation in our results. We were able to assess which aspects of the observed variation might result from genetic differences at a population level by comparing colony

averages within and among populations. Individuals collected from Chantry and Middle Sister Islands belong to a large, panmictic Great Lakes population, and would not be expected to be genetically distinct based on colony of origin. Kent Island herring gulls belong to a reproductively isolated Atlantic population (Weseloh 1984; Chen *et al.* 2001). While we can infer that differences in CYP1A expression or induction observed between the two populations may be related to genetics, considerations such as timing of breeding, diet, and other factors associated with a marine *vs* fresh water environment could also play a role.

It has been speculated that genetic resistance to dioxin-like compounds may have evolved in Great Lakes herring gulls as a result of multi-generational exposure to high levels of contaminants (Kennedy *et al.* 2003a). Previous examples of innate or acquired genetic resistance to dioxin-like compounds have been observed in rodent models and in natural fish populations, but not yet in birds. In many reported cases, dioxin resistance affects CYP1A induction as well as toxicity (Hahn 1998). We hypothesized that acquired resistance to dioxin-like compounds would result in reduced sensitivity to CYP1A induction in Great Lakes herring gulls compared to Kent Island gulls. This is not what was observed. No significant differences in sensitivity to CYP1A induction were detected between colonies in terms of ECthr, or maximal fold induction.

Although we did not find evidence for differential sensitivity to dioxin-like compounds in Great Lakes and Atlantic herring gulls, other differences in CYP1A mRNA expression were apparent between the two populations. Hepatocyte cultures prepared from Kent Island individuals expressed significantly higher basal levels of CYP1A4 and CYP1A5 mRNA than hepatocyte cultures prepared from Chantry or Middle Sister Islands (Table 5.1). Additionally, a larger proportion of Kent Island individuals were not responsive to CYP1A induction at any dose of TCDD tested. We initially speculated that this may have been related to elevated basal expression. This explanation seems unlikely because basal expression was not predictive of fold induction on an individual basis.

Our findings suggest that there may be genetic differences between the Great Lakes and Atlantic populations that result in differential expression of CYP1A isoforms. It is difficult to interpret this result in the context of acquired genetic resistance since it is not clear if basal CYP1A mRNA expression in cultured cells is related to sensitivity to the toxic effects of dioxin-like compounds *in vivo*. More research is required to confirm the existence

of a population level difference in CYP1A expression, and to investigate how this difference might affect sensitivity estimates derived from pooled samples. The question of whether Great Lakes herring gulls are genetically resistant to dioxin-like environmental contaminants might be better addressed by measuring population specific LD50 values for TCDD via egg injection experiments.

5.5.3. Implications for sensitivity estimates

Do EC50 values measured in pooled samples represent the population average?

Inducibility of the EROD response to TCDD in cultured hepatocytes is most easily assessed in pooled samples because individual embryonic livers are small and difficult to work with. The large degree of variation observed among individual samples in this study and others (Davis *et al.* 1997; Sanderson *et al.* 1998), implies that accurate interpretation of CYP1A biomarker data derived from pooled samples may depend on an understanding of how individual responses contribute to the average.

We approached this question by comparing (1) individual CYP1A4 mRNA expression data, (2) average CY1A4 mRNA expression data for all Great Lakes individuals presented as a composite dose-response curve, and (3) previously published EROD data measured in pooled samples. In Chapter 2, we demonstrated that concentration-dependent effects of TCDD on CYP1A mRNA expression and EROD activity are very similar. Dose-response curves measured in chicken embryo hepatocytes are nearly superimposable, with statistically identical EC50 and slope parameters. Measurement of different endpoints in the CYP1A4 induction pathway would therefore not be expected to produce different potency values.

A composite 4 parameter logistic curve was fit to average CYP1A4 mRNA fold induction values assessed in hepatocyte cultures prepared from the 38 Great Lakes herring gull embryos used in this study. This composite dose-response curve was compared to a previously published EROD dose-response curve measured in hepatocyte cultures prepared from a pool of 20 Great Lakes herring gull embryo livers (Figure 5.7). The individuals used in the EROD study were collected from nests in Terrace Bay, Lake Superior in 1992 (Kennedy *et al.* 1996a). Concentration-dependent effects of TCDD on EROD activity in pooled hepatocyte cultures, and average CYP1A4 mRNA expression measured in individual

hepatocyte cultures, were nearly identical (Figure 5.7). EROD activity decreased at concentrations of TCDD above 30 nM TCDD, but this phenomenon has previously been attributed to competitive inhibition of the CYP1A4 enzyme (Petrulis and Bunce 1999).

At first glance, the consistency between EROD data from 1992 and average CYP1A4 mRNA expression data measured in individual samples seems to suggest that EC50 values measured in pooled samples are representative of the population average. On closer inspection, the EC50 value calculated from the composite curve seems to overestimate the population average because it is strongly influenced by a few particularly responsive hepatocyte culture preparations. This observation is illustrated in Figure 5.8. The EC50 values derived from composite Great Lakes dose-response curves including and excluding non-responsive hepatocyte culture preparations are nearly identical. This is revealing since nearly 1/3 of Great Lakes samples were not responsive to TCDD at any dose tested. Furthermore, removing two particularly responsive hepatocyte culture preparations from the overall Great Lakes composite curve increased the EC50 by approximately 2-fold. As previously discussed, the response measured in one of these two individuals, M13, may have been related to exposure to high embryonic TEQs. Unfortunately we were not able to obtain reliable potency estimates from all of the individual hepatocyte preparations, but we speculate that the average of EC50s would be greater than the EC50 of the average. This hypothesis could easily be tested in hepatocytes cultured from more readily available chicken embryos. The correspondence between dose-response curves fit to our data and data from pooled hepatocytes, suggests that the same phenomenon may occur in pools. If this is true, variation in EROD EC50 values between experiments may be related to the strong effect of particularly responsive samples on the average potency value.

Are in vitro EC50 values predictive of in vivo toxicity in individuals?

We used our data to assess whether potency values derived from individual hepatocyte culture preparations are likely to be representative of sensitivity to dioxin-like compounds in each individual, and therefore of the range of *in vivo* sensitivities to dioxin-like compounds in the population. An estimate of the relative potency of TCDD in each hepatocyte culture preparation was obtained by identifying the first dose that significantly

induced CYP1A mRNA expression by at least 2-fold (ECthr). A large degree of variation was detected in these potency values (Figure 5.3). For example, the lowest dose to significantly induce CYP1A4 mRNA expression was 0.1 nM TCDD, and more than 40% of the individuals tested did not respond to any dose up to 100 nM TCDD. If these values are representative of *in ovo* sensitivities, we could expect the lethal dose of TCDD to vary by more than 1000-fold among individuals. This far exceeds the 10-fold factor that is generally applied to account for interindividual variation, and seems improbably large. A review of egg injection studies suggests that the range encompassing the dose that kills a single individual, and the dose that kills all but one individual is consistently less than 10-fold (Nosek *et al.* 1993; Powell *et al.* 1996a, 1996b). These data indicate that the variation observed in this study is unlikely to be representative of the *in vivo* variability in sensitivity to dioxin-like compounds among individuals.

Considerations for experimental design of CYP1A bioassays

A large degree of variation in CYP1A mRNA expression and induction was observed in hepatocyte cultures prepared from 55 individual herring gull embryos. Experimental, environmental, and genetic factors were all suspected to contribute to the observed variation. To minimize effects of these factors, and to improve reproducibility of species sensitivity estimates based on CYP1A EC50 values measured in pooled samples, the following observations should be considered prior to the design of biomarker experiments.

1) Egg contamination of less than 5.1 µg/kg TEQs lipid weight is unlikely to affect CYP1A inducibility in cultured cells. Among 15 colonies monitored annually by the Canadian Wildlife Service, contaminant concentrations measured in pooled egg homogenate ranged from 1.4 to 4.4 µg/kg TEQs lipid weight at 14 colonies, and were 10.7 µg/kg TEQs lipid weight at the 15th colony (Jermyn-Gee *et al.* 2005).[4] These figures suggest that TEQs in eggs collected from clean sites would surpass the 5.1 µg/kg NOEL only in the most contaminated individuals. Embryonic exposure to contaminants is nevertheless an important

[4] TEQs were calculated from reported concentrations of dioxin, dibenzofuran and PCB congeners (Jermyn-Gee *et al.* 2005) using avian TEFs (Van den Berg *et al.* 1998). Chemical concentrations reported on a wet weight basis were converted to lipid weight using percent lipid values for each colony (Jermyn-Gee *et al.* 2005). The data presented here are for 2001.

consideration, because we have shown that hepatocytes cultured from individuals with TEQs above the threshold value are likely to have a large effect on the average EC50 value.

2) Significant differences in basal CYP1A mRNA expression were detected between herring gull colonies belonging to the Great Lakes and Atlantic populations. While it is difficult to interpret the toxicological significance of this finding, it does suggest that CYP1A biomarker responses in the two populations may not be equivalent. This indicates that wherever possible, sensitivity estimates for populations in contaminated environments should be based on data measured in samples collected at a clean site within the same population.

3) Samples prepared from at least one individual per colony were particularly responsive to CYP1A induction by TCDD, and these responses appeared to have a large influence on the composite EC50 value. Further research is needed to determine the optimal number of individuals to be included in a pool, but as a general guideline, we speculate that reproducible EC50 estimates may require hepatocyte cultures from pools comprised of no less than 20 individuals.

5.5.4. Conclusions

Our findings suggest that the CYP1A inducing potency of TCDD assessed in pooled embryo hepatocytes may be strongly influenced by interindividual variation. In particular, hepatocyte cultures prepared from certain individuals are exceptionally sensitive to CYP1A induction by TCDD, and these outliers have a dramatic effect on the average EC50 value. An understanding of the factors that contribute to variation in CYP1A inducibility in cultured hepatocytes may improve species sensitivity estimates based on this biomarker response.

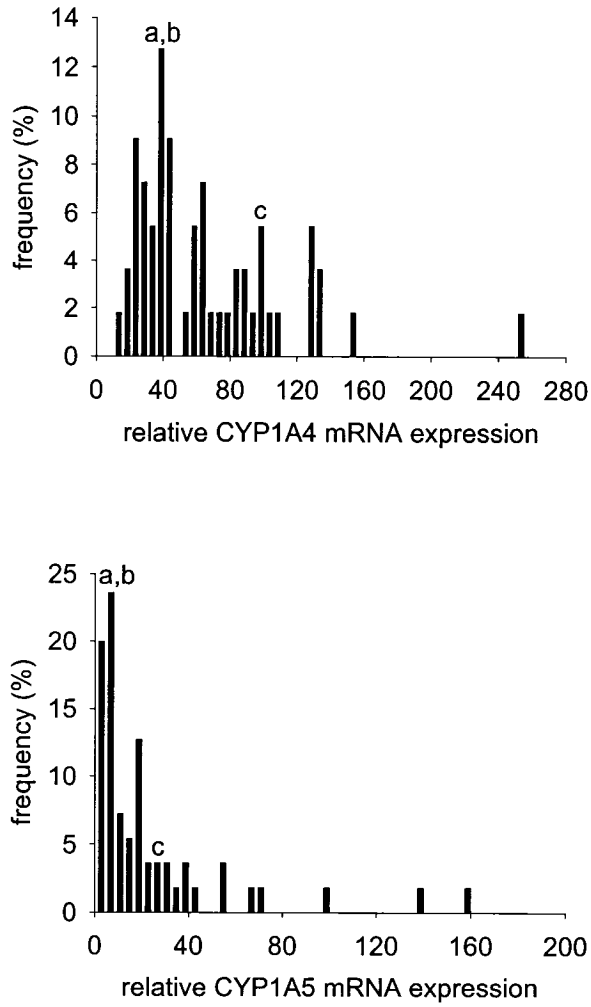

Figure 5.1. Frequency distribution of basal CYP1A mRNA expression in hepatocyte cultures prepared from 55 individual herring gull embryos. Letters mark the bin corresponding to the median response for embryos collected at Middle Sister Island (a), Chantry Island (b), and Kent Island (c). Colony comparisons of CYP1A mRNA basal expression are presented in Table 5.1.

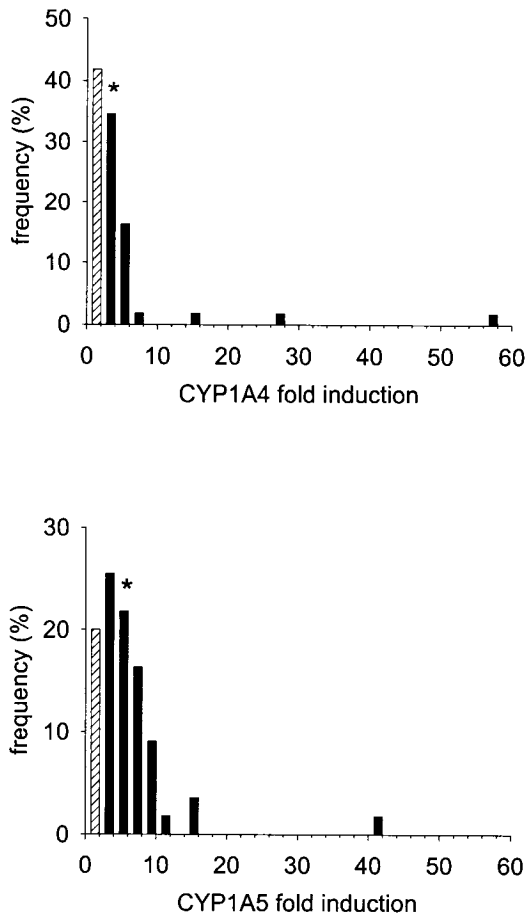

Figure 5.2. Frequency distribution of maximal CYP1A mRNA fold induction values in TCDD-treated hepatocyte cultures prepared from 55 individual herring gull embryos. The hatched bar represents the percentage of hepatocyte cultures that were not responsive to treatment. Included in this group are hepatocyte culture preparations for which the maximal CYP1A fold induction value was not significantly different from basal levels, and cultures that responded with less than 2-fold CYP1A induction, regardless of statistical significance. Asterisks mark the bin that corresponds to the median response for all 55 cell culture preparations, including non-responders. Colony comparisons of CYP1A mRNA induction are presented in Table 5.2.

Table 5.1. Colony comparison of relative basal CYP1A mRNA expression in hepatocyte cultures prepared from 55 individual herring gull embryos. Individuals were collected from Kent Island (KI), Chantry Island (CI), or Middle Sister Island (MSI). Hepatocyte cultures prepared from individuals collected at the two Great Lakes sites (CI and MSI) expressed significantly lower levels of CYP1A4 and CYP1A5 mRNA than the Atlantic site (KI) ($p < 0.05$).

		CYP1A4 relative basal expression				CYP1A5 relative basal expression			
Colony	n[a]	range[b]	median	mean[c]	CV[d]	range[b]	median	mean[c]	CV[d]
KI	17	6.4	98.9	108.3 ± 48.3*	0.4	12.7	26.1	44.1 ± 42.2*	1.0
CI	18	5.0	39.4	44.1 ± 23.0**	0.5	31.2	6.8	14.2 ± 15.8**	1.1
MSI	20	8.6	38.4	43.9 ± 24.3**	0.6	78.2	5.8	12.3 ± 22.3**	1.8
Total	55	19.9	54.0	63.9 ± 44.3	0.7	125.5	11.0	22.7 ± 31.5	1.4

[a]Sample size (n)
[b]'range' refers to the fold difference between hepatocyte culture preparations with the most and least abundant level of basal CYP1A mRNA expression
[c]mean relative basal expression ± standard deviation. Asterisks indicate a statistically significant difference between Great Lakes and Atlantic levels of basal CYP1A mRNA expression ($p < 0.05$)
[d]coefficient of variation (standard deviation/mean)

Table 5.2. Colony comparison of maximal CYP1A mRNA fold induction in TCDD-treated hepatocyte cultures prepared from 55 individual herring gull embryos. Individuals were collected from Kent Island (KI), Chantry Island (CI), or Middle Sister Island (MSI). Fold induction refers to the relative increase in mRNA expression between the maximal response and the basal response. No significant differences in average fold induction values were detected between colonies ($p < 0.05$).

		CYP1A4 fold induction				CYP1A5 fold induction			
Colony	n[a]	NR[b]	median[c]	mean[d]	CV[e]	NR[b]	median[c]	mean[d]	CV[e]
KI	17	59	1.7 (14.7)	3.0 ± 3.3	1.0	29	2.7 (9.2)	3.5 ± 2.2	0.4
CI	18	6	3.8 (27.5)	5.2 ± 5.7	0.7	6	6.0 (15.6)	6.9 ± 3.5	0.5
MSI	20	22	1.9 (56.6)	5.0 ± 12.2	2.2	6	4.1 (40.1)	6.4 ± 8.3	1.5
Total	55	41	2.6	4.5 ± 8.2	1.7	20	4.7	5.7 ± 5.6	1.0

[a]sample size (n)
[b]percentage of non-responsive hepatocyte culture preparations
[c]the median maximal response followed by the largest maximal response at each colony in parentheses
[d]mean fold induction ± standard deviation
[e]coefficient of variation (standard deviation/mean)

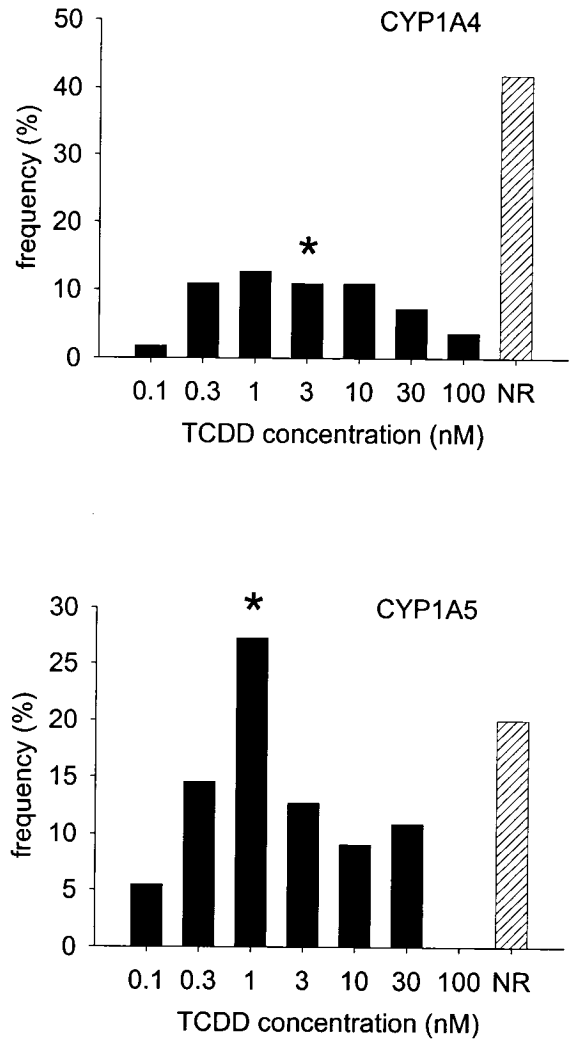

Figure 5.3. Frequency distribution of the CYP1A inducing potency of TCDD in hepatocyte cultures prepared from 55 individual herring gull embryos. Threshold potency (ECthr) is reported as the first concentration of TCDD to cause a statistically significant increase in CYP1A expression that is larger than 2-fold. Non-responders (NR, represented by the hatched bar) were defined as hepatocyte culture preparations for which CYP1A mRNA expression was not significantly induced over basal levels, or was not induced by more than 2-fold at any TCDD dose tested. Asterisks mark the bin corresponding to median ECthr for all individuals tested, excluding non-responders. The median CYP1A4 inducing potencies for Kent, Chantry, and Middle Sister Islands were 1, 3, and 3 nM TCDD respectively. The median CYP1A5 inducing potencies were 1, 1, and 3 nM TCDD respectively.

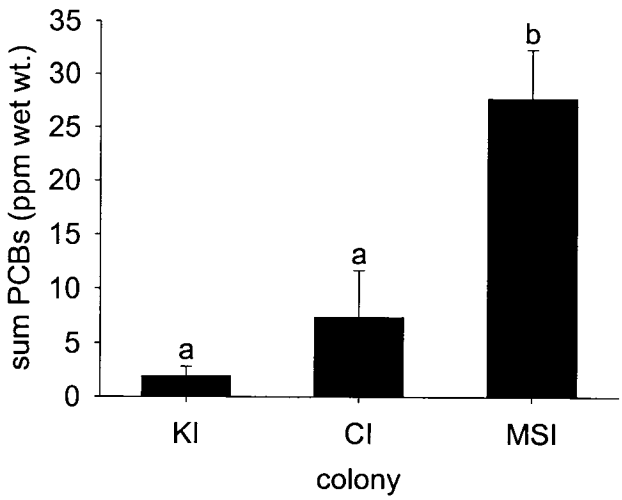

Figure 5.4. Colony comparison of sum PCB concentrations measured in herring gull embryonic yolk sacs. Eggs were collected from at Kent (KI), Chantry (CI), and Middle Sister Islands (MSI). Bars represent the average sum PCB concentration (μg/g wet weight) measured in 5 individuals for each colony ± standard error. Letters indicate statistically significant differences in sum PCB concentrations between colonies ($p < 0.05$).

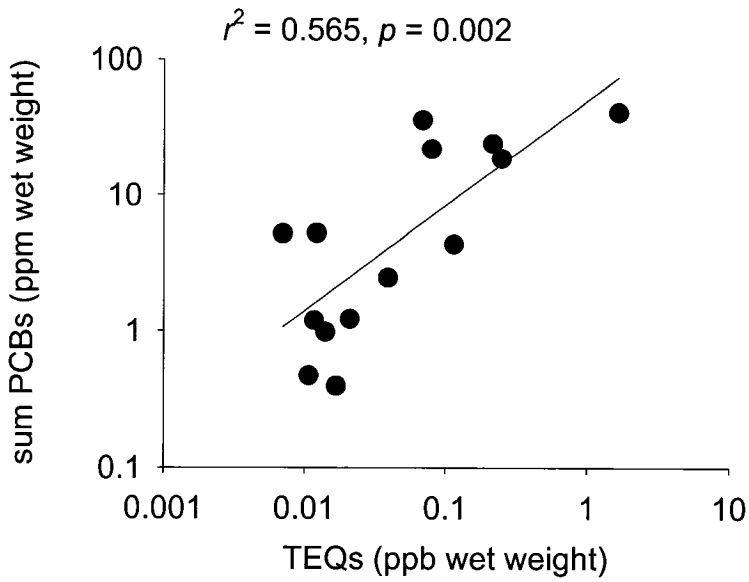

Figure 5.5. Relationship between herring gull yolk sac TEQs, and sum PCB concentrations. Various PCB congeners were measured in embryonic yolk sacs of a subset of 13 individuals (4 or 5 from each colony), and in triplicate samples of a pool of whole egg homogenate. The potency of herring gull yolk sac, and egg homogenate extracts was estimated based on their ability to induce EROD activity in chicken embryo hepatocytes. TEQs were derived from these potency values as described in the methods section. Analytically determined PCB concentrations were correlated with extract TEQs with an r^2 value of 0.565 ($p < 0.05$).

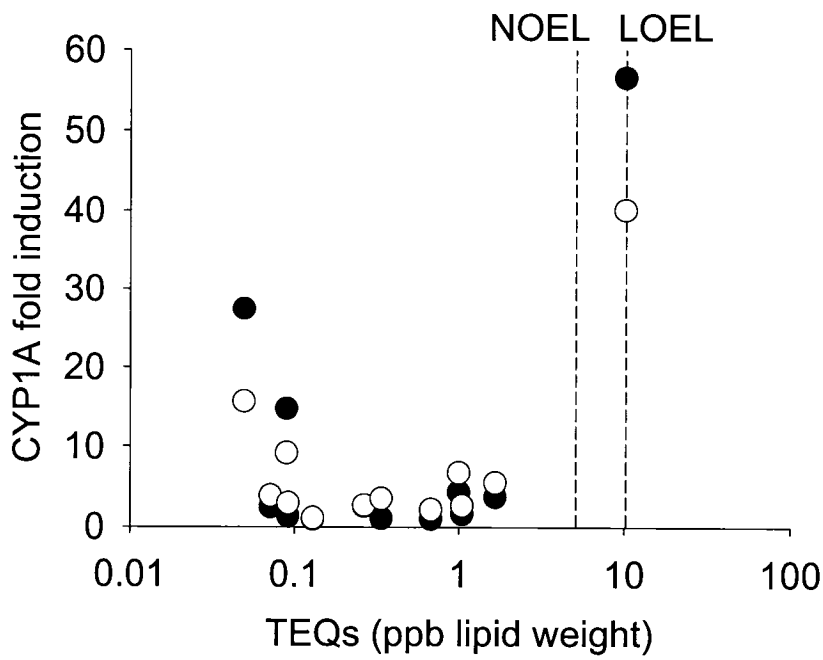

Figure 5.6. Threshold levels for effects of embryonic TEQs on the inducibility of CYP1A mRNA in avian embryo hepatocytes. The potency of herring gull yolk sac extracts (assessed as TEQs), is plotted against maximal CYP1A4 fold induction (closed circles), and CYP1A5 fold induction (open circles) values for a subset of 12 individuals. Fold induction values were assessed in TCDD treated embryo hepatocytes cultures prepared from each individual. Vertical lines represent the no observable effect level (NOEL) and lowest observable effect level (LOEL). These refer to the concentration of embryonic TEQs that would be expected to alter CYP1A fold induction values in cultured hepatocytes (see text for details).

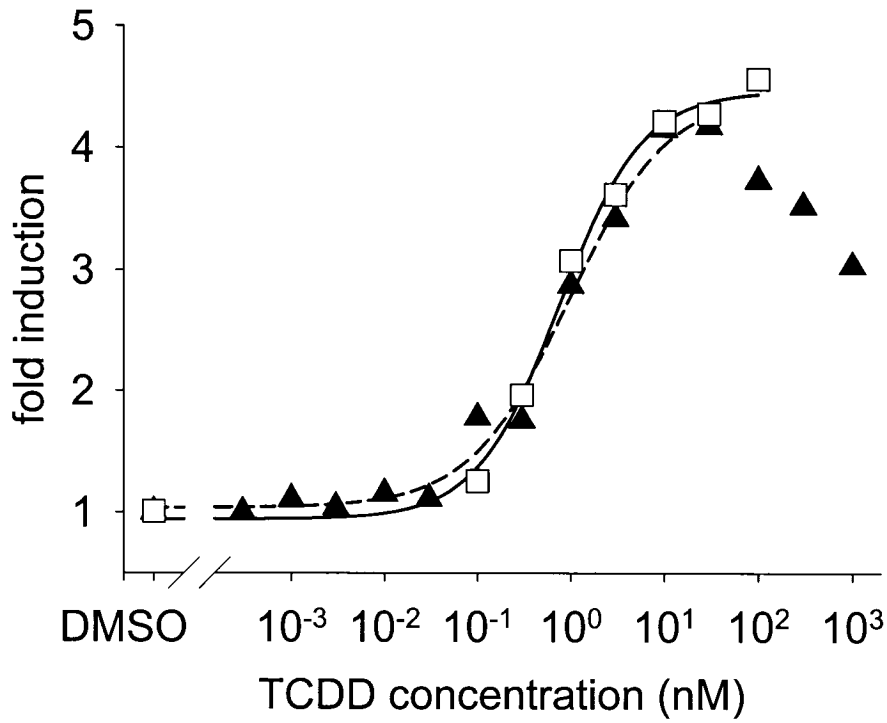

Figure 5.7. Concentration-dependent effects of TCDD on CYP1A4 mRNA induction and EROD activity in herring gull embryo hepatocytes. Open squares represent the mean fold increase in CYP1A4 mRNA expression measured in 38 hepatocyte cultures prepared from Great Lakes herring gull embryos. Error bars are not shown for these data because the degree of variation between individuals was very large (Table 5.1 and 5.2). Black triangles represent the mean fold increase in CYP1A4 enzymatic activity (EROD activity) in TCDD-treated herring gull embryo hepatocytes prepared from a pool of 20 Great Lakes individuals. These data were previously published as EROD activity in pmol/min/mg protein Kennedy *et al.* (1996a).

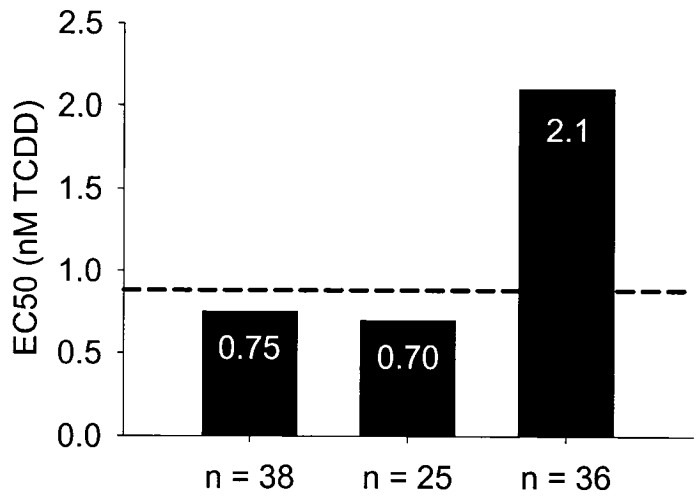

Figure 5.8. Effect of non-responders and outliers on CYP1A4 EC50 values. Composite dose response curves were created for the average CYP1A4 response to TCDD measured in hepatocytes cultures prepared from all 38 Great lakes individuals (n = 38), 25 Great Lakes individuals (n = 25, excludes non-responders), and 36 Great Lakes individuals (n = 36, excludes the two most responsive samples). Bars represent the dose of TCDD causing a half-maximal CYP1A4 mRNA response (EC50) for each composite dose-response curve and values are presented on each bar. The dashed line indicates a previously published EC50 value derived from inducibility of the CYP1A4 enzyme, measured as EROD activity in hepatocytes cultures prepared from a pool of 20 Great lakes herring gull embryo livers (0.88 nM TCDD) (Kennedy *et al.* (1996a).

Chapter 6

Characterization of AHR genotype in birds; a potential biomarker for species sensitivity to dioxin-like compounds

6.1. Abstract

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related dioxin-like compounds are toxic to virtually all vertebrate species, but significant strain and species differences in sensitivity exist. Among avian species, herring gulls, common terns, American kestrels, and eastern bluebirds are between 25 and 330 times less sensitive to the embryotoxic effects of dioxin-like compounds than chickens, the typical avian model. Recent work suggests that two amino acid residues in the ligand binding domain (LBD) of the chicken and common tern aryl hydrocarbon receptor (AHR) contribute to differential sensitivity between these species. Residues Ile-324, and Ser-380 in the high affinity chicken receptor, are replaced by Val and Ala in the low affinity tern receptor. To further our understanding of mechanisms underlying variation in dioxin sensitivity, we sequenced the AHR LBD in 17 avian species from diverse orders, and compared the resulting genotypes to previously published toxicity data. Chickens, unique in having a Ser residue at position 380, were the only species classified as 'very sensitive' according to our review of the literature. Three species of galliforms with a chicken-like Ile-324, and a tern-like Ala-380 were predicted to be 'moderately sensitive'. This prediction is consistent with toxicity data for ring-necked pheasant which also has an Ile-324, Ala-380 genotype. Fish-eating species from several different orders were classified as 'insensitive' to dioxin-like compounds based on toxicity data. Each of these had tern-like Val-324 and Ala-380 substitutions. Interestingly, Japanese quail, the only galliform with tern-like residues at both positions, was also classified as 'insensitive', suggesting that AHR LBD genotype may be predictive of dioxin toxicity both within and between avian orders.

6.2. Introduction

Identifying species that are at risk from exposure to environmental contaminants is a central goal of wildlife toxicology. This task is made difficult by the fact that sensitivities to toxicants can vary widely among species. The molecular basis for variation in sensitivity to dioxin-like compounds is likely to involve multiple processes and to differ among vertebrate classes. Nevertheless, activation of the aryl hydrocarbon receptor (AHR) emerges as a common mechanism underlying differential sensitivity in several rodent models (Poland *et al.* 1994; Ema *et al.* 1994; Pohjanvirta and Tuomisto 1994; Pohjanvirta *et al.* 1998; Tuomisto *et al.* 1999).

The molecular basis for differential sensitivity to dioxin-like compounds in avian species is not well understood, but a recent study suggests that, as in mammalian models, the AHR plays an important role. Karchner *et al.* (2006) identified two amino acid differences in the ligand binding domain (LBD) of chicken and tern AHR that confer a 7-fold higher 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) binding affinity to the chicken receptor. Residues Ile-324, and Ser-380 in the high affinity chicken receptor, are replaced by Val and Ala in the low affinity tern receptor. Using site-directed mutagenesis, the authors demonstrate that these substitutions are responsible for reduced transactivation by the tern AHR expressed in COS-7 cells (Karchner *et al.* 2006). This finding may partially explain the 80-fold differences in cytochrome P4501A (CYP1A) inducibility in cultured hepatocytes (Lorenzen *et al.* 1997b), and the 260-fold difference in embryolethality of dioxin-like compounds (Hoffman *et al.* 1998) in these two species.

We have expanded upon this work by sequencing the AHR LBD in diverse avian species, and comparing these data to species sensitivity estimates derived from *in vitro* and *in vivo* dosing studies. Combined with the findings of Karchner *et al.* (2006), our results suggest that AHR LBD genotype may be useful as a genetic marker for sensitivity to dioxin-like compounds in avian species.

6.3. Materials and Methods

6.3.1. Sample collection

Liver samples from 17 avian species were obtained for this study. Frozen tissue from 14 species was retrieved from the specimen bank at the National Wildlife Research Centre (NWRC, Environment Canada, Ottawa, ON). These species were; America kestrel (*Falco sparverius*), bald eagle (*Haliaeetus leucocephalus*), common eider (*Somateria mollissima*), double-crested cormorant (*Phalacrocorax auritus*), great blue heron (*Ardea herodias*), osprey (*Pandion, halieatus*), ring-billed gull (*Larus delawarensis*), thick-billed murre (*Uria Iomvia*), willow ptarmigan (*Lagopus logopus*), wood duck (*Aix sponsa*), rock ptarmigan (*Lagopus muta*), ruffed grouse (*Bonasa umbellus*), japanese quail (*Coturnix japonica*), and tree swallow (*Tachycineta bicolor*). At least 30 mg of tissue was obtained from three individuals of each species. Fertilized ring-necked pheasant (*Phasianus colchicus*) and wild turkey (*Meleagris gallopavo*) eggs were obtained from a local breeder (Garth Greene, Almonte, ON) and sacrificed at one day pre-hatch. Livers were removed and immediately placed in RNA-later stabilization buffer (Ambion, Austin, TX, USA). Herring gull (*Larus argentatus*) samples were obtained from individual embryo hepatocyte cultures prepared in the experiment presented in Chapter 5. Samples from at least three individuals were obtained for each species, except for wild turkey where two samples were obtained.

6.3.2. Sample treatment prior to storage

The samples obtained from the specimen bank were collected by various Canadian Wildlife Service scientists over several decades, and archived as studies ended. Archived samples were stored at –40°C, or in liquid nitrogen, but in many cases, previous handling was variable or unknown.

6.3.3. Cloning and sequencing

A region of mRNA corresponding to the ligand binding domain of the chicken and common tern AHR was cloned from three individuals of each species. Approximately 30 mg of whole liver tissue from each individual was homogenized using QIAshredder mini columns (Qiagen, Mississauga, ON). Total RNA was isolated from homogenized samples with the RNeasy Mini kit (Qiagen). For herring gull samples, total RNA was isolated from cultured hepatocytes with the RNeasy 96 kit (Qiagen) as previously described (Chapter 5). RNA was quantified by spectrophotometric analysis at 260 nm and 3 μg of RNA was reverse transcribed to cDNA using Superscript II, and random hexamer primers (Invitrogen Canada, Burlington, ON). The cDNA samples were PCR amplified with forward primer 5'-CCAGACCAACTTCCTCCAGA-3' and reverse primer 5'-CACCAGTGGCAAACATGAAG-3', for 35 cycles using 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds. For tree swallow, the same forward primer was used and the reverse primer was 5'-GTAGGAGGGAGTTTGGATCC-3'. PCR products run on an agarose gel resulted in a single band of approximately 600 bp for all species. This band was excised, purified with the QIAquick gel extraction kit (Qiagen) then cloned using the TOPO TA Cloning kit (Invitrogen). Plasmid DNA was purified with the QIAprep spin miniprep kit (Qiagen) and sequenced on an Applied Biosystems 3730 DNA Analyzer at the Ontario Health Research Institute (OHRI, Ottawa, ON). Reported sequences indicate consensus between two out of three individuals at the nucleotide level.

Additional AHR LBD sequences were obtained from 5 herring gull samples that had previously been used in a gene expression study (Chapter 5). Sample preparation and PCR conditions were as previously described, but in this case PCR products were sequenced directly with no cloning step.

6.3.4. Alignments

Predicted avian AHR LBD amino acid sequences were aligned with previously published avian AHR sequences using ClustalW online software (Chenna *et al.* 2003).

6.4. Results and Discussion

6.4.1. Sequence comparisons

A region of mRNA corresponding to the chicken AHR LBD (amino acid residues 235 – 402) was cloned and sequenced from 17 avian species. An alignment with publicly available sequences for chicken (GenBank Accession no. AF192502), common tern (AF192503), great cormorant (AB109545), black-footed albatross (AB106109), and a partial sequence for Pekin duck (AF192501), demonstrated that this region of the AHR is highly conserved among avian species. The percent identity at the amino acid level was above 97% for all species comparisons, and we observed only 6 amino residues in the 168 residue AHR LBD that were variable among the 22 available sequences (Figure 6.1). Full length chicken and common tern share 92% overall amino acid identity, with the highest percent identity in the LBD (98%) (Karchner *et al.* 2006). The AHR LBD domain was 100% conserved within avian orders *Charadriiformes* (4 species), *Falconiformes* (3 species), *Pelecaniformes* (2 species), and *Anseriformes* (2 species). Within the order *Galiiformes* (6 species), we observed 3 different forms of the AHR LBD (Figure 6.1 and Table 6.2).

6.4.2. Review of toxicity data for avian species

A large degree of variation in sensitivity to dioxin-like compounds exists among avian species. For example, herring gulls, common terns, American kestrels, and Eastern bluebirds are between 25 and 330 times less sensitive to the embryotoxic effects of dioxin-like compounds than chickens, the most sensitive species studied to date (Table 6.1). To further our understanding of mechanisms underlying this variation, we compared AHR LBD sequences to a literature review of toxicity data for avian species. Seventeen species of birds were classified according to three broad categories, where '1' indicates very sensitive, '2' indicates moderately sensitive, and '3' indicates insensitive (Table 6.1). Classifications were assigned based on LD50 data from egg injection studies, and/or TCDD EC50 values for EROD induction in cultured avian embryo hepatocytes (see Table 6.1 footnote for numerical ranges of categories). To allow comparisons between egg injection studies, values reported

for PCB 77 and PCB 126 were converted to TCDD equivalents (TEQs) using avian toxic equivalency factors (TEF) of 0.05 and 0.1 respectively as suggested in Van den Berg *et al.* (1998). Where available, the actual LD50 value was reported. In other cases, the LD50 was reported to be above the no observable effect level (NOEL, refers to mortality), or between the NOEL and 100% mortality (further details in footnote to Table 6.1).

6.4.3. A potential biomarker for dioxin sensitivity in avian species

The potential of the AHR LBD as a biomarker for sensitivity to dioxin-like compounds in avian species was assessed by comparing AHR LBD genotype to species sensitivity classifications based on a literature review of toxicity data (Table 6.2, column a). Of the 6 amino acid residues that differed among species, we initially focussed on two that have been shown to be of functional significance. Residues Ile-324 and Ser-380 in the chicken AHR are replaced by Val and Ala in common tern. These substitutions confer 7-fold higher TCDD binding affinity to the chicken receptor. A third substitution (Ala-257 in chicken is replaced by threonine in the tern), did not affect AHR ligand binding affinity (Karchner *et al.* 2006). We found that species with tern-like residues at positions 324 and 380 were consistently classified as insensitive (category 3) based on LD50 and EC50 toxicity data. This included all available species in orders *Charadriiformes, Falconiformes, Pelecaniformes,* and *Anseriformes*. Chickens, unique in having a serine residue at position 380, were the only species to be classified as very sensitive (category 1). The AHR LBD of several other species of galliforms, including ring-necked pheasant and turkey, were found to have a chicken-like Ile-324 and a tern-like Ala-380 genotype. Both are classified as moderately sensitive (category 2) based on LD50 data. Interestingly, Japanese quail, had tern-like residues at both positions and was classified as insensitive based on LD50 data. These findings suggested that the identities of amino acid residues corresponding to Ile-324 and Ser-380 in the chicken AHR are critical for determining species sensitivity both within and between avian orders. We propose that three AHR LBD genotypes; Ile/Ser (very sensitive), Ile/Ala (moderately sensitive), and Val/Ala (insensitive), may be predictive of toxicity classifications in avian species.

For species with no available toxicity data, sensitivity classifications were predicted based on AHR LBD genotype and classifications for related species (Table 6.2, column b). Thick-billed murre, osprey, great blue heron, and great cormorant were predicted to be 'insensitive' to the effects of dioxin-like compounds based on tern-like residues at positions corresponding to chicken 324 and 380 (Val/Ala genotype). Willow ptarmigan, rock ptarmigan, and ruffed grouse, were classified as moderately sensitive (category 2) based on AHR LBD genotype, and their relatedness to ring-necked pheasant and wild turkey. Interestingly, black-footed albatross is also of AHR genotype Ile/Ala, suggesting that it may be moderately sensitive to dioxin-like compounds. The sequence for tree swallow was unique in having a glutamate (E) residue at the position corresponding to chicken residue 341. All other species tested had a lysine residue (K) at this position (Figure 6.1). The functional significance of this substitution has yet to be determined, but based on the sensitivity classification for eastern bluebird, a closely related passerine, we predict that the tree swallow may be moderately sensitive to dioxin-like compounds (category 2). We are currently in the process of cloning AHR LBDs in eastern bluebird and many other species. These sequences will further clarify the accuracy of our species sensitivity predictions based on AHR LBD genotype.

6.4.4. Other factors contributing to species sensitivity

The data presented here suggest a link between AHR LBD genotype, inducibility of EROD activity in cultured cells, and species sensitivity to dioxin-like compounds *in vivo*. Although AHR LBD genotype shows promise as a genetic marker of sensitivity to dioxin-like compounds in avian species, it is clearly not the sole mechanism determining differential sensitivity between species and individuals. For example, common terns are at least 5 times more sensitive to dioxin-like compounds than herring gulls (Table 6.1), yet these species share 100% amino acid identity in the AHR LBD (Figure 6.1 and Table 6.2). Moreover, as pointed out by Karchner *et al.* (2006), the 7-fold difference in ligand binding affinity between the tern and chicken AHRs may not completely account for the 80- to 250-fold difference in dioxin sensitivity between these two species. Differences in sensitivity between individuals within a species may not be associated with AHR LBD genotype. PCR

products sequenced from 5 individual herring gulls were 100% identical at the amino acid level although hepatocyte cultures prepared from these individuals had previously been shown to be differentially sensitive to CYP1A induction by TCDD (Chapter 5).

These observations suggest that mechanisms underlying variation in species sensitivity to dioxin-like compounds are likely to involve multiple pathways and regions of the AHR outside of the LBD. AHR LBD genotype may nevertheless be a powerful biomarker for dioxin sensitivity in avian species. Because more traditional approaches such as egg injection studies are difficult to carry out with wild animals, LD50 values may never be widely available for many ecologically relevant species. Moreover, it would be counter-productive to sacrificed large numbers of animals in threatened ecosystems composed of rare or endangered species for the purpose of obtaining species sensitivity estimates. The advantages of a genetic marker for sensitivity, even one that classifies species according to broad categories, are therefore numerous. The sequence of the AHR LBD can provide a discrete prediction of species sensitivity and requires only a small tissue sample from a single individual. This genetic marker would not be affected by variation between individuals or background levels of contamination in field collected samples, factors that have been shown to affect the outcome of other biomarkers of susceptibility to dioxin-like compounds (Chapter 5). Combined with chemical data, AHR LBD genotype has the potential to be a powerful tool for identifying species at risk from exposure to dioxin-like compounds in contaminated environments.

6.4.5. Conclusions and future directions

Our preliminary results suggest that the sequence of the AHR LBD may be useful for predicting sensitivity to dioxin-like compounds in species from diverse avian orders. Further evaluation of the potential of AHR LBD genotype as a genetic marker for sensitivity to dioxin-like compounds in avian species will depend on clarification of the contribution of all 6 amino acid substitutions to differential sensitivity. We must also determine how unique combinations of these amino acids affect TCDD binding affinity and transactivation potential of the AHR in each species, and strengthen the association between these biochemical endpoints and *in ovo* toxicity.

This research also suggests intriguing hypotheses concerning mechanisms of natural selection contributing to patterns of sensitivity observed in modern birds. For example, based on the literature review presented in this chapter we can hypothesize that species that feed at high trophic levels, and are therefore likely to accumulate significant body burdens of lipophillic contaminants, are also relatively insensitive to the toxic effects of dioxin-like compounds. Future work could focus on assessing relationships between dioxin sensitivity, trophic level and tissue concentrations of lipophillic contaminants in diverse species. We are currently cloning the AHR LBD in additional species from a range of avian orders with goals of a) strengthening the association between AHR LBD genotype and species sensitivity and b) exploring the evolutionary origins of variation in sensitivity to dioxin-like compounds among avian species.

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WODU            AMNFQGRLKFLHGQNKKGKDGTTLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
COEI            AMNFQGRLKFLHGQNKKGKDGTTLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
OSPR            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
AMKE            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
BAEA            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
GRCO            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
HERG            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
TBMU            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
DCCO            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
RBGU            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
COTE            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
TRES            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
GBHE            AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
JAQU            AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
BFAL            AMNFQGRLKFLHGQNKKGKDGATLSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
RUGR            AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
WIPT            AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
WITU            AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
ROPT            AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF
CHICKEN     235 AMNFQGRLKFLHGQNKKGKDGAALSPQLALFAVATPLQPPSILEIRTKNFIFRTKHKLDF 294
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WODU            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
COEI            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
OSPR            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
AMKE            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
BAEA            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
GRCO            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
HERG            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
TBMU            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
DCCO            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
RBGU            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
COTE            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
TRES            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMETGESGMTVFRLLT
GBHE            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
JAQU            TPTGCDAKGKIVLGYTEAELCMRGTGYQFVHAADMLYCAENHVRMMKTGESGMTVFRLLT
BFAL            TPTGCDAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLT
RUGR            TPIGCDAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLT
WIPT            TPIGCDAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLT
WITU            TPIGCDAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLT
ROPT            TPVGCDAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLT
CHICKEN     295 TPTGCDAKGKIVLGYTEAELCMRGTGYQFIHAADMLYCAENHVRMMKTGESGMTVFRLLT 354
                ** *************************:***************:*************

WODU            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
COEI            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
OSPR            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
AMKE            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
BAEA            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
GRCO            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
HERG            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
TBMU            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
DCCO            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
RBGU            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
COTE            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
TRES            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
GBHE            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
JAQU            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
BFAL            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
RUGR            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
WIPT            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
WITU            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
ROPT            KENRWAWVQANARLVYKNGRPDYIIATQRPLTDEEGAEHLRKRNMKL
CHICKEN     355 KENRWAWVQANARLVYKNGRPDYIISTQRPLTDEEGAEHLRKRNMKL 401
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Figure 6.1. Alignment of the predicted amino acid sequence of the avian AHR ligand binding domain. Asterisks indicate a match between all 20 sequences. Four letter species codes are; wood duck (WODU), common eider (COEI), ring-billed gull (RBGU), thick-billed murre (TBMU), American kestrel (AMKE), bald eagle (BAEA), tree swallow (TRES), common tern (COTE), herring gull (HERG), great cormorant (GRCO), osprey (OSPR), double-crested cormorant (DCCO), great blue heron (GBHE), Japanese quail (JAQU), black-footed albatross (BFAL), ruffed grouse (RUGR), willow ptarmigan (WIPT), wild turkey (WITU), and rock ptarmigan (ROPT).

Table 6.1. Dioxin sensitivity classifications for avian species.

Order	Common name	LD50* (µg/kg TEQ)	EC50** (nM TCDD)	Sensitivity*** LD50	EC50
Charadriiformes	Herring gull	>50[a]	0.88 – 2.1[h,i]	3	3
	Ring-billed gull	-	1.60[j]	-	3
	black-headed gull	> 50[a]	0.71[i]	3	3
	Common tern	10.4[b]	0.12 - 0.32[i,k]	3	2
Falconiformes	American kestrel	6.5 - 15.8[b]	-	3	-
	Bald eagle	-	2.1 - 2.5[l]	-	3
Pelecaniformes	Double-crested cormorant	4.0 - 17.7[c]	1.1[j]	3	3
Anseriformes	Wood duck	> 4.6[d]	-	3	-
	Common eider	> 50[a]	-	3	-
	Pekin duck	-	1.9[h]	-	3
	Goldeneye	> 50[a]	-	3	-
	Domestic goose	> 50[a]	-	3	-
Galiiformes	Chicken	0.15 - 0.3[e]	0.004 - 0.06[h,i,j,k]	1	1
	Japanese quail	> 24[a]	-	3	-
	Ring-necked pheasant	1.3 - 2.2[f]	0.14[h]	2	2
	Turkey	> 10[a], 2.0 - 6.0[a]	0.63[h]	2	3
Paseriformes	Eastern bluebird	1.0 – 10[g]	-	2	-

*Lethal dose for 50% of the population (LD50) expressed in terms of µg/kg TCDD equivalents (TEQs). Studies cited involved injection of PCB 77, PCB 126, or TCDD into the yolk sac, albumin or air cell on day 0 or 4 of incubation. Values reported for PCB 77 and PCB 126 were converted to TEQs using avian toxic equivalency factors (TEFs) of 0.05 and 0.1 respectively as suggested by Van den Berg *et al.* (1998). A no observable effect level (NOEL), or the range from the NOEL to 100% mortality, is reported where an LD50 value was not available. In all cases the NOEL refers to mortality.

**The effective median concentration (EC50) refers to the dose of TCDD causing a half-maximal EROD response in cultured avian hepatocytes.

***Sensitivity classifications based on LD50 and EC50 values as follows:

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

[a]Brunström and Reutergardh 1986; Brunström and Lund 1988; Brunström 1988; Brunström 1989; Brunström *et al.* 1990; Brunström and Halldin 1998. No observable effect levels (NOEL) are reported for herring gull, black-headed gull, common eider, goldeneye, domestic goose, and Japanese quail. For turkey, values refer to the NOEL for PCB 77, followed by the range of doses between the NOEL and the dose causing 100% mortality for PCB 126.

[b]Hoffman *et al.* 1998. The range reported for American kestrel is derived from LD50 values for PCB 77 and PCB 126.

[c]Powell *et al.* 1998. The range reported for double-crested cormorant is derived from LD50 values for PCB 126 and TCDD.

[d]Augspurger 2006. The value reported for wood duck is a NOEL.

[e]reviewed in Powell *et al.* 1996a.

[f]Nosek *et al.* 1993. The range reported for ring-necked pheasant is derived from LD50 values for TCDD injected into the yolk and albumin.

[g]Thiel *et al.* 1988. The values reported for eastern bluebird are derived from the range of TCDD doses between the NOEL and the dose causing 100% mortality.

[h]Kennedy *et al.* 1996a

[i]Bosveld 1995

[j]Sanderson *et al.* 1998

[k]Lorenzen *et al.* 1997b

[l]Kennedy *et al.* 2003b

Table 6.2. Predicted sensitivity classification for avian species based on AHR genotype. Genotype refers to the identity of the 6 amino acids in the AHR ligand binding domain that differ among known species. These correspond to residues 256, 257, 297, 324. 341, and 380 of the chicken AHR. Amino acid residues presented in bold are of special interest, having been shown to cause differential TCDD AHR binding affinity in chicken and common tern.

Order	Common name	Genotype	Sensitivity	
			a	b
Charadriiformes	Herring gull	ATTVKA	3	
	Ring-billed gull	ATTVKA	3	
	Black-headed gull	-	3	
	Common tern*	ATTVKA	3	
	Thick-billed murre	ATTVKA		3
Falconiformes	American kestrel	ATTVKA	3	
	Bald eagle	ATTVKA	3	
	Osprey	ATTVKA		3
Pelecaniformes	Double-crested cormorant	ATTVKA	3	
	Great cormorant*	ATTVKA		3
Anseriformes	Wood duck	TTTVKA	3	
	Common eider	TTTVKA	3	
	Pekin duck*	TTTV..	3	
	Goldeneye	#	3	
	Canada goose	#		3
Ciconiiformes	Great blue heron	AATVKA		3
Galiiformes	Chicken*	AATIKS	1	
	Japanese Quail	AATVKA	3	
	Ring-necked pheasant	TAIIKA	2	
	Turkey	TAIIKA	2	
	Willow ptarmigan	AAIIKA		2
	Rock ptarmigan	AAVIKA		2
	Ruffed grouse	AAIIKA		2
Paseriformes	Eastern bluebird	#	2	
	Tree swallow	ATTVEA		2
Procellariiformes	Black-footed albatross*	ATTIKA		2

[a]Sensitivity classifications based on toxicity data presented in Table 6.1. In cases where classifications differ between LD50 and EC50 values, the LD50 classification was used.
[b]Sensitivity classifications predicted from AHR ligand binding domain genotype and toxicity data for related species.
#Liver samples have been obtained, and sequencing is currently underway for these species
*Sequences for the common tern, great cormorant, chicken, and black-footed albatross AHR LBD are publicly available on the NCBI website. A partial Pekin duck sequence is also available. All other sequences were generated for this project.

Chapter 7

General discussion & conclusions

The experiments presented in this thesis provide a detailed description of the effects of several experimental factors on the cytochrome P4501A (CYP1A) response to 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD) in cultured avian embryo hepatocytes. This research addresses both biological and technical aspects of the CYP1A *in vitro* bioassay. The findings have provided insight into mechanisms underlying variation in dioxin sensitivity in avian species, and have resulted in the development of novel *in vitro* methods for obtaining species sensitivity estimates.

The relationship between the CYP1A response to TCDD at the level of gene expression and enzymatic activity was examined in chicken embryo hepatocytes. Concentration-dependent effects of TCDD on CYP1A mRNA expression and EROD activity were nearly identical under the conditions used in the experiments presented in this thesis (Figure 2.4). When scaled to account for differences in the magnitude of the response, EROD, CYP1A4 mRNA, and CYP1A5 mRNA dose-response curves were superimposable, with statistically equivalent EC50 values (Figure 2.4). This result suggests that dioxin sensitivity estimates derived from EROD and CYP1A mRNA dose-response curves can be used interchangeably. Because mRNA abundance can be measured in a very small quantity of tissue, the CYP1A mRNA bioassay may be particularly useful when applied to risk assessment for dioxin-like compounds in ecologically sensitive species where sample collection is difficult. Routine application of the CYP1A mRNA bioassay to risk assessment in avian wildlife will require further validation of the concepts presented in this thesis. For example, a comparison of the potency of the CYP1A mRNA and EROD responses to environmental mixtures of dioxin-like contaminants would be of interest.

Both the CYP1A mRNA and EROD bioassays can be used to predict *in vivo* toxicity to dioxin-like compounds, but particular advantages are associated with each method. These are summarized in Table 7.1. We exploited the strengths of each bioassay,

by developing a novel method for assessing CYP1A mRNA abundance in hepatocytes that had previously been analyzed for EROD activity. For wildlife studies, this approach represents a significant improvement over measuring each endpoint separately because it minimizes sample requirements. Moreover, the ability to measure CYP1A abundance in post-EROD hepatocytes may occasionally allow the researcher to salvage data from a valuable experiment in cases where EROD activity is below the detection limit of the assay. An additional advantage is that enzyme activity and mRNA expression are measured in the exact same cells, eliminating the contribution of variance between replicate samples to the overall response. To the best of our knowledge, the EROD/CYP1A mRNA bioassay presented here is the first published method for measuring a biomarker response at the level of both enzymatic activity and mRNA expression in a single sample. Further research is required to determine if this novel sequential approach can be applied to other enzyme assays and target genes. Such approaches would be particularly suited to studies using tissues collected from wild species where it is important to maximize the amount of data that can be obtained from each sample.

A chicken model was used to investigate how embryonic exposure to contaminants might affect CYP1A bioassay results (Chapter 4). We found that injection of 3,3',4,4',5-pentachlorobiphenyl (PCB 126) into the air cell of chicken eggs prior to incubation resulted in a large CYP1A response in whole liver tissue, and that this response was diminished in cultured hepatocytes. Hepatocytes cultured from PCB 126-treated embryos were more responsive to CYP1A mRNA induction by TCDD, but this effect was only observed at concentrations of PCB 126 that were above typical environmental levels. We concluded that embryonic exposure to dioxin-like compounds would be unlikely to affect EROD bioassay results except in hepatocytes cultured from particularly contaminated individuals, or in particularly sensitive species. Our findings support the use of a primary cell culture model for studying effects of dioxin-like compounds in wild species and may also have broader implications for *in vitro* bioassays in general. Future research should focus on whether the general principles outlined in Chapter 4 also apply to mixtures of dioxin-like compounds, or to other classes of lipophillic environmental contaminants.

A large degree of variation in basal and TCDD-induced CYP1A mRNA abundance was observed among hepatocyte cultures prepared from 55 individual embryos (Chapter 5).

Factors contributing to this variation, and the resulting impact on EC50 values were investigated. Statistically significant differences in basal CYP1A4 and CYP1A5 mRNA expression were detected in hepatocytes cultures prepared from herring gull embryos originating from two separate populations, and this suggested that there might be a genetic component to the observed variability. Further research is required to determine how population level differences in baseline expression values relate to *in vivo* sensitivity to dioxin-like environmental contaminants.

The chicken model presented in Chapter 4 suggested that embryonic exposure to dioxin-like compounds at current environmental levels would be unlikely to affect sensitivity estimates based on EROD induction in cultured cells for all but the most contaminated herring gulls. This result was confirmed in hepatocytes cultured from individual herring gull embryos. No relationship between embryonic TCDD equivalents (TEQs) and CYP1A induction was detected, and only one individual was contaminated with TEQs over the no observable effect level (NOEL) for effects on CYP1A endpoints in cultured cells. Interestingly, hepatocytes cultured from this individual were exceptionally responsive to CYP1A mRNA induction by TCDD (Figure 5.6). We speculated that this observation may be related to up-regulation of the aryl hydrocarbon receptor (AHR) in the embryonic liver, and this hypothesis warrants further investigation.

Many factors were thought to contribute to the large degree of variation in CYP1A expression and induction in hepatocyte cultures prepared from individual herring gull embryos. Regardless of the source, the observed variation had a clear impact on EC50 values derived from composite CYP1A dose-response curves. Particularly responsive hepatocyte culture preparations had a strong influence on the EC50 value, while non-responsive hepatocyte cultures had a minimal influence (Figure 5.8). The close correspondence between the composite CYP1A4 dose-response curve, and a previously published EROD dose-response curve assessed in pooled herring gull embryo hepatocytes (Figure 5.7), suggests that the same phenomenon may occur in pooled samples. These findings indicate that interindividual variation in CYP1A expression and induction is a confounding factor for obtaining reproducible EROD EC50 values, and is likely to have an important impact on the predictive power of this *in vitro* bioassay for estimating dioxin sensitivity in avian species. Taken together, the results from Chapters 4 and 5 suggest that

CYP1A endpoints are likely to be affected by embryonic exposure to dioxin-like compounds in hepatocytes cultured from only the most contaminated individuals, but that these outliers can have a large impact on EROD EC50 values.

These findings gave rise to several recommendations for more effective application of the CYP1A bioassay in herring gulls and by extension, in other avian species. Namely; (1) eggs used for hepatocyte culture experiments should be collected from sites with an average contamination level well below 5.1 μg/kg TEQ (the threshold for effects observed in cultured cells), (2) the effect of genetic variation in the CYP1A response can be minimized by sampling individuals from within populations, and (3) due to a large degree of interindividual variation, reproducible EC50 estimates may require hepatocyte cultures prepared from pools comprised of no less than 20 individual livers.

Investigating mechanisms underlying species differences in sensitivity to dioxin-like compounds led to the development of a genetic biomarker for dioxin sensitivity in avian species. Previously published research identified two key amino acids in the ligand binding domain (LBD) of chicken and common tern aryl hydrocarbon receptors (AHR) that were associated with differential dioxin sensitivity in these two species (Karchner *et al.* 2006). We expanded upon this work by comparing a literature review of avian toxicity data with AHR LBD sequences cloned from 18 avian species. This comparison revealed that the key amino acids identified by Karchner *et al.* (2006) are predictive of sensitivity to dioxin-like compounds in diverse avian orders (Figure 7.1). This preliminary finding raises the intriguing possibility that a simple genetic test could be used to identify species that are particularly sensitive to dioxin-like compounds within contaminated environments. AHR LBD genotype represents a significant improvement over traditional approaches for estimating species sensitivity because it requires sample from only a single individual and would not be influenced by many of the environmental and genetic sources of variation described in this thesis. More research is required to identify the precise effect of additional AHR amino acid substitutions on TCDD binding affinity, CYP1A induction, and dioxin toxicity. Furthermore, the list of species for which toxicity data and AHR LBD sequence data exists must be expanded to include a wider range of avian orders. This may strengthen the association between AHR LBD genotype and *in vivo* dioxin sensitivity, and

may also provide insight into processes governing the evolutionary origins of variation in dioxin sensitivity in birds.

In conclusion, the experiments presented in this thesis provide a detailed description of factors contributing to variation in the CYP1A response to TCDD in avian embryo hepatocytes. By examining the CYP1A response at the level of the individual, population, and species, we were able to assess factors contributing to a lack of reproducibility in EROD EC50 values, and describe mechanisms underlying differential dioxin sensitivity. Additionally, our findings led to the development of two novel *in vitro* approaches for assessing sensitivity to dioxin-like environmental contaminants in avian wildlife. Together, this research provides a more complete understanding of variation in dioxin sensitivity in birds.

Table 7.1. Summary and assessment of *in vitro* methods for estimating dioxin sensitivity. Each method can be used to predict *in vivo* toxicity of dioxin-like compounds in avian species. Methods marked with asterisks were developed based on the results presented in this thesis.

method	endpoint	advantages
EROD bioassay	EROD activity in cultured embryo hepatoyctes	▪ Minimal cost and labour ▪ Ease of application to multiple species
CYP1A mRNA bioassay*	CYP1A4 and CYP1A5 mRNA abundance in cultured embryo hepatoyctes	▪ Both isoforms assessed in a single multiplex assay ▪ Magnitude of the response is larger than EROD ▪ Preferred endpoint for environmental mixtures ▪ Low limit of quantitation; response is detectable in small samples and at low cell densities
EROD/CYP1A sequential bioassay*	CYP1A4 mRNA. CYP1A5 mRNA, and EROD activity in the same sample	▪ Exploits advantages of EROD and CYP1A mRNA ▪ Maximizes the amount of data obtained from a single sample
AHR genotype*	Nucleotide sequence of the AHR ligand binding domain	▪ Simple genetic test ▪ Minimal sample requirements ▪ Possibility for non-destructive sampling

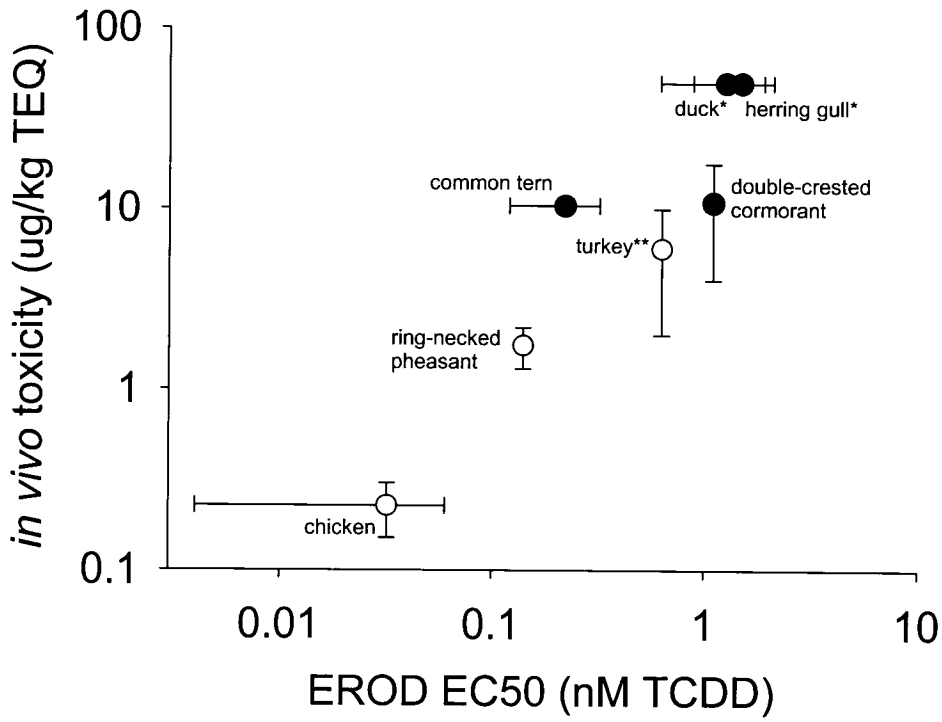

Figure 7.1. Relationship between *in vitro* and *in vivo* measures of dioxin toxicity, and AHR ligand binding domain genotype in avian species. This figure is adapted from figure 1.6, and a description of the data can be found in the original caption. AHR LBD genotype is predictive of EROD EC50 and *in vivo* toxicity of dioxin-like compounds in avian species, where Ile/Ser indicates very sensitive (○), Ile/Ala indicates moderately sensitive ( ), and Val/Ala indicates insensitive (●).

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